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In Vitro Anticancer Activity on 3T3, PC3, and HeLa Cell Line and GC-MS Assay of Oil Fractions of *Alstonia Scholaris* Flower Obtained by Column Chromatography

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Abstract: Several plant-derived compounds are currently successfully employed in cancer treatment. Various studies have demonstrated the anticancer or cytotoxic potential of different extracts of *Alstonia scholaris* at the highest doses. This study aimed to analyze the anticancer potential of oil fractions obtained from *Alstonia scholaris* flower isolation. The oil fractions were characterized using gas chromatography–mass spectrometry (GC-MS) assay. The (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) (MTT) colorimetric assay was used for 3T3, PC3, and HeLa cell line cytotoxic potential of oil fractions. The results showed no cytotoxic activity of oil fractions, i.e., Oil I and Oil II were at a minimum dose of 30 µg/ml. However, the GC-MS assay depicted saturated (palmitic acid, ethyl ester, caproic acid, stearic acid, ethyl ester, octacosanoic acid, etc.), unsaturated (oleic acid, 3-(octadecyloxy)propyl ester, ethyl oleate and some other hydrocarbons such as betulin, ethyl iso-allocholate, lupenyl acetate, phthalic anhydride, phytol, etc. Thus, the reported chemical constituents showed no significant anticancer effect in the scientific literature. This research suggested that *Alstonia* has no potential as an anticancer agent. The findings of this study provide insight into maintaining the optimum data for future aspects of plant-derived phytoconstituents with potential cytotoxic effects on 3T3, PC3, and HeLa cell lines.

Keywords: *Alstonia scholaris*, GC-MS assay, MTT colorimetric assay, cytotoxicity.

對3吨3、个人电脑3和海拉細胞系的體外抗癌活性以及通過柱色譜法獲得的洋槐花的油組分的氣相色譜-質譜法測定

摘要：幾種植物來源的化合物目前已成功用於癌症治療。各種研究已經證明了最高劑量的洋槐不同提取物的抗癌或細胞毒性潛力。本研究旨在分析從洋槐花分離物中提取的油分的抗癌潛力。使用氣相色譜質譜測定法對油餾分進行表徵。(3-[4,5-二甲基噻唑-2-基]-2,

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5二苯基溴化四唑比色法用於3吨3、个人电脑3和海拉細胞系油餾分的細胞毒性潛力。結果顯示油組分沒有細胞毒活性，即油I和油II的最小劑量為30微克/毫升。然而，色譜質譜法分析描繪了飽和（棕櫚酸、乙酯、己酸、硬脂酸、乙酯、二十八酸等）、不飽和（油酸、3（十八烷氧基）丙酯、油酸乙酯和其他一些物質）烴類如樺木腦、異異膽酸乙酯、醋酸羽苯酯、鄰苯二甲酸酐、葉綠醇等。因此，科學文獻中報導的化學成分沒有顯著的抗癌作用。這項研究表明，阿爾斯托尼亞沒有作為抗癌劑的潛力。這項研究的結果提供了關於維持植物來源植物成分未來方面的最佳數據的見解，這些植物成分對3吨3、个人电脑3和海拉細胞系具有潜在的細胞毒性作用。

关键词：洋槐，质谱联用仪测定，MTT比色测定，细胞毒性。

1. Introduction

Diseases like carcinoma (cancer) are the leading causes of death worldwide. Malignancy rates in Asia are lower than in Western countries, but they are increasing as the rural population drifts to the urban. Urban areas increase various factors such as health outcomes and changing lifestyles [1, 2]. The MTT/MTS in vitro tumorigenesis assay is among the most widely used methods for investigating the experimental anticancer potential of both chemical derivatives and biological source and natural product isolates. The colorimetric-based test is highly reliable and may be used on an extensive range of cell lines such as 3T3, HepG2, PC3, A-549, and HeLa Cell Line, etc. [3-6]. The previously used chemotherapeutic medications were relatively harmful not just to cancer cells, but also to healthy cells in the body area where the malignancy had formed. The quest for new anticancer treatments is currently being done in both terrestrial plants and marine habitats [7–12]. An estimated 60% of the medications already used to treat cancer are derived from natural sources. Camptotheca alkaloids, Podophyllum lignans Vinca alkaloids, and Taxus diterpenes, are some of them. Meanwhile, 13 novel plant-derived drugs are in phase I or II clinical trials, and three are in phase III [13–15].

The genus Alstonia (Apocynaceae) is widespread in Africa and Asia in tropical climates [16, 17]. A. scholaris has long been used to treat human illnesses in numerous ethnomedicine. It's high in alkaloids, polyphenols, and phenolics, as per mythology. Antibacterial, antiamoebic, anthelmintic, antiprotozoal, hepatoprotective, antiproliferative, anti-cancer, anti-asthmatic, superoxide radicals scavenging, antioxidant, anesthetic, anti-inflammatory, anti-ulcer, anti-fertility, and collagen synthesis activities have all been documented. [18–21]. This study focused on the anticancer potential of oil fractions extracted from Alstonia scholaris Flower against 3T3, PC3, and HELA Cell Line. 3T3 (fibroblast line) [22], PC3 (prostate tumours) are adenocarcinomas with glandular development and androgen receptor (AR) luminal differentiation markers and prostate-specific antigen (PSA) [23, 24] and HELA Cell Line (cervix cancer) one of the most recurrent forms of malignancy among feminine in developing countries [25].

2. Materials and Methods

2.1. Collection of Plant Material

Plant material (*Alstonia scholaris*) flower was collected from the field of the HEJ Research Institute of the Chemical and Biological Center (ICCBS). A taxonomist from the University of Karachi's Department of Botany identified the plant. A voucher sample of the flowers of *Alstonia scholaris* has been preserved. Voucher number - # G.H. 94482. This reference sample has been deposited in the herbarium.

2.2. Extraction and Fractionation

Plant extracts (ASF-EtA) were prepared in the following manner. The first stage was solvent extraction using ultrasonic waves. The flower bud of A. scholaris was extracted with 95 percent ethanol (20 L 3 times, AR grade, Thailand) at room temperature for 30 min with ultrasonic assistance. The *A. scholaris* ethanolic extract (ASF-EtOH) was kept refrigerated until it was used. Ethyl acetate was used to achieve liquid-liquid partitioning of ASF-EtOH. It was labeled ASF-EtA (ethyl acetate layer) and kept in the fridge until required. Isolation of ASF- EtA (ethyl acetate layer) was performed.

2.3. Isolation by Column Chromatography

ASF-EtA is isolated by column chromatography, eluent using hexane, dichloromethane (DCM), DCM, and DCM: Methanol (CH_3OH) mixtures (CC, silica gel, HEX, DCM, and CH_3OH in order of increasing polarity). 11 fractions designated as F1—F11, respectively. Fraction F1 and Fraction F2 were

obtained from hexane (100%) and hexane: DCM (9:1) eluting two oil fractions, respectively, were named Oil I and Oil II. The Oil I was pale yellow oily appearance, while Oil II appeared ghee-like.

2.4. Identification

The oil fractions eluted after isolation would be subjected to GC-MS analysis for the identification of the present compounds [27, 28].

2.4.1. GC-MS Analysis

A 7890B gas chromatograph with a 7693 autosampler and a 5977B mass-selective detector was used for the GC–MS study. Agilent's HP-5MS capillary column, 30 m 0.25 mm 0.25 mm, was employed (All – Agilent Santa Clara, CA, USA). At 1.5 mL/min, helium was employed as the carrier gas. Pulsed pressure was used to operate the split–splitless injector. After 1.5 min, the purging valve will open. The sample volume was set at 5 liters. The GC system conditions were: injector T = 290°C; transfer line T = 280°C; oven temperature program: 50°C (1.5 min)–30°C/min–180°C–20°C/min–280°C (20 min) [28]. The MS detector (quadrupole) was employed in the +EI mode.

2.4.2. Data Interpretation

For Windows-based applications, several virtual machines with 12–20 CPUs (Intel Xeon E312xx or Intel Core i7 9xx, Santa Clara, CA, USA) and 16–32 GB RAM were employed. A 64-bit Windows 8.1 Enterprise OS was used to run all of the software.

2.5. Anticancer Activity of 3T3, PC3, and HeLa Cell Line

The standard MTT (3-[4, 5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) colorimetric test was used to assess the cytotoxic activity of substances in 96-well flat-bottomed microplates [37]. 3T3 (mouse fibroblast) cells, PC3 (prostate cancer) cells, and HeLa (cervical cancer) cells were cultivated in 75 cm^2 flasks in Dulbecco's Modified Eagle Medium, supplemented with 5% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 g/ml streptomycin, and incubated at 37°C in a 5% CO2 incubator. The stepwise methodology is depicted in Fig. 1. Cytotoxicity was measured as the concentration that caused 50% growth inhibition in 3T3, PC3, and HeLa cells (IC50). The percentage inhibition was calculated using the following formula:

% inhibition = 100 – (mean of O.D of test compound – mean of O.D of negative control)/(mean of O.D of positive control – mean of O.D of negative control) × 100

Soft-Max Pro software was used to process the results (percent inhibition) (Molecular Device, USA).

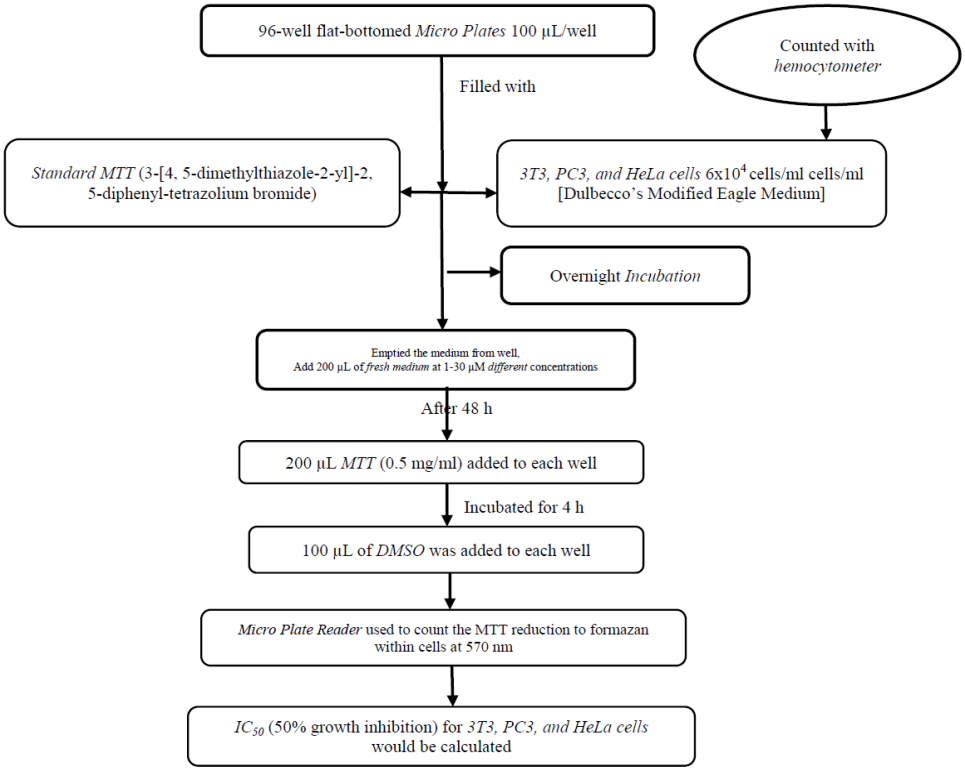

Fig. 1 Methodology of MTT colorimetric assay for 3T3, PC3, and HeLa cell culture

3. Results

3.1. GC-MS Analysis

The components found in Oil I and Oil II, as well as their retention time, molecular weight, and their reversed match factor, are presented in Tables 1 and 2 and depicted in Figs. 2 and 3.

Table 1 Components of the Oil I obtained from *Alstonia scholaris* flower analyzed by GC-MS

No.	Compounds	RT (Retention time), min	RMFs (Reversed Match Factor)	MW (Molecular weight)
1	Heptane, 3-methyl-1	5.70 min	868	114
2	Hexadecanal, 2-methyl-	5.83	778	254
3	β-Methylcrotonaldehyde	5.97	929	84
4	2-Ethylhexene	6.10	929	112
5	Cyclopentanol, 2-methyl-, trans-	6.35	817	100
6	Dodecadien-2-one, 6,10-dimethyl-, (E,E))-	7.85	846	208
7	Heptanone	8.62	867	114
8	Hexen-1-ol, acetate, (Z)-	9.37	818	142
9	Furanone, 5,5-dimethyl-	10.36	829	112
10	Heptenal, (E)-	10.46	814	112
11	Betulin	77.32	792	442
12	Ethyl iso-allocholate	80.28	768	436
13	Lupenyl acetate	79.25	768	468
14	12-Oleanen-3-yl acetate, (3α)-	74.07	902	468
15	24-Methylenecycloartan-3-one	73.39	857	438
16	13,27-Cycloursan-3-one	71.50	823	424
17	Oleic acid, 3-(octadecyloxy) propyl ester	68.09	781	592
18	Ethyl iso-allocholate	67.50	769	436
19	Vitamin E	65.62	751	430
20	cis-13-Eicosenoic acid	63.46	776	310
21	Octadecanoic acid, phenyl ester	60.10	815	360
22	Ethyl tetracosanoate	58.92	832	396
23	Terephthalic acid, di (2-ethylhexyl) ester	58.46	878	390
24	Phthalic acid, 6-ethyloct-3-yl 2-ethylhexyl ester	58.17	855	418
25	Phenyl palmitate	57.66	781	332
26	1,2-Benzenedicarboxylic acid, diisooctyl ester	56.70	963	390
27	Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	55.75	971	278
28	Palmitic acid, ethyl ester	52.92	825	284
29	Heptacosane	50.60	836	380
30	Stearic acid, ethyl ester	46.83	812	312
31	Ethyl Oleate	45.32	879	310
32	Ethyl 9.cis.,11.trans.-octadecadienoate	44.93	843	308
33	Z-(13,14-Epoxy)tetradec-11-en-1-ol acetate	39.24	813	268
34	Aspidospermidin-17-ol, 1-acetyl-19,21-epoxy-15,16-dimethoxy-	30.82	788	414
35	Cholestan-3-ol, 2-methylene-, (3β,5α)-	28.87	809	400
36	Ar-tumerone	25.17	857	216
37	Nonanoic acid, 9-oxo-, ethyl ester	22.19	877	200
38	Octanal	11.62	922	128
39	2(5H)-Furanone, 5-ethyl-	12.49	879	112
40	Epoxy-linalooloxide	18.02	774	186
41	Phthalic anhydride	18.84	902	148

Table 2 Components of the Oil II obtained from *the Alstonia scholaris* flower analyzed by GC-MS

No.	Compounds	RT (Retention time), min	RMFs (Reversed Match Factor)	MW (Molecular weight)
1	2-Hexanone	6.13	922	100
2	3-Hexanol	6.27	801	102
3	Cyclopentanol, 2-methyl-, trans-	6.40	813	100
4	2-heptenal, (E)-	10.47	902	112
5	Octanal	11.63	950	128
6	2(3H)-Furanone, 5-ethenyldihydro-5-methyl-	12.63	878	126
7	Caproic acid anhydride	13.35	863	214
8	Nonanal	14.07	885	142
9	Ethyl caprylate	16.09	851	172
10	Undecenal	19.61	909	168
11	Dodecane, 2,6,10-trimethyl-	19.79	929	212
12	Tetradecane	20.22	938	198
13	1-Hexadecene	23.75	934	224
14	Palmitic acid, ethyl ester	78.30	782	284
15	Lupenyl acetate	75.62	772	468
16	Stigmasta-3,5-dien-7-one	72.71	851	410
17	Pentatriacontane	68.15	912	492
18	Hentriacontane	63.86	918	436
19	Ethyl iso-allocholate	62.24	802	436
20	Octacosanoic acid	61.76	751	424

Continuation of Table 2				
21	Tetratetracontane	60.52	921	618
22	Ethyl tetracosanoate	59.02	821	396
23	Octacosane	57.97	919	394
24	Docosanoic acid, ethyl ester	56.42	847	368
25	Mono (2-ethylhexyl) phthalate	55.73	909	278
26	Hexacosane	55.07	938	366
27	cis-13-Eicosenoic acid	52.73	855	310
28	cis-11-Eicosenoic acid, methyl ester	52.41	849	324
29	Stearic acid, ethyl ester	47.34	842	312
30	Ethyl Oleate	46.56	913	310
31	Oleic Acid	43.71	781	282
32	Phytol	41.53	917	296
33	Heptadecanoic acid, ethyl ester	40.19	909	298
34	Palmitic acid, ethyl ester	35.53	914	284
35	2(3H)-Furanone, dihydro-4,4-dimethyl-	10.79	782	114
36	Caproic acid	11.44	865	116
37	Farnesan	12.11	868	212
38	Levulinic acid	13.10	842	
39	Aspidospermidin-17-ol, 1-acetyl-19,21-epoxy-15,16-dimethoxy-	23.09	795	414
40	Estradiol, 3-deoxy-	36.19	827	256
41	Geranyl isovalerate	47.69	782	238
42	9-Tricosene, (Z)-	49.89	835	322
43	Erucic acid	50.29	832	338
44	Cerotic acid methyl ester	60.75	760	410

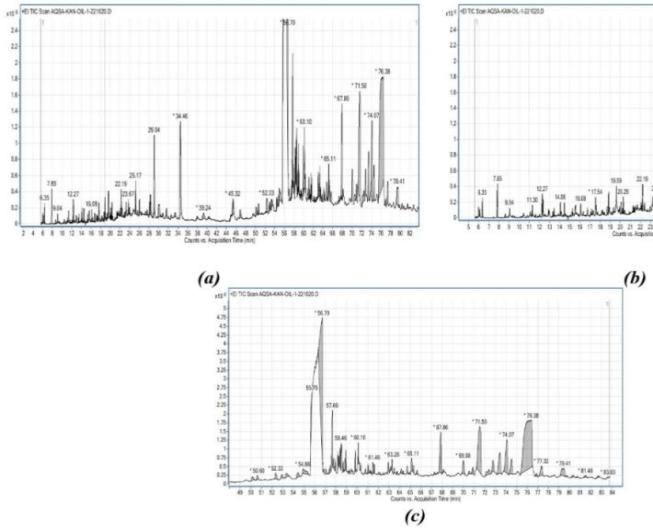

Fig. 2 Chromatogram of oil I fraction obtained by GC-MS with +EI source (a), (b) and (c)

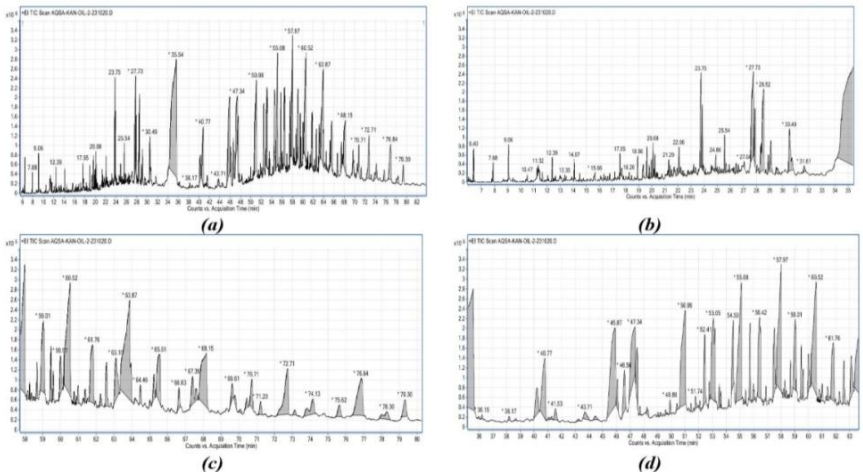

Fig. 3 Chromatogram of oil II fraction obtained by GC-MS with +EI source (a), (b), (c) and (d)

3.2. Anticancer Activity

The cytotoxic potential of *Alstonia scholaris* 3T3, PC3, and HeLa Cell Line with their % inhibition and IC_{50} is presented in Tables 3, 4, and 5, respectively.

Table 3 IC_{50} of oil fractions of *A. scholaris* for 3T3 cell line

No.	Sample	Conc. µg/ml	% Inhibition/stimulation	$IC_{50} \pm SD$
1.	Oil I	30	2.81	Inactive
2.	Oil II	30	5.9	Inactive
3.	Standard (Doxorubicin)	30	96.2	0.1 ± 0.02

Table 4 IC_{50} of oil fractions of *A. scholaris* for PC3 cell line

No.	Sample	Conc. µg/ml	% Inhibition/stimulation	$IC_{50} \pm SD$
1.	Oil I	30	12.9	Inactive
2.	Oil II	30	11.0	Inactive
3.	Standard (Doxorubicin)	30	89.9	1.9 ± 0.4

Table 5 IC_{50} of oil fractions of *A. scholaris* for HeLa cell line

No.	Sample	Conc µg/ml	% Inhibition/stimulation	$IC_{50} \pm SD$
1.	Oil I	30	11.7	Inactive
2.	Oil II	30	6.2	Inactive
3.	Standard (Doxorubicin)	30	101.2	0.9 ± 0.14

4. Discussion

GC-MS Assay of both Oil I and Oil II depicted saturated (palmitic acid, ethyl ester, caproic acid, stearic acid, ethyl ester, octacosanoic acid, etc.), unsaturated (oleic acid, 3-(octadecyloxy) propyl ester, ethyl oleate and some other hydrocarbons such as Betulin, Ethyl iso-allocholate, Lupenyl acetate, Phthalic anhydride, phytol, details of the constituents already presented in Tables 1 and 2, respectively.

The findings of this investigation showed that the oil fractions of ethyl acetate extract of *Alstonia scholaris* do not have anticancer potential at the concentration (30 µg/ml) examined for 3T3, PC3, and HeLa Cell Line (Tables 3, 4 and 5) compared with standard doxorubicin. This has been proved by GC-MS analysis since it does not depict any compound that has cytotoxic evidence in the literature. However, based on literature evidence, various extracts of *Alstonia scholaris* showed a synergistic or moderate cytotoxic activity. As a result, MTT (3-[4, 5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) colorimetric assays for 3T3, PC3, and HeLa cell lines were performed on freshly extracted oil fractions that had not been previously reported.

The prevalence of anticancer activity has been reported in various articles, such as the chemomodulatory effect of *Alstonia scholaris* extract (ASE) in Ehrlich ascites carcinoma-bearing mice when combined with berberine hydrochloride (BCL) (topoisomerase inhibitor). The anticancer effect was best when 180 mg/kg of ASE was combined with 8 mg/kg of BCL [29]. Furthermore, [30] found that a dosage of 25 g/mL of *Alstonia scholaris* ethanolic extract showed a time-dependent rise in antineoplastic activity in HeLa cells treated for 24 h.

[31] showed that *Alstonia scholaris* (bark extract) has chemopreventive efficacy in DMBA-induced skin carcinogenesis in Swiss albino mice. ASE given experimental groups had significantly higher levels of reduced glutathione, catalase, and superoxide dismutase but lower levels of lipid peroxidation than the carcinogen-treated control. In the monsoon, winter, and summer, the seasonal variation of various fractions of ASE was also obtained. When HeLa cells were exposed to different extracts prepared from stem bark collected during the monsoon, winter, and summer, the cell-killing effect of ASE increased dose-dependently, with the extract prepared from the summer collections having the maximum cell-killing effect [32]. AgNPs mediated by nanoparticles demonstrated better efficacy against HepG2 cells (27.01 g/ml) and PC3 cells (32.15 g/ml) [33].

The recent study [34] found that the methanolic extract inhibited lung, colorectal and prostate cancer cells by 82–90 percent at 50 g/mL and 73–78 percent at 10 g/mL. However, against prostate (PC-3) and lung (A-549) cancer cells, 1 g/mL of the extract only inhibited proliferation by 52 percent. [35] investigated the effect of *Alstonia scholaris* on HSI human sarcoma and benzo(a)pyrene-induced forestomach cancer (Mice). [36] investigated the cytotoxicity of three distinct Alstonia scholaris bark extracts: ethanol, n-hexane, and chloroform. Thus, with an IC50 of 50 (125.06 g/mL), chloroform fraction was the most hazardous to HeLa cells, followed by the ethanol fraction (200.07 g/mL), and n-hexane fractions (238.47 g/mL). Furthermore, no research has been conducted on 3T3 cells, and only a small amount of work has been done on PC3 cells against the *Alstonia scholaris* plant.

5. Conclusion

The study indicates that the flower of *A. scholaris* specifically oil fractions at a dose of 30 µg/ml showed no cytotoxic potential. However, the preceding evidence shows that various extracts of *A. scholaris* moderately or synergistically effective along with another extract of plants such as berberine hydrochloride [29] and either at higher dose. As a result, the findings confirmed that Alstonia flowers have limited application for anticancer agent. Thus, it is recommended to investigate the anticancer potential of *A. scholaris* achieved by Bio Guided assay or specific targeted isolation such as alkaloid, flavonoids and polyphenolic compounds.

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