

***In-vitro* ANTI-CERVICAL CANCER ACTIVITY OF ISOLATE ASE 3.3.3 FROM ETHYL ACETATE EXTRACT OF *Annona squamosa* L. LEAF**

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ABSTRACT

The increasing burden of cervical cancer has urged medicinal scientists to innovate novel anticancer which can be derived from ethnomedicinal plants. Herein, ethyl acetate isolate of *Annona squamosa* L. leaf (ASE 3.3.3) was investigated for its anti-cervical cancer activity in vitro using HeLa cell lines. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) scavenging assay was employed to measure the antioxidant potential of the extract. The results suggest that ASE 3.3.3 was active in scavenging the free radical ($IC_{50}=84.2$ mg/L). The brine shrimp lethality test suggested its potential as an anticancer ($LC_{50}<30$ mg/L). ASE 3.3.3 is potent in suppressing the proliferation of HeLa cell lines with $IC_{50}=1.003$ mg/L. Squalene was detected in the isolate which could facilitate the anticancer activity. Taken altogether, ASE 3.3.3 is worth further exploration as an anti-cervical cancer agent.

Keywords: HeLa, DPPH, BSLT, Sugar Apple.

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INTRODUCTION

Women, especially those with immunocompromised conditions, are at risk of developing cervical cancer.¹ A report from 2021 published an estimation of 604,000 newly emerged cervical cancer cases, where 342,000 related mortalities were reported.² Infection by human papillomavirus (HPV) has been reported as an associated factor for cervical cancer, yet its causative-ness should be combined with others including cigarette smoking, long-term oral contraceptives uptake, and sexually transmitted diseases (*i.e.* chlamydia trachomatis and human immunodeficiency virus, HIV).²⁻⁴ In Aceh, women working as oyster collectors have a greater risk of developing cervical cancer due to long-term exposure to heavy metal-contaminated water.⁵ To reduce the increasing burden of cervical cancer, innovation in its management is urgently needed. A review article has summarized 64 medicinal plants reported to possess anti-cervical cancer activities.⁶ The plant extracts could act as pro-apoptotic agents and angiogenesis antagonists to reduce the development of cancer cells.⁶ A review article has endorsed 199 anti-cancer plant species, obtained from a systematic literature search, while also stressing the potential of novel anticancer drug development.⁷ Among many ethnomedicinal plants, *Annona squamosa* (also known as sweetsop or sugar apple) has been reported to possess multiple health benefits.⁸ Of which are treating tumors, wounds, dysentery, diarrhea, rheumatism, eczema, skin eruption, intoxication, malaria, hypertension, and diabetes mellitus.⁹ In tumor promotion and progression, reactive oxygen species (ROS) has a moderate increase associated with a higher rate of metabolism and gene mutation.¹⁰ Thus, antioxidant properties are important in preventing and curing cancer. The methanolic extracts from *A. squamosa* L. were found to be rich in antioxidants enabling its application as a bio-reduction in Co nanoparticle synthesis.¹¹ Antioxidant potential of *A. squamosa* has been reported for its fruit pulp,¹² seeds,¹³ and leaves.¹⁴ Moreover, *A. squamosa* has been suggested to attenuate the proliferation of various cancer cells.¹⁵ Based on these potentials of using *A. squamosa* as a therapeutic agent against cancers, we performed an investigation of exploring the isolates of its leaf.

EXPERIMENTAL

Materials

A plant specimen of *Annona squamosa* L. was collected from the area around Universitas Syiah Kuala (latitude:5.576020; longitude:95.380189). Chemicals used in this study were ethyl acetate (99%), n-hexane

(99%), methanol (70%), dimethyl sulfoxide, and 2,2-diphenyl-1-picrylhydrazyl (DPPH). All materials were analytical grade and procured from Merck (Selangor, Malaysia), except for those which were not stated.

Extraction and isolation of *A. squamosa* L. leaf

The priorly air-dried fine powder of *A. squamosa* leaf has undergone a maceration at room temperature with methanol 70% (1:1), which this process lasted for 24 h. Partition was performed on the concentrated extract using n-hexane as well as ethyl acetate, sequentially. The ethyl acetate extract was stored at 4°C. The obtained ethyl acetate extract of *A. squamosa* leaf (ASE) was weighed 4.8 g and chromatographed through a gravitational column with silica gel and n-hexane: ethyl acetate (2:8) using gradient elution. In this system, silica gel acted as a stationary phase while the solvent mixture was – a mobile phase. Fractions with the same patterns in the thin layer chromatography (TLC) were combined resulting in 4 major fractions and labeled ASE 1—4, respectively (Fig.-1). Re-chromatography was carried out on the ASE 3 using the same system resulting in another 4 subfractions (ASE 3.1—3.4) and analyzed for their retention patterns through TLC (Figure 1). Re-chromatography was carried out once again using the procedure on ASE 3.3, resulting in 4 sub-subfraction labeled ASE 3.3.1—3.3.4. The sub-subfraction ASE 3.3.3 was observed using TLC and analyzed using GC-MS. The sample was re-crystallized using methanol and n-hexane for further screenings.

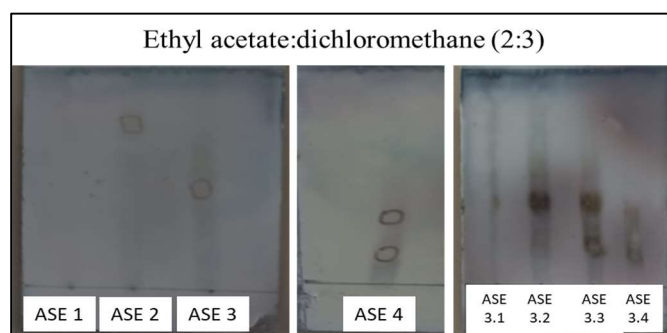


Fig.-1: TLC chromatograms of Fractions (ASE) and Sub-Subfractions of the Ethyl Acetate Extract from the *Annona Squamosa* L. Leaf

In-vitro Screenings

The antioxidant activity was assessed based on ASE 3.3.3's ability in scavenging the free radical DPPH. BLST assay was employed to determine the cytotoxicity of ASE 3.3.3 using *Artemia salina* larvae. Anti-cervical cancer activity of ASE 3.3.3 was evaluated based on its inhibition against HeLa cell lines under MTT assay. Detailed descriptions of the protocols used for the in vitro screenings have been published previously.¹⁶⁻²² Dissolution of ASE 3.3.3 was conducted using dimethyl sulfoxide. All observations were carried out with three times repetition, where the data were presented as averaged values.

RESULTS AND DISCUSSION

Antioxidant Activity

Free radical DPPH scavenging activity of the ASE 3.3.3 has been presented in Table-1. 56.4% inhibition was reached at a concentration of 100 mg/L, with 34.2% and 26.5% inhibition reached at 50 and 25 mg/L, respectively. At the lowest tested concentration, the DPPH inhibition was 9.8%. By using linear regression, the IC_{50} was obtained to be 84.2 mg/L. As a comparison, the positive control ascorbic acid yielded IC_{50} =3.03 mg/L. In our previous study, ASE had around 2 times lower IC_{50} (31.55 mg/L).²³ *A. squamosa* fruit pulp extract was reported to be active in scavenging not only DPPH but also nitric oxide (NO), lipid peroxidation, superoxide anion (O_2^-), as well as hydroxyl radical ($OH^\bullet$).¹² Extracts from *A. squamosa* seeds obtained with various solvents were revealed to contain total phenolic contents of 0.49—0.66 mg/L which is responsible for the DPPH scavenging activity of IC_{50} =66—70 mg/L.¹³ In a study investigating its leaves, *A. squamosa* hydroalcoholic extract was revealed to have IC_{50} =132.96 mg/L in DPPH scavenging with high content of total phenolic and flavonoid contents.¹⁴ Antioxidant activity may suggest its use in ameliorating oxidative stress during cancer cell progression.¹⁰ Moreover, anticancer phytochemicals have been suggested to target pro-inflammatory Nuclear factor- κB .^{24, 25} In addition, extracts with strong antioxidant activities could be exploited for the bio-synthesis of nanoparticles.^{11, 22, 26}

Cytotoxicity

The BSLT-based cytotoxicity profile of ASE 3.3.3 has been presented in Table-1. A mortality of 76% was obtained after the exposure of 6.25 mg/L ASE 3.3.3. A higher mortality rate of 90% was found at 25 as well as 50 mg/L. The highest concentration, 100 mg/L, caused 100% mortality in the *A. salina* larvae. The LC_{50} obtained from this assay was 25.11 mg/L. Fermented polysaccharides isolated from *A. squamosa* had BSLT LC_{50} of 58.44–63.05 mg/L.²⁷ Extracts from any parts of *Annona reticulata* have been studied for their cytotoxic effects which were associated with caspases regulation.²⁸ The BSLT cytotoxic profile could indicate antiproliferative activity.²⁹ Hence, the next investigation was carried out on HeLa cervical cancer cell line through MTT assay.

Table-1: Results of DPPH and BSLT Assays

Concentration (mg/L)	DPPH		BSLT	
	Inhibition (%)	IC_{50} (mg/L)	Mortality (%)	LC_{50} (mg/L)
6.25	9.8	84.2	76.7	25.11
12.5	18.3		73.3	
25	26.5		90	
50	34.2		90	
100	56.4		100	

Note: Ascorbic acid was used as the positive control for DPPH Assay

Median inhibitory concentration, IC_{50} ; median lethal concentration, LC_{50}

Anti-Cervical Cancer Activity

ASE 3.3.3 at all concentrations has succeeded in the inhibition rate of over 90% on the HeLa cervical cancer cell line (Fig.-2). The highest inhibition was resulted from the exposure of ASE 3.3.3 at 250 mg/L, whilst the lowest – at 62.5 mg/L. The IC_{50} value was obtained very low (1.003 mg/L), suggesting potent anticancer activity of ASE 3.3.3 (Fig.-2). Acetogenins isolated from *A. squamosa* L. seeds namely squamotin A-D, muricin O, squamosten B, and annosquatin IV-V have been suggested to exert high activity toward hepatocarcinoma, breast cancer, along with cervical cancer cell lines.³⁰ Selective anticancer activity was found in acetogenins isolated from *A. squamosa* barks against human pancreatic carcinoma cell line—PACA-2.³¹ *A. squamosa* seeds extracted with petroleum ether had relatively high inhibitory effects against breast cancer (MCF- 7) cells, nasopharyngeal cancer (KB) cells, leukemic (K-562) cells, as well as lung cancer (A-549) cells.¹⁵ In a recently published report, *A. squamosa* leaf extract could induce an apoptosis cascade in breast cancer cells.³² The methanolic extract has been reported to have a cytotoxic effect against the HeLa cell line with IC_{50} of 70.9 mg/L.³³ Taken altogether, the ASE 3.3.3 has the potential to be used as a therapy for cervical cancer with an IC_{50} value even higher than the previous study.

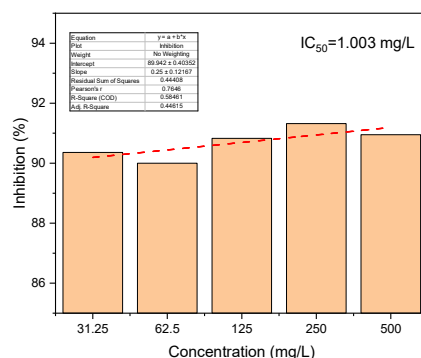


Fig.-2: HeLa Cervical Cancer Line Inhibition by ASE 3.3.3. No Inhibition was Observed on the Control (without ASE 3.3.3)

Phytochemical Profile

The data indicating the phytochemicals contained in ASE 3.3.3 based on GC-MS analysis have been presented in Table-2. 9-Octadecenoic acid (Z)-, methyl ester was a compound with the highest peak area percentage (43.83%), followed by 9,12-Octadecadienoic acid (Z,Z)-, methyl ester with a peak area of 10.08%. Squalene was detected twice at retention times of 46.65 and 46.75 minutes with a total relative

concentration of 7.44%. An intermediate compound for sterol biosynthesis in plants, squalene, has been popularly known for its high antioxidant activity, hence being used for the treatment of oxidative stress-related disorders.³⁴ Squalene has been investigated for its superior properties as a drug carrier for anticancer drug—gemcitabine.³⁵ As compared with our previous research, cyclooctasiloxane, hexadecamethyl, and cycloheptasiloxane, tetradecamethyl- were the predominating compounds with relative content of 27.43% and 22.39%, respectively.²³

Table-2: Phytocompound Profile of ASE 3.3.3 Based on GC-MS

Compound	Retention time (min)	Relative content (%)	Similarity Index (%)
Cyclohexane, 1,4-dimethyl-	4.08	0.40	75.3
Cyclopentane, 1-ethyl-3-methyl-,trans	4.23	0.47	78.3
Cyclopentane, 1-ethyl-3-methyl-,trans	4.29	0.47	78.4
Hexadecanoic acid, methyl ester	25.14	6.18	94.6
Hexadecanoic acid, methyl ester	29.39	6.93	83.5
Hexadecanoic acid, methyl ester	31.97	4.31	81.3
4-Trifluoroacetoxypentadecane	32.42	1.37	81.3
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	32.61	10.08	93.1
9-Octadecenoic acid (Z)-, methyl ester	32.84	43.83	94.7
Methyl stearate	33.67	2.16	82.2
Trans-13-Octadecenoic acid, methyl ester	34.15	1.77	87.2
Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]methyl]-, methyl ester	35.17	0.57	82.3
Trans-13-Octadecenoic acid, methyl ester	35.27	2.95	88.2
Octadecane, 6-methyl-	35.64	1.87	80.5
Tributyl acetyl citrate	37.12	1.04	74.1
1-Hexadecanol, 2-methyl-	37.92	1.17	74.0
2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	38.21	1.04	75.4
Tetradecane, 2,6,10-trimethyl-	39.81	0.91	78.7
Estra-1,3,5(10)-trien-17 β -ol	41.66	0.43	72.9
Phthalic acid, di(2-propylpentyl) ester	42.40	2.13	82.1
Tetradecane, 2,6,10-trimethyl-	43.22	0.49	73.5
Octocrylene	44.41	0.92	74.9
Squalene	46.65	3.93	84.5
Squalene	46.75	3.51	81.9
Ethyl iso-allocholate	50.55	1.06	68.4

CONCLUSION

ASE 3.3.3 has potent cytotoxic activity against *A. salina* larvae with $LC_{50} < 30$ mg/L. The anticancer activity against HeLa cervical cancer cell line was observed to be strong with IC_{50} of 1.003 mg/L. Squalene and anti-free radical activity of the ASE 3.3.3 might mediate the anticancer potential. Investigation on anticancer potential of *A. squamosa* leaf worth further investigation.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved

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