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Ethyl acetate extract of *Clausena execavata* promotes growth inhibition of Burkitt's lymphoma cell line via apoptotic activities

[El extracto de acetato de etilo de *Clausena excavata* promueve la inhibición del crecimiento de la línea celular del linfoma de Burkitt a través de actividades apoptóticas]

Shaymaa Fadhel Abbas Albaayit

Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq

Reviewed by:
Mario Simirgiotis
Universidad Austral
Chile

Ibrahim Aktas
Adiyaman University
Turkey

Correspondence:
Shaymaa Fadhel Abbas **ALBAAYIT**
shaymaa_albaayit@yahoo.com

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Abstract: *Clausena excavata* is a famous folklore medicinal plant in Asian region that is being used for the treatment of different disorders. This study investigated the cytotoxic effects of leaf extracts via MTT assay, as well as the *in vitro* apoptotic activities of the ethyl acetate *C. excavata* leaf extract (EACE) on human Burkitt's lymphoma, Raji, cell line using annexin-V-FITC/propidium iodide flow cytometric assays. Pro-apoptotic (BAX) and anti-apoptotic (BCL-2, c-MYC) gene expressions were determined via real-time quantitative PCR. Phytochemical screening was done by Gas chromatography-mass spectrometry (GC-MS). EACE has the lowest IC_{50} (19.3 ± 0.35 µg/mL) among extracts. EACE-treated Raji cells after 48 h underwent apoptosis as evident by loss of cell viability and increase in the percentage of early and late apoptotic cells. The results also showed EACE mediated decreased in the BCL-2 and c-MYC gene expressions and increased in the BAX gene. *C. excavata* is a potential treatment for Burkitt lymphoma through activation of apoptosis

Keywords: *Clausena excavate*; Burkitt lymphoma; Raji cell line; c-MYC; Apoptosis

Resumen: *Clausena excavata* es una planta medicinal tradicional famosa en la región asiática que se utiliza para el tratamiento de diferentes trastornos. Este estudio investigó los efectos citotóxicos de los extractos de hojas a través del ensayo MTT, así como las actividades apoptóticas *in vitro* del extracto de hoja de acetato de etilo de *C. excavata* (EACE) en la línea celular de linfoma de Burkitt humano, Raji, usando citometría de flujo de yoduro de anexina-V-FITC/propidio. Las expresiones génicas proapoptóticas (BAX) y antiapoptóticas (BCL-2, c-MYC) se determinaron mediante PCR cuantitativa en tiempo real. El cribado fitoquímico se realizó mediante cromatografía de gases-espectrometría de masas (GC-MS). EACE tiene el IC_{50} más bajo (19,3 ± 0,35 µg/mL) entre los extractos. Las células Raji tratadas con EACE después de 48 h sufrieron apoptosis como es evidente por la pérdida de viabilidad celular y el aumento en el porcentaje de células apoptóticas tempranas y tardías. Los resultados también mostraron una disminución mediada por EACE en las expresiones de los genes BCL-2 y c-MYC y un aumento en el gen BAX. *C. excavata* es un tratamiento potencial para el linfoma de Burkitt a través de la activación de la apoptosis.

Palabras clave: *Clausena excavata*; Linfoma de Burkitt; línea celular Raji; c-MYC; Apoptosis

INTRODUCTION

Human Burkitt's lymphoma (BL) is also known as B cell non-Hodgkin's lymphoma derived from germinal B cells (Wright, 1963). BL is highly aggressive due to its aggressively fast-growing nature (doubling time 24-48 h) which is caused due to upregulation and translocation of MYC gene located on chromosome 8 to chromosome 14 (Schmitz *et al*., 2014), resulting excessive cell proliferation. BL has three epidemiological subtypes (sporadic, endemic, and immunodeficiency related). Out of these three subtypes, endemic BL is mostly confined to Brazil, Papua New Guinea, and Equatorial Africa) where 3-6 children out of 100,000 children per year (Magrath, 2012) and approximately male: female ratio of 2:1 is reported (Ogwang *et al*., 2008). Sporadic BL is mostly seen in Western Europe and USA (Morton *et al*., 2006). About 20% cases of immunodeficiency associated BL variants are found in HIV positive individuals (Shield *et al*., 2011), showing the higher chances of developing BL in immunocompromised patients. Recent study showed approximately 50% cases of all BL patients age were below 40 years (Dozzo *et al*., 2017). In Papua New Guinea, study conducted during 1958-1987 found four instances of familial BL cases indicating the possibility of transferring to offspring (Winnett *et al*., 1997). In 2005, it is reported that 16% cases are BL out of all childhood malignancies and still remain childhood malignancies in Papua New Guinea due to late diagnosis and reporting (Lavu *et al*., 2005).

In developed countries, the sporadic BL cases in pediatrics and young adults are successfully treated with cure rate of 90%. The cure rate for pediatrics in stage I and II reached 98% (Molyneux *et al*., 2012), due to combination therapy of surgical removal and two cycles of moderate-intensity chemotherapy (cyclophosphamide, doxorubicin, vincristine, prednisolone) while the stage III disease patients are advised four cycles of dose-intensive chemotherapy (cyclophosphamide, doxorubicin, vincristine, prednisolone, and high-dose methotrexate) (Graham & Lynch, 2020). Currently, the National Comprehensive Cancer Network has recommended advanced chemotherapy regimens with CNS prophylaxis named as CODOX-M/IVACA regimen (Magrath *et al*., 1996; Mead *et al*., 2002). However, in Africa, the cure rate of BL has not gone beyond 30% due to limited resources and capacity (Mbulaiteye *et al*., 2010). Late diagnosis and reporting and then abandoning the chemotherapy treatment without complete course contributed to the poor outcomes and high mortality (Albaayit *et al*., 2021a). Due to life threatening side effects of intensive chemotherapy, and lack of good intensive care services, its use in Africa has limited. Therefore, it is dire need to find new safer drugs with lower side effects which could be found in native lands and bring lifesaving benefits to people from Africa, Papua New Guinea, and Brazil (Schmitz *et al*., 2012; Albaayit *et al*., 2021b).

In pursuance of finding native medicinal plants for BL treatment, we targeted the plant *Clausena excavate* Burm. f. (Family: Rutaceae) due to its abundant prevalence in South-East Asia (from India, China to Papua New Guinea) and in Africa (Madagascar). This plant is used in traditional medicine for numerous disorders like wound healing, gastric ulcer (Albaayit *et al*., 2016; Albaayit, 2021). *C. excavata* has been reported for its anti-cancer properties in several cancers like hepatocellular cancer (Waziri *et al*., 2016), cervical cancer (Peng *et al*., 2013a), oral cavity cancer, small-cell lung cancer (Sripisut *et al*., 2012), breast cancer (Bruni *et al*., 2019), and non small-cell lung cancer (Albaayit *et al*., 2021c). The anticancer compounds reported from this plant are O-demethylmurrayanine, clausine D (Jiang *et al*., 2014), clausines E, murrayanine, clauszoline J (Sripisut *et al*., 2012), clausine TY (Taufiq-Yap *et al*., 2007), excavatine A, nordentatin (Peng *et al*., 2013b), clausarin (Su *et al*., 2009), dentatin (Manosroi *et al*., 2004), and clauslactone (A-J) (Peng *et al*., 2013b).

In this study, we investigated the ethyl acetate fraction of *C. excavata* leaves for its possible apoptosis inducing effect on the Raji Burkitt lymphoma cancer cell line by evaluating through MTT assay; PI/annexin V pathway and expression of apoptotic and anti-apoptotic related genes.

MATERIALS AND METHODS

Preparation of Ethyl acetate extract

The protocol of Shaymaa Fadhel Abbas Albaayit *et al*. (2021d), was followed to prepare the ethyl extract of *C. excavata*.

Cell viability through MTT assay

The human B lymphocyte carcinoma, Raji (ATCC CCL-86) cell line was obtained from cell culture bio-bank of Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi. These cells were cultured and passaged using RPMI medium. The cytotoxic effect of the

solvent extracts on the Raji cell lines was investigated using the MTT assay. Cells were trypsinized and counted the cell numbers and approximately, 5 × 103 Raji cells/well were placed in 96-well plate and incubated at 37°C, 5% CO_2 for 24 h, before treatment with 12.5 to 100 µg/mL of the petroleum ether (PT), chloroform (CH), ethyl acetate (EA), and methanol (MOH) fractions for 48 h. After incubation, 20 µL of MTT reagent was added to each well and the plate incubated further for 4 h at 37°C, 5% CO_2. The purple formazan crystals formed at the bottom were dissolved with 100 µL dimethyl sulfoxide (DMSO) and the absorbance was measured at 570 nm using an ELISA plate reader (Tecan, California, USA). The cancer cell growth inhibition was expressed as IC_{50} (half-maximal inhibitory concentration) value.

Apoptosis

Annexin-V/propidium iodide (PI) assay

To determine the necrosis, early and late apoptosis in Raji cells were determined by using the Annexin-V/PI staining technique (ThermoFisher Scientific). Briefly, 1 × 106 Raji cells/well suspension were seeded into 6-well plate and incubated for 24 h at 37°C. Ethyl acetate *C. excavate* extract (EACE) at 19.3 and 100 µg/mL was added to the respective wells and the plate incubated for 48 h. After the treatment, the cells were harvested by trypsinization, washed thrice with phosphate buffered saline (PBS), and resuspended in annexin binding buffer. In this suspension, 5 µL of fluorescein isothiocyanate (FITC)-conjugated annexin-V and 5 µL of PI was added and incubated for 15 min at room temperature in the dark. The percentage of cells in necrosis, early and late apoptosis were determined by flow cytometry on FACSCalibur™ (Becton Dickinson).

Quantitative Real-time polymerase chain reaction (qRT-PCR)

qRT-PCR was used to determine expression of apoptosis-related genes in treated Raji cells. The Raji cell were seeded into each well of a 6 well plates at a density of 1 × 106 Raji cell/well and treated with EACE at IC_{50} concentration for 48 h. Cells were then transferred to nuclease free eppendorf and RNA was isolated from these cells by using the TRIzol® Reagent (Bio basic BS410A, Canada). From 1 µg of total RNA, complementary DNA (cDNA) was synthesized by using cDNA synthesis kit (ThermoFisher Scientific K0221). Then, 1 µL of cDNA was amplified by using primers listed in Table No. 2 and by using SYBR Green Master Mix (ThermoFisher Scientific), real time-PCR was performed. LightCycler® 480 Gene Scanning Software (Agilent Technologies StratageneMx 3000P, Santa Clara, CA) was used to generate the results and calculate the amount of transcripts relative to the normal control. GAPDH was used as the housekeeping gene control (Hidayat *et al*., 2016).

Phytochemical Screening

The ethyl acetate *C. excavata* showed good activity, therefore this fraction was sent for GC-MS analysis to screen for the presence of phytochemicals (Advance Chemistry Solution, GHOD SdnBhd ACD/Labs Inc., Malaysia) by using standard procedures as described in Albaayit *et al.* (2014).

Statistical analysis

Data was expressed as mean ± SD and level of significance at $p<0.05$ was determined by one-way analysis of variance (Dunnett's test) using SPSS (Version 19.0; IBM Corporation, Armonk, NY, USA) by comparing treated group with untreated group.

RESULT

The yield (weight of crude extract/weight of fresh plant) of the PT, CH, EA, and MOH *C. excavata* leaf extracts were of 1.56, 2.57, 0.38, and 0.94% respectively.

Cytotoxicity assay

MTT assay results showed the ethyl acetate *C. excavata* extract (EACE) as the most active extract with the lowest mean IC_{50} of 19.3 µg/mL against Raji cells, and hence it was chosen for subsequent studies (Table No. 1). This was followed by chloroform extract whose IC_{50} value was found to be 36.1 µg/mL. However, it wasn't selected for mechanistic studies. Remaining petroleum ether and methanolic extracts were not active against this cell line.

Raji cell apoptosis

Under Annexin-V/PI flowcytometric analysis, the EACE (100 µg/mL) treated Raji cells showed early and late apoptosis induction (12.3 and 85.1%, respectively) after 48 h as compared to the negative control (Figure No. 1), whereas at 19.3 µg/mL it showed 27.1 and 52.4 % early and late apoptosis respectively. On the contrary, the non-treated control cells were mostly intact with no fluorescence. This shows that cells had undergone apoptosis with increase in incubation time.

Expression of apoptotic and anti-apoptotic genes in Raji cell using qRT-PCR

After 48 h-treatment, the Raji cell expression of BAX gene was significantly (p<0.001) upregulated by ~3.3-fold, while that of anti-apoptotic (BCL-2 and c-MYC genes) were down-regulated by ~1.5- and 2.7-fold, respectively, in comparison with the untreated control (Figure No. 2). Since there was upregulation of BAX gene and downregulation of anti-apoptotic (BCL-2 and c-MYC genes), it is concluded that EACE leads cell death through apoptosis process.

Table No. 1
IC_{50} values of *Clausena excavate* leaf extracts treatment on Raji cell line after 48 h.

Fraction	IC_{50} (µg/mL)
Petroleum ether	>100
Chloroform	36.1 ± 0.47
Ethyl acetate	19.30 ± 0.53
Methanol	>100

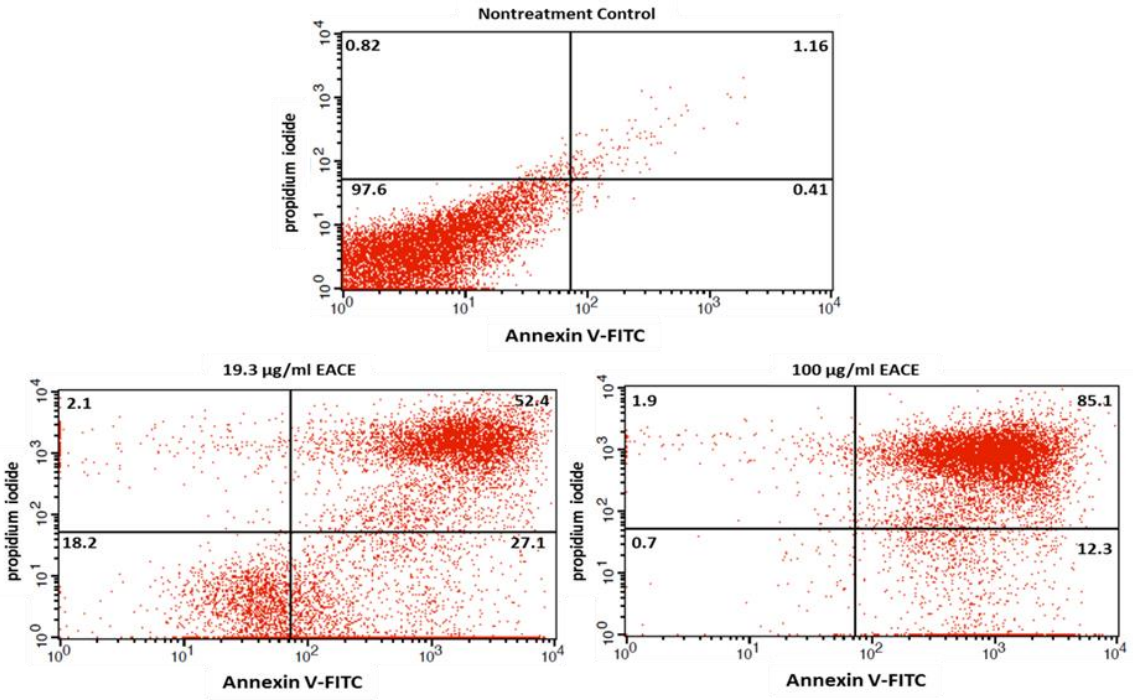


Figure No. 1
Annexin-V/PI Flowcytometric analysis of the Raji cells
Cells treated with ethyl acetate *C. excavata* leaf extract (EACE) at 19.3 and 100 µg/mL for 48 h. Control show low number of apoptotic cells. Significantly high apoptotic cells treated with EACE especially at the higher dose

Table No. 2
Genes and primer sequences

Gene	Forward primer sequence	Reverse primer sequence
BCL2-associatedX (*BAX)*	AAGAAGCTGAGCGATGTC	GGCCCCAGTTGAAGTTGC
B cell lymphoma -2 (*BCL2*)	GGCATTCAGTGACCTGACATC	AGTCATGCCCGTCAGGAAC
C-MYC	CACAGCAAACCTCCTCACAG	GGTGCATTTTCGGTTGTTGC
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	CCAGAACATCATCCCTGCCT	CCAGAACATCATCCCTGCCT

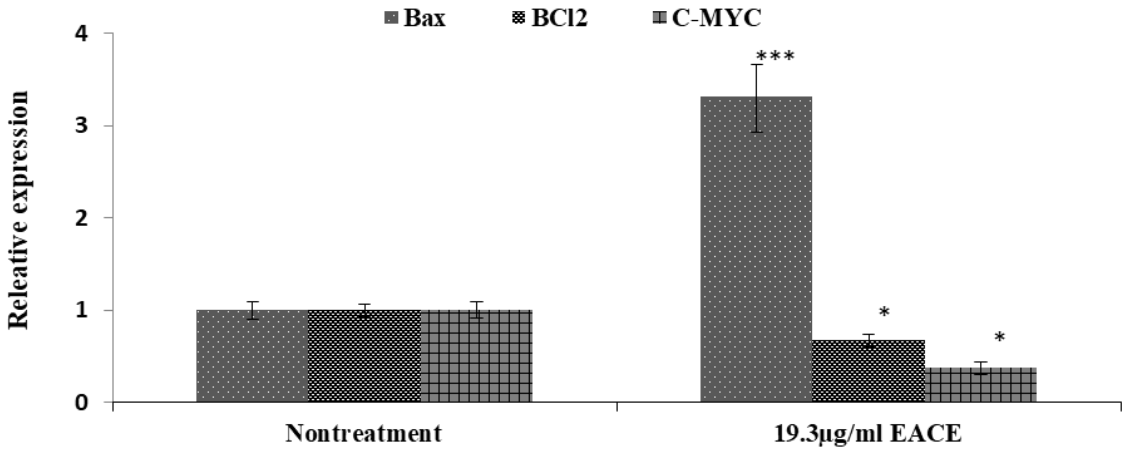


Figure No. 2
qRT-PCR result of Raji cell line
mRNA expression of BAX, BCL2 and c-MYC genes in B cell lymphocyte carcinoma, Raji, cell line treated with 19.3 µg/mL (IC_{50}) ethyl acetate *C. excavata* leaf extract (EACE). Values are mean ± standard deviation. (*) Mean significantly different ($p<0.05$) as compared to untreated control

Phytochemical analysis

Based on GC-MS analysis, the EACE contains several chemical groups as shown in Figure No. 3 and Table No. 3.

Table No. 3
Major phytochemical components from EACE via GC-MS analysis

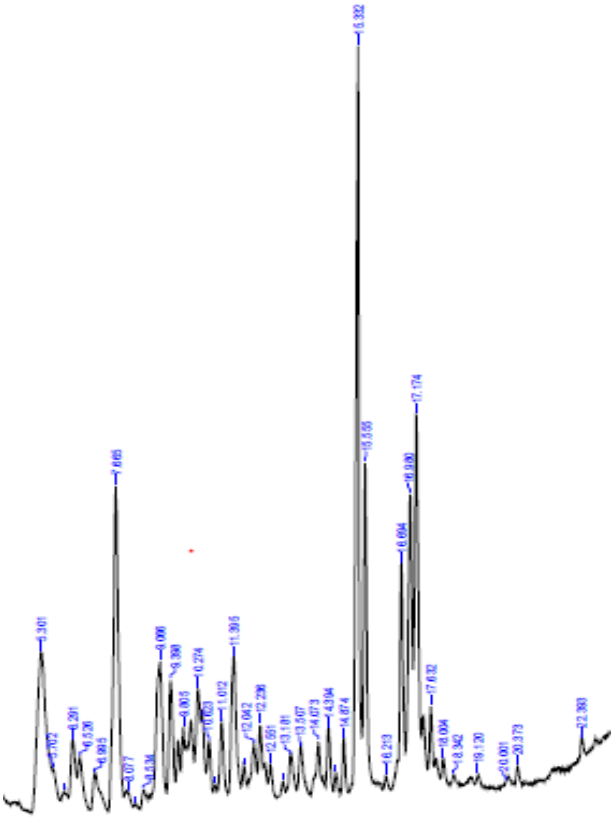
No.	Compound	Rt (min)	Area %	Formula	Remarks
1	Benzenemethanol, α,α,4-trimethyl-	6.3	1.04	$C_{10}H_{14}O$	Antimicrobial, antioxidant, neuroprotective
2	2,6-Dimethyl-1,3,5,7-octatetraene, E,E-	6.5	0.83	$C_{10}H_{14}$	Antioxidant
3	1,3-Benzodioxole, 5-(2-propenyl)	7.6	4.82	$C_{10}H_{10}O_2$	Anti-inflammatory
4	benzene 1 2-dimethoxy-4-(2-propenyl)-	9.06	2.8	$C_{11}H_{14}O_2$	Antioxidant, antibacterial
5	Caryophyllene	9.4	1.54	$C_{15}H_{24}$	Antibacterial antioxidant, anti-cancer

6	*Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl*	10.6	0.72	$C_{15}H_{24}$	Antioxidant
7	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-	10.8	0.19	$C_8H_{10}O_2$	Antioxidant, anti-cancer
8	1,6,10-Dodecatrien-3-ol, 3,7,11-trimethyl-	11.02	1.0	$C_{15}H_{26}O$	Anti-oxidant, Antimicrobial
9	Longifolenealdehyde	12.0	0.86	$C_{15}H_{24}O$	Antibacterial , antioxidant
10	1H-Cycloprop(e)azulene, 1a,2,3,4,4a,5,6,7b-octahydro-	12.2	1.18	$C_{15}H_{24}$	Antibacterial
11	*Tetradecanoic acid*	13.2	0.67	$C_{14}H_{28}O_2$	Antimicrobial, anti-inflammatory
12	2-Pentadecanone, 6,10,14-trimethyl	14.07	0.94	$C_{18}H_{36}O$	Antimicrobial, anti-inflammatory
13	9-octadecenoic acid (Z) methyl ester	14.2	0.5	$C_{19}H_{36}O_2$	Cancer preventive, anti-inflammatory
14	Octadecanoic acid methyl ester	16.79	0.44	$C_{19}H_{38}O_2$	Antimicrobial, anti-inflammatory, anticancer
15	Cis-vaccenic acid	17.06	18.86	$C_{18}H_{34}O_2$	Antibacterial, hypolipidemic
16	Petroselinic acid	18.35	0.75	$C_{18}H_{34}O_2$	Immunoregulatory, anti-cancer, antimicrobial
17	cis-11-Eicosenoic acid	18.55	0.84	$C_{20}H_{38}O_2$	Antioxidant, antiarthritic, anticoronary, Anti-inflammatory
18	Hexadecanoic acid, methyl ester	18.91	3.97	$C_{17}H_{34}O$	Antimicrobial, anti-inflammatory, anti-tumor
19	n-Hexadecanoic acid	15.3	6.82	$C_{16}H_{32}O_2$	Antibacterial, Antioxidant
20	Hexadecanoic acid, ethyl ester	15.5	3.3	$C_{18}H_{36}O_2$	Antimicrobial, Antioxidant
21	Octadecanoic acid	15.9	0.2	$C_{18}H_{36}O_2$	Antimicrobial, Antioxidant
22	Phytol	16.7	2.4	$C_{20}H_{40}O$	Antinociceptive, antioxidant Antimicrobial
23	9,12,15-Octadecatrienoic acid	16.9	3.4	$C_{18}H_{30}O_2$	anti-inflammatory, cancer preventive, hepato protective, Antioxidant and hypo cholesterolemic
24	Methyl 17-methyloctadecanoate	17.3	0.83	$C_{20}H_{40}O_2$	Antimicrobial
25	7-Pentadecene	18.3	0.31	$C_{15}H_{30}$	*Antimicrobial*, anti-cancer
26	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl	25.13	1.29	$C_{21}H_{40}O$	*Antimicrobial*, anti-inflammatory

DISCUSSION

Till date, various studies related to C. excavata reported many beneficial biological activities (Albaayot *et al*., 2020a). Among them, anticancer property had been extensively studied and reported on many types of cancer like hepatocellular, oral, breast, lungs, colorectal, cervical cancers (Sripisut *et al*., 2012; Peng *et al*., 2013b; Waziri *et al*., 2016; Bruni *et al*., 2019). However, no any study was carried out on Burkitt lymphoma and reported before, so we studied this plant and first time reported its anti-Burkitt lymphoma anticancer effect by using different molecular techniques. Due to expensive chemo-therapy for this disease, many African and Papua New Guinea patients could not afford it and some patients who started its therapy abandoned its treatment therapy due to its side effects and unaffordable prices leading to higher mortality. It seems like this plant has been neglected as its native people didn't know about its potential use for this disease. As this plant grows abundantly in South East Asia and some parts of Africa therefore it could be potential treatment to endemic Burkitt lymphoma and help native people who couldn't afford expensive chemotherapy.

Figure No. 3
GC-MS chromatogram of ethyl acetate extract of *C. excavate*



C. excavata leaves were subjected for the extraction process using various solvents to evaluate the extract with potentially the best anticancer activity. Among different extracts, MTT assay showed that EACE exhibited the most potent anticancer activity with IC_{50} value of 19.3 µg/mL. The chemical composition of the leaves of *C. excavata* reported (Phillips, 2006; Sripisut *et al*., 2012), showed higher phenolic contents which could be the main contributor for its apoptotic effects on cancer cells. Here, we investigated the apoptosis inducing properties of this extract by using annexin V/PI staining of Raji cell line. Results showed there was significant rise in late apoptosis phase with increase in concentration. There was significant upregulation of pro-apoptotic BAX gene while there was significant downregulation of anti-apoptotic BCL-2 and C-MYC gene. The translocation and dysregulation of C-MYC gene on chromosome 8 was the main reason behind its aggressive growth proliferation (Schmitz *et al*., 2014). As our results showed downregulation of C-MYC gene, it therefore signifies the important role of compounds present in this extract which could specifically target this gene and turn it down causing less cell proliferation. Since increase in BAX expression and downregulation of BCL-2 and C-MYC genes are mitochondria-mediated (Huang *et al*., 2017; Yao *et al*., 2017), it is suggested that the intrinsic apoptosis pathway is involved in the anti-proliferative effect of ethyl acetate extract on the Raji cells. Phenols from plant, including *C. excavata* can cause cell arrest, and premature aging, facilitating the anticancer effects (Losuwannarak *et al*., 2018). It was also suggested that phenolic compounds inhibit the stress-activated NF-κβ and AP-1 signaling cascade (Albaayit *et al*., 2020b). Phenolic compounds also enhance the immune system in the destruction of cancer cells, inhibit angiogenesis, and reduce the metastatic potential of tumors (Mohammad & Mahdi, 2017; Atiyah & Kadhum, 2021). Excavatine A, carbazole alkaloid is reported for its anticancer activity against alveolar basal epithelial cell and cervical cancer (Peng *et al*., 2013a). Clausenidin crystals isolated from this plant is reported to induce apoptosis in hepatocellular carcinoma cells (Waziri *et al*., 2016). Nordentatin, murrayanine, and

heptaphylline were reported to have anticancer effects against oral cavity, breast, and small cell lung cancer respectively (Sripisut *et al*., 2012). Ethyl acetate extract were also reported to have anticancer effect on non-small cell lung cancer (Albaayit *et al*., 2021a). It could be possible that these compounds were responsible for anticancer activity in Raji cell line, which further need confirmation by testing pure compound against Raji cell line at different doses.

CONCLUSION

The results collectively provided evidence that the ethyl acetate extract of *C. excavata* leaves is toxic to Burkitt Lymphoma and the anticancer effect of the extract is through the activation of apoptosis-induction with concomitant inhibition of the anti-apoptosis mechanisms and encourage the researchers to isolate and find the compound responsible for its specific anticancer activity and also recommend traditional practitioners to further evaluate its clinical benefits in endemic Burkitt Lymphoma.

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