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The comparative antimicrobial and anticancer of chemical extract from *in vitro* and *in vivo Peperomia pellucida* plantlet

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ABSTRACT

Plant tissue culture is a promising technique to produce valuable metabolites for treatments against various diseases. In this study, we assessed the chemical compound and medical properties of *Peperomia pellucida* L. Kunth with special attention toward antibacterial and anticancer activities. The detection of major compounds such as apiol, phytol, phenol, 9-octadecenoic acid (z), and caryophyllene from methanol and ethanol extracts of both *in vivo* and *in vitro* grown plants was done through gas chromatography-mass spectrometry analysis. All plant extracts were found to be effective against *Staphylococcus aureus, Salmonella typhi, Escherichia coli,* and *Pseudomonas aeruginosa.* Among these, the methanol extracts of *in vitro* grown plants showed the highest inhibition zone (12.5 mm) against *S. aureus* followed by ethanol extracts of *in vitro* grown plants (12 mm) against *S. typhi* and *P. aeruginosa.* Upon testing the extracts on human breast adenocarcinoma cell line (MCF-7) and human lung adenocarcinoma cell lines (A549), ethanol extracts of *in vitro* grown plants were inhibitive against the proliferation of MCF-7 cells while methanol extracts of *in vitro* grown plants inhibited the proliferation of A549. The reproducible protocol in this study has the potential for the establishment of selected and standardized plants suitable for the exploitation in various industries such as pharmaceutical industries, agrochemical industries, and food industries.

1. INTRODUCTION

Plants have been a very important source of medicine for thousands of years. Lately, the interest in utilizing herbal drugs for treatments has increased as herbs belong to natural sources and is generally considered harmless. In many developing countries, the demand and popularity of herbal medicines from plants or animals as an alternative to modern medicine are increasing day by day as they are readily accessible to rural folk in addition to having an advantage in cost and safety factors. In herbal medicine, all parts of the plant such as the seeds, stems, flowers, leaves, roots, barks, as well as their respective extracts are explored for medicinal purposes [1-3].

Conventionally, many people forage herbal plants through open spaces and forests. This practice may damage the ecological site if not performed correctly. Alternatively, many of the medicinal plants can and have been commercially propagated through plant cell and tissue culture [4]. To this end, plant tissue culture techniques offer an alternative potential sources of producing valuable bioactive compounds through manipulations of the culture environment and plant growth hormones [5,6].

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The most researched natural products are the phenolic compounds, studied primarily for their bioactive properties, which have immense structural and chemical diversity and that are produced naturally by plants. Through plant tissue culture techniques, generating and growing *in vitro* cultures in a controlled environment could challenge the plant biochemical systems to produce novel metabolites. Due to this reason, choosing phenolic compounds from *in vitro* cultures will provide the required stress conditions for their production and increase the secretion of secondary metabolites through plant tissue culture activities [7].

Peperomia pellucida L. Kunth. is a heart-shaped leaf plant that belongs to the Piperaceae family. This plant can be found in various parts of the world, throughout tropical and subtropical regions. It generally grows in clumps and thrives in loose humid soils [8,9]. This family comprises more than 12 genera and this flowering plant is one of the most widely distributed families [10]. *P. pellucida* is a common fleshy tropical annual shallow-rooted herb. It typically grows to a height of about 15–45 cm [11].

P. pellucida has been highly regarded for its medicinal value and is used as a traditional medicine worldwide. The whole plant parts of *P. pellucida* are used to treat different diseases [12]. Many believe that the whole plant contains the therapeutic value to treat many diseases such as fever, headache, gout, fatigue, colic, acne, abscesses, skin diseases, and eye inflammation [13-15]. Studies have reported that *P. pellucida* has additional biological properties

such as antibacterial [16,17], antifungal [18], antioxidant [19], anti-amoebic [20], anti-ulcer, anti-neoplastic, and anti-inflammatory properties [11,21]. Studies have revealed hundreds of chemical compounds from this herb [22]. Many chemical compounds were isolated from the essential oil of the Piperaceae family. It is also known that *P. pellucida* contains flavonoids, steroids, saponin, tannin, triterpenoid, and phytol [9,23,24].

Despite all the attractions this plant receives, there are inadequate reports on scientific investigation and plant tissue culture to determine the therapeutic potential and medicinal property of this plant as compared to other medicinal plants. Therefore, the present study reports the activities of bioactive compounds from *P. pellucida* obtained through naturally grown plantlets (*in vivo*) and plantlets generated through tissue culture (*in vitro*). We identified the compounds through gas chromatography-mass spectrometry (GC-MS) and tested for antimicrobial and anticancer properties.

2. MATERIALS AND METHODS

2.1. Plant Material

P. pellucida plants were collected and identified from a population growing at the Institute of Biological Science's garden at the University of Malaya (UM) as well as from various areas around the UM. The plants were then maintained at the Institute of Biological Sciences garden at the Faculty of Science, UM, Kuala Lumpur, Malaysia. The voucher specimens were deposited in the UM Herbarium (KLU), Kuala Lumpur, Malaysia, for identification. The intact plants without the roots were collected in the year 2017 from the ISB garden at the UM, and the *in vitro* regenerated plantlets of *P. pellucida* grown on MS medium were used as a comparison.

2.2. *In vitro* Regeneration of *P. pellucida*

Above ground, vegetative shoots of *P. pellucida* were collected from the ISB garden, UM. The plants were first washed under running tap water for 30 min. Then, the plants were soaked and washed with three drops of teepol with sterile distilled water for 2 min and rinsed thoroughly 5 times with sterile distilled water. Next, the plants' surfaces were sterilized with 50% sterile sodium hypochlorite for 2 min, followed by a 50% disinfectant solution (Dettol, Reckitt Benckiser) for 10 min. This was followed by rinsing the plant 5 times with sterile distilled water. Finally, the *P. pellucida* plants were treated with an antifungal, 0.01% (w/v) carbendazim diluted in 70% (v/v) ethanol for 30 s and rinsed 5 times with sterile distilled water. Then, the sterilized internodes were tapped dry using sterile tissue paper and intersected small pieces (10 mm) were cultured onto the MS basal medium [25] comprised of 30 g/L sucrose (System), 4.4 g/L MS powder (Duchefa Biochemie), 3.3 g/L gelrite (Duchefa Biochemie), and 0.01% carbendazim, adjusted to pH 5.7 before being autoclaved at 121°C for 20 min. Cultures were maintained at 25 ± 2°C, 16 h/8 h (light/ dark) photoperiod with 25 µmol m-2s-1 of light intensity.

2.3. Extraction of Plant Material

The harvested *in vivo* or *in vitro* generated plants were separately washed clean from soil or media. The clean, intact plants were freeze-dried, after which the dried samples were ground to a uniform powder and sorted out according to size using 250 µm sieves. In brief, dry powdered plant material (10 g) was weighed out and filled into a cellulose extraction thimble (Whatman), then inserted into a Soxhlet apparatus and extracted with 300 mL ethanol (R&M Chemicals) for 48 h or methanol (R&M Chemicals). Separately, all the extracts were then concentrated using a rotary evaporator under reduced pressure at 20°C, relatively low temperature and kept in an airtight container and preserved at −20°C until further use.

2.4. Analysis of Plant Extracts Chemical Composition

The phytochemical components of the *P. pellucida* extractions were identified using Agilent GC-MS. GC-MS was carried out on a 7890A GC/MS Agilent 5975, Shimadzu QP2010 ULTRA GCMS equipped with RTX-5 column. Sample injected: 2 µl. The oven column temperature was pre-set at 60°C, with an initial hold time of 2 min, to 280°C at the rate of 5°C/min with a final hold time of 9 min. The injector temperature was set to 240°C. The total GC running time was 47 min. Interpretation of GC-MS was conducted, using the National Institute Standard and Technology Version – Year 2011 library. The name, retention time, molecular weight, and molecular structure of the compounds of the test substances were ascertained using the database.

2.5. Antimicrobial Activity

All the isolated bacteria were provided by the Microbiology Laboratory at the Institute of Biological Sciences, Faculty of Science, UM. The microorganisms used in this study are as follows: *Salmonella typhi, Pseudomonas aeruginosa, Escherichia coli,* and *Staphylococcus aureus.* All the bacteria were cultured in Nutrient broth (Difco) and incubated for 24 h at 37°C. The disk diffusion method by Bauer *et al*. [26] was used with slight modification. A crude extract of *in vivo* and *in vitro* grown plants was diluted in distilled water to a concentration of 10 mg/mL. 10 mg/mL of chloramphenicol (Duchefa Biochemie) was also prepared as a positive control for bactericidal activity. Sterile blank disks sized 6 mm in diameter (Oxoid) were impregnated with 20 µL of each diluted extract and chloramphenicol. The impregnated disks were allowed to fully dry in the laminar flow before the application on the bacterial lawn. The 10^{-9} serially diluted bacteria were aseptically swabbed on the surface of sterile 4% nutrient agar (Difco) prepared plates using sterile cotton swabs. Using sterile forceps, aseptically, the prepared antibiotic disks were placed over the bacteria seeded agar plates. The disks were separated sufficiently from each other to avoid overlapping of inhibition zones. The plates were then incubated at 37°C for 24 h. Finally, antibacterial activity was expressed as the mean zone inhibition diameter (mm) produced by the extract.

2.6. Anticancer Activity

To test the anticancer properties, both the methanol-extracted and ethanol-extracted compounds from *in vitro* and *in vivo* conditions were prepared in seven different concentrations, serially diluted with a dilution factor of two. As such, the final concentrations of each condition were as follows: 30 µg/mL, 15 µg/mL, 7.5 µg/mL, 3.75 µg/mL, 1.875 µg/mL, 0.9375 µg/mL, and 0.469 µg/mL.

The cell culture works were performed as described previously with a slight modification [27]. The anticancer properties of extracted compounds were tested on two different types of commercially available cancer cell lines, namely adenocarcinomic human alveolar basal epithelial cells (A549, ATCC, USA) and human breast adenocarcinoma cell lines (MCF-7, ATCC, USA). They were seeded at 1000 cells/100 uL/well in a 96 well plate format (Corning, USA). The anticancer effects of the aforementioned compounds were evaluated by utilizing a viability assay using 10% Alamar Blue reagent (Thermo Fisher Scientific, MA, USA). Cell viability was analyzed through absorbance at 450 nm with a reference wavelength set at 590 nm by the Tecan microplate reader (Infinite 200 PRO Tecan, Life Sciences,

Switzerland). From the viability profile, the respective half-maximal inhibitory concentration (IC_{50}) was calculated from the general equation y =mx + c to indicate the toxicity level of each compound. All assays were performed in triplicates to ensure consistency.

2.7. Statistical Analysis

For the antimicrobial study, data were collected and statistically analyzed using Duncan's Multiple Range Test. It should be noted that the mean with different letters in the same column indicates a significant difference with $P < 0.05$. For the anticancer study, data were collected and analyzed using two-way ANOVA with Tukey's post-hoc.

3. RESULTS AND DISCUSSION

3.1. Analysis of Plant Extracts Chemical Composition

In the recent study, GC-MS analysis had identified the presence of phytochemical compounds in both ethanol and methanol extracts of *in vivo* and *in vitro* grown *P. pellucida*. The ethanol extract of the *in vitro* grown *P. pellucida* yielded 24 constituents [Table 1], while the ethanol extract of the *in vivo* grown *P. pellucida* only yielded 23 constituents [Table 2]. For the methanol extract of *in vitro* grown, *P. pellucida* gave rise to 13 constituents from the chromatogram of the extract [Table 3], while the methanol extract of *in vivo* grown *P. pellucida* yielded a total of 15 constituents [Table 4]. At present, no studies are reporting the antibacterial activity using extracts from tissue cultured (*in vitro*) *P. pellucida* plants. Our study is the first ever to report the constituents of phytochemical compounds from tissue cultured (*in vitro*) *P. pellucida* plants. Since methanol and ethanol are efficient in traversing cell walls and seed pods which have more unipolar character and would cause cells to release their chemical compounds, thus they are chosen for extraction. The number of chemical compounds extracted varied depending on the usage of different materials and solvents. The chemical constituents obtained showed that solvent polarity influenced the outcome of the extraction process as the solubility of components depends on the number, type, and linkage position of the compound [28,29].

The ethanol extracts from *in vivo* and *in vitro* grown plants showed a greater number of chemical compounds as compared to methanol extracts and these identified compounds possessed biological properties. This shows that *P. pellucida* contains more non-polar compounds that are more readily extracted with ethanol compared to methanol. A comparison was done between the extracts and it was found that certain compounds that are identified in ethanol and methanol extracts of *in vitro* and *in vivo* grown plants are nearly similar. For instance, in this study, apiol was found in all the extracts. However, the apiol content was found to be higher in ethanol extracts of *in vitro* and *in vivo* grown plants compared to methanol extracts. Previous studies have reported the presence of apiol in the ethanolic extracts [29] and in the essential oil of *in vivo* grown plants [9,18,30,31]. Apiol was reported to have antifungal activities against *Trichophyton mentagrophytes* [21,32] and interestingly, it is also used as birth control as well as to treat menstrual disorder [33].

Table 1: GC-MS of ethanol extract analysis revealed the presence of phytochemical components in *in vitro* grown *Peperomia pellucida.*

No	RT	Compound	Molecular formula	Area	MW
1	8.004	Heptadecane, 2,6,10,15-tetramethyl-	$C_{21}H_{44}$	14930	296
2	8.299	Phenol, 3,5-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	10933	206
3	8.573	Dodecane, 1-iodo-	$C_{12}H_{25}I$	15065	296
4	9.819	Apiol	$C_{12}H_{14}O_4$	13615	222
5	10.056	1,1,1,3,5,7,7,7-Octamethyl-3,5-bis(trimethylsiloxy)tetrasiloxane	$C_{14}H_{42}O_5Si_6$	21897	458
6	10.631	Heneicosane	$C_{21}H_{44}$	38616	296
7	11.125	Heptadecane, 2,6,10,15-tetramethyl-	$C_{21}H_{44}$	24149	296
8	11.903	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$	23116	578
9	12.147	Phytol, acetate	$C_{22}H_{42}O_2$	11401	338
10	12.452	6-Octen-1-ol, 3,7-dimethyl-, propanoate	$C_{13}H_{24}O_2$	9386	212
11	12.685	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	4074	296
12	13.195	Heneicosane	$C_{21}H_{44}$	31253	296
13	13.740	Eicosane	$C_{20}H_{42}$	16281	282
14	13.829	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	$C_{18}H_{52}O_7Si_7$	21128	576
15	14.146	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	15246	312
16	14.391	3,5-Decadien-7-yne, 6-t-butyl-2,2,9,9-tetramethyl-	$C_{18}H_{30}$	957	246
17	14.713	1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-	$C_{30}H_{50}O$	15834	426
18	15.893	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	$C_{12}H_{38}O_5Si_6$	16124	430
19	16.088	Octadecane, 1-iodo-	$C_{18}H_{37}I$	20998	380
20	16.681	Tridecanol, 2-ethyl-2-methyl-	$C_{16}H_{34}O$	8105	242
21	16.681	7,9-Di-tert-butyl-1-oxaspiro (4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	15201	276
22	16.963	2-Bromotetradecane	$C_{14}H_{29}Br$	4178	276
23	25.175	Pentanedioic acid, 3-phenyl-, dibenzyl ester	$C_{25}H_{24}O_4$	77051	388
24	33.837	1,3,5-Cycloheptatriene, 2,4-di-t-butyl-7,7-dimethyl	$C_{17}H_{28}$	5528	232

Retention time (RT) and molecular weight (MW). GC-MS: Gas chromatography-mass spectrometry

Table 2: GC-MS of ethanol extract analysis revealed the presence of phytochemical components in *in vivo* grown *Peperomia pellucida.*

No	RT	Compound	Molecular formula	Area	MW
1	9.780	Apiol	$C_{12}H_{14}O_4$	26511	222
2	10.620	Heneicosane	$C_{21}H_{44}$	24850	296
3	14.395	4-(3,5-Di-tert-butyl-4-hydroxyphenyl)butyl acrylate	$C_{21}H_{32}O_3$	14673	332
4	15.507	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	$C_{16}H_{50}O_7Si_8$	78017	578
5	17.849	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	$C_{18}H_{52}O_7Si_7$	79189	576
6	18.209	2-Propenoic acid, pentadecyl ester	$C_{18}H_{34}O_2$	31124	282
7	19.053	Azetidine, 3-methyl-1-(phenylmethyl)-	$C_{11}H_{15}N$	222955	161
8	19.855	1-Benzylazetidine	$C_{10}H_{13}N$	93849	147
9	20.058	Heptasiloxane, hexadecamethyl-	$C_{16}H_{48}O_6Si_7$	83943	532
10	21.865	Cyclopentanone, 2-(4-benzyloxy-3-methoxybenzylidene)-	$C_{20}H_{20}O_3$	399631	308
11	22.189	1,1,1,3,5,7,7,7-Octamethyl-3,5-bis(trimethylsiloxy)tetrasiloxane	$C_{14}H_{42}O_5Si_6$	84704	458
12	22464	Benzene, (1-ethylpropyl)-	$C_{11}H_{16}$	113270	148
13	24.241	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	$C_{18}H_{52}O_7Si_7$	81360	532
14	24.973	Oxazolo[4,3-a]isoquinolin-3-one, 1-benzyl-8,9	$C_{22}H_{25}NO_4$	646578	367
15	25.149	Naphtho[2,3-b]norbornadiene	$C_{15}H_{12}$	97222	192
16	25.316	trans,trans-Dibenzylideneacetone	$C_{17}H_{14}O$	20241	234
17	26.498	3-Pyrrolidinone, 1-(phenylmethyl)-	$C_{11}H_{13}NO$	372102	175
18	27.200	2-Benzylaminonicotinonitrile	$C_{13}H_{11}N_3$	85393	209
19	27.839	2-Propenoic acid, 3-phenyl-, phenylmethyl ester	$C_{16}H_{14}O_2$	27490	238
20	28.788	1-Imidazolidinecarboxylic acid, 2-(1,1-dimethylethyl)-4-oxo-3-(phenylmethyl)-, phenylmethyl ester, (s)-	$C_{22}H_{26}N_2O_3$	77422	366
21	30.000	1,3-Dibenzyl-2-(3-nitro-phenyl)-hexahydropyrimidine	$C_{24}H_{25}N_3O_2$	339136	387
22	32.987	9-Octadecenoic acid, 1,2,3-propanetriyl ester, (E,E,E)-	$C_{57}H_{104}O_6$	754734	884
23	34.303	beta-Phenylpropiophenone	$C_{15}H_{14}O$	325594	210

Retention time (RT) and molecular weight (MW). GC-MS: Gas chromatography-mass spectrometry

Table 3: GC-MS of methanol extract analysis revealed the presence of phytochemical components in *in vitro* grown *Peperomia pellucida.*

No	RT	Compound	Molecular formula	Area	MW
1	6.939	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-	$C_{15}H_{24}$	920	204
2	7.295	Caryophyllene	$C_{15}H_{24}$	9467	204
3	8.031	Octadecane, 1-chloro-	$C_{18}H_{37}Cl$	15936	288
4	8.336	(7a-Isopropenyl-4,5-dimethyloctahydroinden-4-yl)methanol	$C_{15}H_{26}O$	15000	222
5	8.612	Heptadecane, 2,6,10,15-tetramethyl-	$C_{21}H_{44}$	5219	296
6	9.053	1,2-Dimethoxy-4-(2-methoxyethenyl)benzene	$C_{11}H_{14}O_3$	20096	194
7	9.829	Apiol	$C_{12}H_{14}O_4$	1994	222
8	10.663	Octadecane	$C_{18}H_{38}$	9479	254
9	11.156	Dodecane, 1-iodo-	$C_{21}H_{44}$	15003	296
10	13.227	Hexadecane, 1-iodo-	$C_{12}H_{25}I$	14503	352
11	13.772	Eicosane	$C_{20}H_{42}$	11266	282
12	16.116	Tridecanol, 2-ethyl-2-methyl-	$C_{16}H_{34}O$	3024	352
13	16.710	Pentadecane, 8-hexyl-	$C_{21}H_{44}$	9511	296

Retention time (RT) and molecular weight (MW). GC-MS: Gas chromatography-mass spectrometry

Apart from apiol, the present study identified the presence of phenolic compounds in the ethanol extracts of *in vitro* grown plants and methanol extracts of *in vivo* grown plants, as reported previously [34]. The current study reported that the detection of cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, and caryophyllene was in the methanol extracts of *in vitro* and *in vivo* grown *P. pellucida*. Narayanamoorthi *et al.* [35] had reported the presence of cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, and caryophyllene in the ethanol extract of an *in vivo* grown *P. pellucida* whole plant. These two compounds have shown to be effective as an anticancer, analgesic, antibacterial, anti-inflammatory, sedative, and also as a fungicide [22,35].

Furthermore, detected was the presence of 9-octadecenoic acid in the ethanol extract of *in vivo* grown *P. pellucida.* Omotoso *et al.* [36] had reported that 9-octadecenoic acid (z) contains anticancer properties, acts as a lubricant, as an anti-inflammatory agent, able to inhibit hemolytic

5-α reductase, acts as a diuretic agent, able to stimulate immune system, antiandrogenic, antifungal, antibacterial, and lipoxygenase inhibitor activities. Decane is normally used in chemical tests as a function of concentration and temperature [37]. In the present study, the detection of phytol is positive in both the ethanol extract of *in vitro* grown plants and methanol extract of *in vivo* grown *P. pellucida*. Narayanamoorthi *et al*. [29] reported the presence of phytol in the ethanolic extract of the *in vivo* grown plant, while Wei *et al*. [38] reported phytol in the methanol extract of an *in vivo* grown plant. Whereas, Okoh *et al*. [39] reported the presence of phytol in essential oil. Previous research has shown that phytol is known to have antimicrobial, anticancer, diuretic, and anti-inflammatory activities [22].

In a previous study, Wei *et al*. [38] had reported the presence of phytol, 2- naphthalenol, decahydro-, hexadecanoic acid, 9,12-octadecadienoic acid, and methyl ester from methanol extraction of *in vivo* grown *P. pellucida.* Verma *et al*. [9] has reported the detection of a total of 50 compounds from the essential oil of *P. pellucida.* Among the 50 compounds, apiol was also present in the essential oil. A separate study reported the presence of dillapiole in *P. pellucida* extract and tested on Wistar rats for gastroprotection. The findings have concluded that dillapiole is the most active gastroprotective agent of *P. pellucida* [8].

3.2. Antibacterial Activity

P. pellucida has known to be a medicinal plant and is widely used for skin, eye, throat, and gout as well as for antibacterial activity. In the present study, ethanolic and methanolic extracts of *in vivo* and *in vitro* grown plants were found to be effective against the following bacteria: *S. typhi, P. aeruginosa, E. coli,* and *S. aureus* [Table 5]. Tests of the

Table 4: GC-MS of methanol extract analysis revealed the presence of phytochemical components in *in vivo* grown *Peperomia pellucida*.

No	RT	Compound	Molecular formula	Area	MW
1	6.813	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-	$C_{15}H_{24}$	11775	204
2	7.251	Caryophyllene	$C_{15}H_{24}$	5400	204
3	8.006	Decane, 2,3,5,8-tetramethyl-	$C_{14}H_{30}$	26432	198
4	8.321	Phenol, 3,5-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	18982	206
5	8.595	Pentadecane, 2,6,10,14-tetramethyl-	$C_{19}H_{40}$	2448	268
6	9.017	1,2-Dimethoxy-4-(2-methoxyethenyl)benzene	$C_{11}H_{14}O_3$	21853	194
7	9.810	Apiol	$C_{12}H_{14}O_4$	6059	222
8	10.631	Heptadecane, 2,6,10,15-tetramethyl-	$C_{21}H_{44}$	17031	296
9	11.127	Heneicosane	$C_{21}H_{44}$	22904	296
10	12.143	Phytol, acetate	$C_{22}H_{42}O_2$	4197	338
11	12.696	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	6933	296
12	13.199	Hexadecane, 1-iodo-	$C_{16}H_{33}I$	17462	325
13	13.746	Heneicosane	$C_{21}H_{44}$	16601	296
14	16.087	2-Bromotetradecane	$C_{14}H_{29}Br$	13482	276
15	16.685	5-Butyl-5-ethylpentadecane	$C_{21}H_{44}$	7061	296

Retention time (RT) and molecular weight (MW). GC-MS: Gas chromatography-mass spectrometry

Table 5: Antibacterial activities of ethanolic and methanolic extracts of *in vivo* and *in vitro Peperomia pellucida.*

Solvent	Zone of inhibition (mm) and concentration				Reference
	Staphylococcus aureus	*Escherichia coli*	*Salmonella typhi*	*Pseudomonas aeruginosa*	
Methanol	11.00 (10 mg/mL) 12.50 (IVT) (10 mg/mL)	9.50 (10 mg/mL) 10.00 (IVT)(10 mg/mL)	11.75 (10 mg/mL) 11.25 (IVT) (10 mg/mL)	10.00 (10 mg/mL) 7.50 (IVT)(10 mg/mL)	Current study
	NI (1 mg/mL)	8.00 (1 mg/mL)	25.00 (1 mg/mL)	20.00 (1 mg/mL)	[42]
	10.00 (200 mg/mL) NI (25–100 mg/mL)	10.00 (200 mg/mL) NI (25–100 mg/mL)	10.00 (200 mg/mL) NI (25–100 mg/mL)	10.00 (200 mg/mL) NI (25–100 mg/mL)	[48]
	10.00 (200 mg/mL) NI (6.25–100 mg/mL)	10.00 (200 mg/mL) NI (6.25–100 mg/mL)	10.00 (200 mg/mL) NI (6.25–100 mg/mL)	12.00 (200 mg/mL) NI (6.25–50 mg/mL)	[34]
	8.00 (4 mg/disc)	12.00 (4 mg/disc)	10.00 (4 mg/disc)	10.00 (4 mg/disc)	[49]
Ethanol	6.75 (10 mg/mL) 10.75 (IVT) (10 mg/mL)	11.50 (10 mg/mL) 8.00 (IVT) (10 mg/mL)	9.25 (10 mg/mL) 12.00 (IVT) (10 mg/mL)	10.75 (10 mg/mL) 12.00 (IVT) (10 mg/mL)	Current study
	6.00 (100 mg/mL)	5.00 (100 mg/mL)	Not performed	Not performed	[19]
	Not performed	10.00 (100ug/mL)	Not performed	14 (100ug/mL)	[50]
	6.33 (250 mg/mL)	NI (250 mg/mL)	6.33 (250 mg/mL)	Not performed	[18]
	Not performed	12.00 (10 mg/mL)	Not performed	19.6 (10 mg/mL)	[10]
Chloramphenicol (Control)	16.75 (10 mg/mL)	17.00 (10 mg/mL)	15.75 (10 mg/mL)	17.00 (10 mg/mL)	Current study

In vitro (IVT), No inhibition (NI)

extracts against several organisms have displayed comparable results that of the standard antibiotics, chloramphenicol. The GC-MS chemical analysis result of *P. pellucida* shown it to contain bioactive compounds and has been reported to exhibit antibacterial activities [22,35,38]. Based on Table 5 in the current study, the methanol extract from an *in vitro* plant showed the highest inhibition zones (12.5 mm) against *S. aureus* with a lower concentration compared to the *in vivo* grown plant extracts from other studies. The ethanol extracts from an *in vivo* grown plant (6.75 mm) and *in vitro* grown plant (10.75 mm) showed a contrasting result with the methanol extract from an *in vitro* grown plant, which was less effective against *S. aureus*. This result is also highly comparable to previous studies [Table 5]. *S. aureus* is a major human pathogen that affects the skin and soft tissues, pleuropulmonary, bloodstream respiratory tract, and can also contaminate medical devices [40,41]. In treating multiple resistant *S. aureus* (MRSA), there are proposals for the usage of the methanol extract of the *in vitro* grown plant. The methanol extract of the *in vitro* grown *P. pellucida* can be a promising potential inhibitor against MRSA. GC-MS analysis of the methanol extract from the *in vitro* grown plant has detected the presence of previously described antimicrobial agents such as Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methylethenyl)-, and Caryophyllene. However, in the current study, the ethanol extract from the *in vitro* plant showed the highest inhibition zones (12 mm) against *S. typhi* and *P. aeruginosa* compared to methanol extracts from *in vivo* and *in vitro* grown plants or the ethanol extract from *in vivo* grown plants. In the current study, the ethanol extract from the *in vivo* grown plant showed greater activity (11.5 mm) against *E. coli* compared to the ethanol extract from an *in vitro* grown plant (8.00 mm). A study conducted by Edewor-Kuponiyi [42] showed that the crude methanol extract from the *in vivo* grown leaves at 1 mg/mL concentration showed a higher inhibition zone compared to the current study. Edewor-Kuponiyi used dimethyl sulfoxide (DMSO) to dissolve the extracts. A study reported DMSO could inhibit the growth of bacteria [43]. This implies that the presence of DMSO can enhance the effect of the inhibition zone. At present, no studies are reporting the antibacterial activity using extracts from tissue cultured (*in vitro) P. pellucida* plants. The results obtained in this study on the antimicrobial efficacy of dried crude extracts of *in vitro* regenerated *P. pellucida* showed the highest inhibitory activity using ethanol and methanol as the medium of extraction. The plant extracts showed bacteria inhibition. This could be due to the presence of various active compounds such as polyphenols and flavonoids from the plant extracts that could be the antimicrobial components [17]. The topical application of the ethanolic and methanolic extracts of *in vivo* and *in vitro* grown plants is possible as both extracts displayed as antibacterial agents. All parts of *P. pellucida* have potent antibacterial activity and it has also been reported that crude methanol extracts of *P. pellucida* have a broad spectrum of antimicrobial activity [23]. *In vivo* and *in vitro* grown plants produce various compounds which show different bioactivity prospectives [44].

Biotechnological methods through plant tissue culture provide a promising bio-production platform for desired natural products. Cultures developed from shoots or roots revealed an undifferentiated metabolic characteristic compared to their parent plant. Tissue culture offers an alternative potential for the fabrication of high-value natural products in the plant [6]. It is important to note that the antimicrobial activities differ in *in vivo* and *in vitro* grown plant extracts, probably due to the inherent characteristics of the fully grown plants and the maturity of their chemically active constituents.

3.3. Anticancer Activity

Apart from the aforementioned characteristics of *P. pellucida,* the anticancer properties of the said extracts were tested on two commercially available cancer cell lines, namely; adenocarcinomic human alveolar basal epithelial cells (A549) and human breast adenocarcinoma cell lines (MCF-7). As shown in Figure 1, with increasing concentration of both ethanol extracts of *in vitro* and *in vivo* grown plants shows a general decrement of the viability of the cells. For instance, at 30 µg/mL, the viability of A549 cells has reduced from 100% to 45.3%. The IC_{50} of this extract was found to be 13.02 µg/mL based on the equation $y = -7.335\ln(x) + 68.828$ [Figure 1a]. Similarly, the viability of A549 cells was also checked upon exposure to both methanol extracts of *in vitro* and *in vivo* grown plants [Figure 1c and d]. In particular, the viability of A549 cells at 30 µg/mL for both methanol extracts of *in vitro* and *in vivo* grown plants were 35.8% and 33.3%, respectively, hereby indicating the potency of this extract, especially upon methanol extraction. The IC_{50} for the extracts was found to be 8.11 µg/mL and 6.76 µg/mL, respectively.

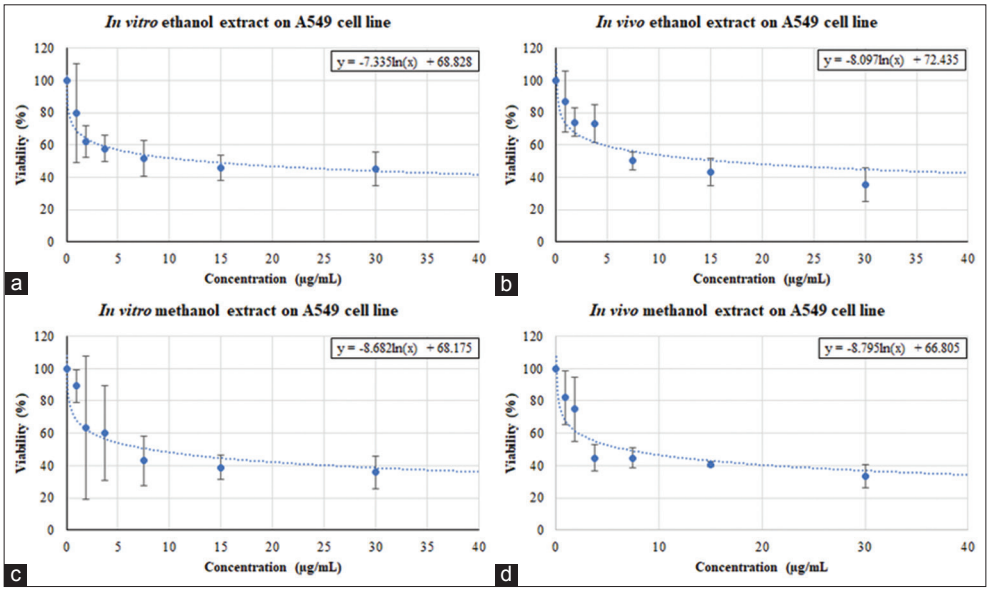

Figure 1: (a-d) Viability assay of A549 cells response

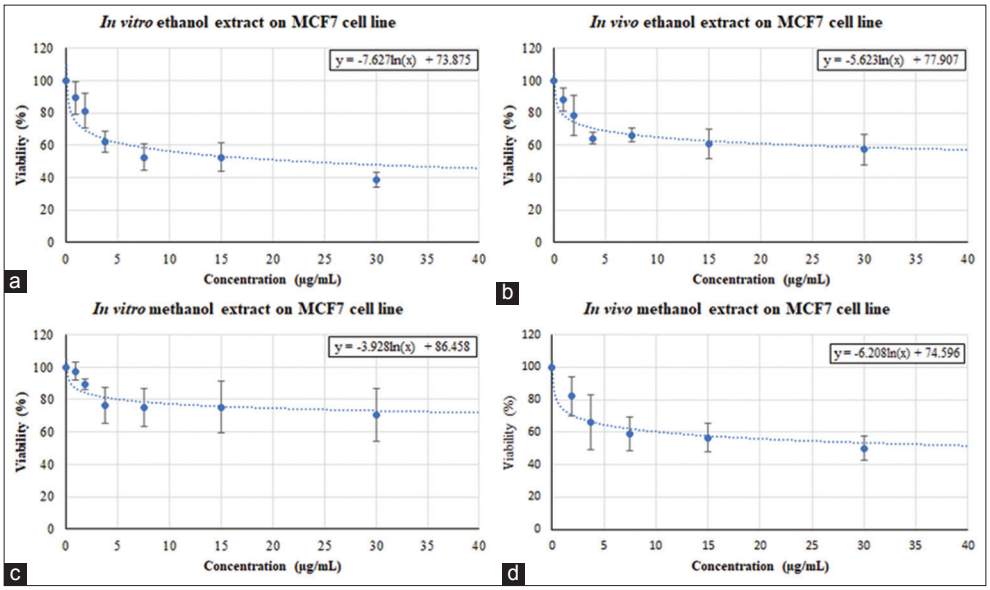

Figure 2: (a-d) Viability assay of MCF7 cells response

In addition to assessing the viability of A549, a similar assay was also conducted on another robust cancer cell line, MCF-7 [Figure 2]. Upon exposure to the ethanol extract from an *in vitro* grown plant, the viability trend showed a typical decrement with increasing concentration. The observation for the exposure of the 30 g/ml extract shows the viability to be at 38.6% compared to 45.3% in its A459 counterpart with IC_{50} of 22.88 g/mL [Figure 2a]. Finally, the viability of MCF7 upon exposure to the methanol extracts from an *in vivo* grown plant also displayed an augmented survival pattern as compared to its A549 counterpart. For instance, the exposure to 30 µg/mL of the extract revealed a viability percentage of 49.94%, as compared to 33.3% in A549 cells with IC_{50} 52.56 µg/mL [Figure 2c]. A similar study by Wei *et al*. [38] also confirmed the anticancer findings, thus strengthening the possibility that *P. pellucida* extract can be commercially harnessed for anticancer treatment. Although the study reported a much lower IC_{50} than our findings, it is believed that this variation could be due to the number of plants used. In this study, two different types of cancer cells, namely adenocarcinomic human alveolar basal epithelial cells (A549) and human breast adenocarcinoma cell lines (MCF-7), were chosen based on the frequent occurrence of such cancers in human [45]. In general, an increase in extract concentration resulted in the poor survival of these cancer cells over time. However, the total effect varied among the type of cancers as well as the origin of the extract being used. For instance, in A549 cells, extracts from methanol resulted in a lower survival rate upon exposure at 30 µg/mL for 24 h. This shows that the presence of more active compounds in the methanolic extracts had displayed higher cytotoxicity on A549 cells as compared to those from ethanol extraction. This was also supported with IC_{50} values found alongside. Contrastingly, in MCF7 cells, compounds through ethanol extraction presented higher cytotoxicity coupled with lower IC_{50} values, as compared to those of methanol extraction.

Although this study did not address the possible mechanism behind the cytotoxicity effect of these compounds, it is speculated that key cellular pathways such as TGF-β/Smad and PI3K/Akt/mTOR signaling pathways could be one of which the active compounds exert their effects [46]. Further studies could clarify therapeutic potentials and toxicity issues should these compounds move toward clinical and therapeutic usages. Anticancer activities differ in *in vivo* and *in vitro* grown plant extracts, probably due to the inherent characteristics of the fully grown plants and the maturity of its chemically active constituents.

4. CONCLUSION

Plant tissue culture is a promising technology for producing valuable plant metabolites. GC-MS analysis revealed a total of 24 compounds from *in vivo* and *in vitro* grown *P. pellucida*. Both ethanol and methanol extracts of *in vivo* and *in vitro* grown *P. pellucida* contained apiol which has proven to contain medicinal properties. Both ethanol and methanol extracts of *in vivo* and *in vitro* grown *P. pellucida* contain numerous chemical compound that may possess medicinal properties. Further investigation is necessary to justify the rationale behind the wide applicability of the plants in herbal medicine.

A comparison was made between extracts from *in vitro* regenerated plants through tissue culture system and *in vivo* plants for antibacterial activities. The results obtained indicated that dried crude extracts of *in vitro* regenerated *P. pellucida* displays antibacterial activities.

Cancer is the most devastating disease and the leading cause of death in many countries. The methanol extract of an *in vitro* regenerated plant showed better anticancer activity on A549 cells, even though it was not as significant as compared to methanol and ethanol extracts of *in vivo* grown plants. In MCF-7, the ethanol extracts of *in vitro* regenerated plants showed the best anticancer activity. The results of this study suggest that extracts from *P. pellucida* contain anticancer properties and have the potential to treat breast and lung cancer.

5. AUTHORS' CONTRIBUTIONS

TL: participated in the acquisition of data, analysis, interpretation, prepared figures, and wrote the manuscript; NG, AFMA, and RMT: designed the experiments, participated in data analysis, revised the manuscript critically for important intellectual contents, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work had been investigated and resolved appropriately. All authors read and approved the final manuscript.

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7. CONFLICTS OF INTEREST

The authors declare no competing financial interests exist.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. PUBLISHER'S NOTE

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