RESEARCH ARTICLE

In vitro shoot regeneration and analysis of biochemical, antioxidant and anticancer properties of *Ananas comosus* var. MD2

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Abstract

Pineapple (*Ananas comosus* (L.) Merr.) is one of the most important crops in the world. Although the phytochemical compounds of pineapple fruits have been extensively studied, little attention was paid on other parts of the plant. In this study, we developed an efficient *in vitro* regeneration procedures for *A. comosus* var. MD2 as well as analyzed the phytochemical composition, antioxidant and anticancer activities in field-grown and *in vitro* leaf samples. Leaf base explants cultured on Murashige and Skoog medium containing 1.0 mg/L IBA and 2.0 mg/L BAP produced the highest frequency of microshoot induction (100%) with D-leaf length to width ratio of 6.3. Biochemical analysis showed that *in vitro* leaf samples contained higher amount of carotenoids and anthocyanins but lower total phenolic content and DPPH radical scavenging activity than field-grown leaves. Flavonoids, tannins, sterols and alkaloids were detected in both field-grown and *in vitro* leaf samples. The methanolic extracts from both samples exhibited low cytotoxic activity against human ovarian SKOV-3 and human breast MCF-7 cancer cells. These results suggested that pineapple leaves might not be suitable to serve as chemotherapeutic agent and further exploitation on other secondary metabolites is required.

Keywords: bioactivity, cytotoxicity, field-grown, *in vitro, Ananas comosus*

INTRODUCTION

Pineapple (*Ananas comosus* (L.) Merr.) is one of the most important crops worldwide (FAOSTAT 2015). Over 13 million metric tons of pineapple were harvested globally. In Malaysia, *A. comosus* variety MD2 has been listed as one of seven high-values and economically important crops under Malaysia's National Key Economic Areas (NKEA). MD2 is highly demanded in the international market due to its aroma, sugar and vitamin contents as well as its longer post-harvest shelf life. Conventionally, pineapple is propagated vegetatively through suckers. However, this method is slow and may take up to 8 years to generate propagules from single mother plants (Zuraida, Shahnadz et al. 2013). Moreover, it can easily transmit numerous diseases from old to new pineapple plantations. Therefore, an alternative method using plant tissue culture to mass produce disease-free pineapple plantlets in order to satisfy the increasing demand for pineapple fruits is essential.

Harvesting pineapple produces a large amount of waste material (Asim, Abdan et al. 2015). In 2008, about 384,673 metric tons of waste materials, such as leaves, were either burnt or discarded which can later cause pollution and landfill problems. Very small portions of pineapple leaf fibers are being used as feedstock and energy production (Asim, Abdan et al. 2015). Therefore, full utilization of these materials by amplifying industrial usage to minimize the wastage of renewable materials is vital. Instead of treating it as wastes, it is of great effort to evaluate its pharmacological properties for medicinal purposes.

Similar to many plant species, pineapple has many applications in folk medicines. Various parts of the pineapple plants have been used to treat numerous diseases and disorders. Apart from the fruit, stem, leaf, rhizome, root and latex of pineapple have been found to contain numerous medicinal properties. For instance, pineapple leaves have been traditionally used in Chinese medicine as anti-dyspepsia and anti-diarrheal agents (Song, Zhao et al. 2012). Other properties, such as anti-diabetic, anti-dyslipidemic and anti-oxidative, have also been reported (Xie, Xing et al. 2005), indicating its potential to serve as medicine. Besides being utilized as a traditional medicine, pineapple leaves are also used in textiles industry in the Philippines.

In this study, we aimed to mass propagate *in vitro* pineapple variety MD2 and to evaluate the chlorophyll content, total carotenoids, anthocyanin content, phytochemicals constituent, antioxidant activity and cytotoxicity of *in vitro*- and field grown-pineapple leaves. This study might provide a new insight into the bioavailability of using pineapple waste material for a new health supplement.

EXPERIMENTAL

Plant materials

Leaf base of i*n vitro* grown *A. comosus* var. MD2 initiated on Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium supplemented with 1.0 mg/L NAA and 3 mg/L BAP (Hamid *et al.*, 2013) were used as explants for shoot proliferation.

Shoot proliferation and maintenance

The leaf base explants (1 cm in length) were cultured on MS medium supplemented with different concentrations and combinations

of indole-butyric acid (IBA; 0.0-2.0 mg/L) and 6-benzylaminopurine (BAP; 0.0-2.0 mg/L). All cultures were maintained at 25 ± 1°C under a photoperiod of 16 h light and 8 h dark with an illumination of 1000 lux. The frequency of microshoot induction, length and width of the D-leaf were measured after 8 weeks of culture.

Preparation of methanolic extracts

Leaves of *in vitro* plantlets and 2 month-old field grown (Fig. 1) plants collected from the Plant Biotechnology Research Centre (PPBGL), University of Malaya, Glami Lemi, Jelebu, Negeri Sembilan, Malaysia, were cleaned, freeze-dried (Labconco, USA) and ground into fine powder using a commercial blender before stored in airtight containers. Fine powder (1 g) were soaked in 30 mL absolute methanol for 7 days at 4 °C and filtered through a Whatman filter paper. Methanolic filtrate was subjected to pigment analysis and phytochemical analysis. For the phytochemical analysis, the methanolic filtrate was evaporated under reduced pressure (from 760 torr to 5 torr) at 60 °C using a rotary evaporator (Buchi Rotavapor R-215, Switzerland). The concentrated methanolic extracts were dissolved in methanol to give stock solutions of 20 mg/mL and stored at -20 °C freezer until further use.

Determination of chlorophyll (Chl) and carotenoid contents

The absorbance of the methanolic filtrate was measured using a UV-Vis spectrophotometer (Perkin Elmer™, USA) and the content of Chl (a and b) and total carotenoids (666, 653 and 470 nm at which observed maximum absorbance were observed) were calculated according to (Lichtenthaler and Buschmann, 2001):

$$C_a \text{ (µg/mL)} = 16.72 \, A_{665.2} - 9.16 \, A_{652.4}$$
$$C_b \text{ (µg/mL)} = 34.09 \, A_{652.4} - 15.28 \, A_{665.2}$$
$$C_{(x+c)} \text{ (µg/mL)} = (1000 \, A_{470} - 1.63 \, C_a - 104.96 \, C_b) / 221$$

Determination of anthocyanin content

Total anthocyanin content was determined using pH-differential method (Giusti and Wrolstad, 2001). The pH of the extract was adjusted to pH 1 using 0.025 M potassium chloride buffer and pH 4.5 using 0.4 M acetate buffer. The absorbance was measured at 510 nm and 700 nm using a Lambda 25 UV-Vis Spectrophtometer (Perkin Elmer™, USA). The monomeric anthocyanin pigment concentration (cyanidin-3-glucoside) in the samples was calculated using formula described by (Giusti and Wrolstad, 2001).

Anthocyanin content (mg/L) = (A x MW x DF x1000) / (ε x 1)

where,

$A = (\text{Abs}_{\lambda\text{vis-max}} - \text{Abs}_{700})_{pH1} - (\text{Abs}_{\lambda\text{vis-max}} - \text{Abs}_{700})_{pH4.5}$
MW (cyanidin-3-glucoside) = 449
DF = dilution factor
ε (molar absorptivity of cyanidin-3-glucoside) = 26,900
$\lambda_{\text{vis-max}}$ (of cyanidin-3-glucoside in aqueous buffer at pH1) = 510nm

Determination of total phenolic content

Total phenolic content was determined according to (Folin and Ciocalteu, 1927). The methanolic extracts (0.2 mL) were pipetted into a 10.0 mL volumetric flask containing 4.0 mL water and 0.5 mL Folin-Ciocalteu's reagent. After 1 min, 2.0 mL of 20 % aqueous solution of sodium carbonate were added to the mixture and made up to 10.0 mL with distilled water before incubated for 30 min. Absorbance of total phenolics was measured at 760 nm and calculated from a calibrated curve ($r^2 = 0.9954$) using gallic acid as standard (0.001 – 0.006 mg/mL). The results are expressed as gallic acid equivalent (mg GAE/g).

Determination of DPPH free radical scavenging activity

The free radical scavenging activity towards 1, 1-diphenyl-2-picryl-hydrazil (DPPH) was assessed according to published method (Khalaf *et al.*, 2008) with minor modifications. Briefly, about 10 mL of different concentrations (10, 50, 100, 200, 300, 500, 700 and 1000 µg/mL) of each extract were added to 1.0 mL of DPPH. An equal amount of ascorbic acid and DPPH was served as control. The mixture was mixed and incubated at room temperature in the dark for 30 min. Absorbance was measured at 517 nm and the DPPH radical scavenging activity was calculated according to the following formula (Gawron-Gzella *et al.*, 2012, Khalaf *et al.*, 2008).

DPPH scavenging activity (%) = $[(A_0 - A_1) / A_0]$ x 100
where,

A_0 = absorbance of control
A_1 = absorbance of sample

The percentage of DPPH radical scavenging activity against concentration was plotted by performing non-linear regression (third degree polynomial) as previously described (Abourashed, 2005, Samarakoon *et al.*, 2011). The results were interpreted as 50 % inhibition concentration (IC_{50}) in µg/mL.

Determination of ferric reducing antioxidant power (FRAP)

FRAP assay was performed based on the method described by Benzie et al. (Benzie & Strain, 1999) with slight modifications. About 300 µL extracts or methanol (control) were mixed with 10 µL FRAP reagent and incubated in microplate wells at room temperature in the dark for 30 min. The absorbance was recorded at 593 nm. A series of stock solution at 100, 10, 1, 0.1 and 0.01 mM of aqueous ascorbic acid were prepared to generate a standard curve ($r^2 = 0.9944$). The results obtained were expressed as mM of ascorbic acid equivalent per gram of dried sample

Analysis of phytochemical compounds

Phytochemical compounds were analyzed as previously described (Solihah *et al.*, 2012) with slight modifications:

Test for phenols
Methanolic extract (2 mL) was warmed at 45–50 °C before adding 2 mL of 3 % (w/v) of $FeCl_3$. Formation of green or blue color indicates the presence of phenols.

Test for flavonoids (I)
Methanolic extract (1 mL) was added to 1 mL of 10 % $(CH_3COO_2)_2Pb$ (w/v) and gently shaken. A muddy brownish precipitate indicates the presence of flavonoids.

Test for flavonoids (II)
One (1) mL methanolic extract was added to 10 % $FeCl_3$ (w/v) and gently shaken. A wooly brownish precipitate indicates the presence of flavonoids.

Test for tannins
One (1) mL methanolic extract was added to 1 mL of 3 % (w/v) of $FeCl_3$. A greenish black precipitate signifies the presence of tannins.

Test for phlobatannins
One (1) mL methanolic extract was boiled with 2 mL 1 % (v/v) HCl. The formation of red precipitate signifies the presence of phlobatannins.

Test for alkaloids
Methanolic extract (1 mL) was stirred with 5 mL of 1 % (v/v) HCL on a steam bath (60 °C) for 15 min and filtered. Test for alkaloids I: 1 mL of Dragendorff reagent was added to 1 mL methanolic filtrate. The formation of cloudy orange indicates the presence of alkaloids. Test for alkaloids II: 1 mL of Mayer reagent was added to 1 mL of filtrate. Appearance of slight yellow color indicates the presence of alkaloids. Test for alkaloids III: 1 mL of Wagner reagent was added to 1 mL of filtrate. The formation of turbid brown color indicates the presence of alkaloids.

Test for terpenoids
A total of 5 mL methanolic extract was mixed with 2 mL $CHCl_3$ before adding 3 mL concentrated H_2SO_4. Formation of the reddish

brown coloration between upper and lower layer indicates the presence of terpenoids.

Test for saponins

Approximately 0.2 mL methanolic extract was mixed with 5 mL distilled water and shaken vigorously for 5 min. Persistence of foams appearance is the indicator for saponins.

Test for sterols (Salkowski's test)

Two (2) mL of concentrated H_2SO_4 was added to 2 mL methanolic extract. A red precipitate formation indicates the presence of steroidal ring compound(s) in the sample.

Test for xanthoprotein

A few drops of HNO_3 were added by the side of the test tube containing 1 mL methanolic extract. A yellow color is formed indicates the presence of xanthoprotein.

Test for cardiac-glycosides

Methanolic extract (100 mg) was dissolved in 1 mL of CH_3COOH containing 1 drop of 3 % (w/v) $FeCl_3$. One (1) mL of concentrated H_2SO_4 was then slowly added to the mixture. The formation of brown ring at the interface indicates the presence of de-oxy sugar characteristics of cardenolides compound(s).

Neutral red (NR) uptake assay

The cytotoxicity was measured by Neutral Red Uptake assay based on the uptake and subsequent lysosomal accumulation of the supravital dye, neutral red, in viable and uninjured cancer cells. Human ovarian SKOV-3 and breast MCF-7 cancer cell lines detached from conical flasks with 1.0 mL solution of accutase in phosphate buffer solution, pH 7.4 were centrifuged at 120 $x\ g$ for 5 min. Viable cells was counted by 0.4 % (w/v) of trypan blue exclusion method using a haemocytometer (Marienfield, Germany) as described by (Freshney, 1994). The cells were seeded in a 96 well micro titer plate (Nunc, China) at a concentration of 30 000 cells/mL and incubated at 37 °C for 3 h in a humidified 5% CO_2/95% air incubator. After incubation, the dye-containing medium was discarded and six different concentrations of extracts (1, 10, 25, 50, 75 and 100 µg/mL) were added. Untreated cells under the same conditions were used as controls. The plates were incubated at 37 °C for 72 h. The plates were re-incubated at room temperature for 3 h in medium containing 50 µg/mL NR solution and cells were subsequently washed with a mixture of calcium chloride: formaldehyde in distilled water (1:10:89, v/v/v) solution to remove NR solution (Ramasamy *et al.*, 2013). The incorporated dye was solubilized using 1% acetic acid and 50% ethanol in milli-Q water (0.2 mL). The plates were agitated on a micro titer plate shaker (LT BioMax 500) for 30 min.

The extracted dye absorbance was measured at 540 nm using a microplate reader (Emax, Molecular Devices USA). Three independent experiments were performed for each extract. NR uptake, proportional to the number of viable cells within the well, was expressed as a percentage of NR uptake by control cells, arbitrarily set to 100%:

NR uptake (%) = [($OD_{cont} - OD_{samp}$) / OD_{cont}] x 100
*cont: control, samp: sample

Statistical analysis

All experiments were carried out in triplicates following a randomized complete block design (RCBD). Data were statistically analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) at 5% significance level using SPSS version 22. All results were reported as mean ± SE of three replicated experiments.

RESULTS AND DISCUSSION

Shoot induction and proliferation of *A. comosus*

Leaf base explants demonstrated differential responses on the different concentrations of plant growth regulators in the culture media after one week of culture. The highest frequency of microshoot induction (100 %) was recorded on the medium containing 1.0 mg/L IBA and 2.0 mg/L BAP (Fig. 1a). The medium containing low concentrations of BAP (0.5 mg/L) alone was found to promote shoot elongation and number of leaves per shoot (data not shown) with D-leaf width of 1 cm (Table 1). BAP has been considered as one of the most effective cytokinins for shoot induction and regeneration (Tan *et al.*, 2011).
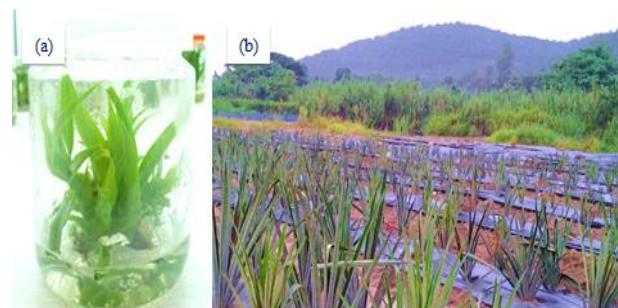

Fig. 1 *In vitro* and field-grown pineapple plants (a) Proliferation of multiple shoots from leaf base explants after 2 months of culture (b) 2 month-old field-grown plants.

Table 1 Effects of different concentrations and combinations of plant growth regulators on frequency of microshoot induction, length of D-leaf, width of D-leaf and D-leaf size (length to width ratio).

Plant growth regulator (mg/L)	Frequency of microshoot induction (%)	Length of D-leaf (cm)	Width of D-leaf (cm)	D-leaf size (length to width ratio)
Control (MS basal)	73.3 ± 3.3[ef]	5.1 ± 0.3[c]	0.5 ± 0.0[ab]	10.2
1.0 IBA	46.7 ± 3.3[bc]	5.3 ± 0.2[c]	0.6 ± 0.0[ab]	8.8
2.0 IBA	43.3 ± 3.3[ab]	5.2 ± 0.2[c]	0.6 ± 0.0[bc]	8.6
0.5 BAP	80.0 ± 0.0[fg]	7.5 ± 0.3[f]	1.0 ± 0.0[f]	7.5
1.0 BAP	66.7 ± 3.3[de]	5.0 ± 0.2[c]	0.8 ± 0.0[e]	6.3
2.0 BAP	70.0 ± 5.8[ef]	4.8 ± 0.6[c]	0.8 ± 0.0[e]	6.0
1.0 IBA + 0.5 BAP	86.7 ± 3.3[g]	6.4 ± 0.2[e]	0.8 ± 0.0[e]	8.0
2.0 IBA + 0.5 BAP	66.7 ± 3.3[de]	5.4 ± 0.1[d]	0.7 ± 0.0[de]	7.7
1.0 IBA + 1.0 BAP	56.7 ± 3.3[cd]	5.0 ± 0.1[c]	0.7 ± 0.0[e]	7.1
2.0 IBA + 1.0 BAP	46.7 ± 3.3[bc]	4.2 ± 0.1[b]	0.5 ± 0.0[ab]	8.4
1.0 IBA + 2.0 BAP	100.0 ± 0.0[h]	5.0 ± 0.2[c]	0.8 ± 0.1[e]	6.3
2.0 IBA + 2.0 BAP	43.3 ± 3.3b[ab]	3.5 ± 0.1[a]	0.5± 0.0[a]	7.0

Data represent mean value ± standard error (SE) with 30 explants in each treatment. Means with different letters in the same column are significantly different at *p* < 0.05 according to ANOVA and Duncan's multiple range test (DMRT).

Similar to our findings, Zuraida et al. (Zuraida *et al.*, 2013) reported that MS medium supplemented with 1.0 or 5.0 mg/L BAP was able to produce the highest number of shoots for *A. comosus* var. Maspine. The authors indicated that supplementation of BAP alone in culture medium was sufficient to produce shoot multiplication. This was in agreement with previous report (Kiss *et al.*, 1995), where incorporation of NAA in culture medium is not necessary for pineapple *in vitro* shoot multiplication. Continuous application of NAA might cause detrimental effects to growth and multiplication of pineapple (Wakasa, 1989). Taken together, MS medium supplemented with 1.0 mg/L IBA and 2.0 mg/L BAP was selected for subsequent shoot multiplication.

Distribution of Chl and carotenoids

The chlorophyll and carotenoid contents for the leaves of field-grown and *in vitro A. comosus* var. MD2 were investigated. The amounts of Chl (*a* and *b*) and carotenoids in *in vitro* leaf samples were at least 10-fold higher than field-grown samples with Chl a/b ratio of 0.21 (Table 2). Differences of Chl amounts between field-grown and *in vitro* samples might be due to edaphic and climatic factors, such as

light, water stress and salt stress (Sevik *et al.*, 2012). Chl a/b ratio was low when compared to C3 plants (Chl a/b ratio of 2.5-3.5). Typically, shade plants have lower Chl a/b ratio than sun-exposed plants as shade plants containing more light-harvesting protein LHC-II in photosystem II (PSII) than sun-exposed plants (Lichtenthaler and Buschmann, 2001). Leaves exposed to- and shaded from- sun light are differ in internal and external structures and photosynthetic rate, probably due to the amount of active ray received (Kurtar, 2012). Direct sun light may contain about 35 % of active ray that can penetrate leaves, whereas diffuse light may have 50-60 % of active ray (Kurtar, 2012).

In terms of carotenoid contents, *in vitro* leaves were 11.8-fold higher than field-grown leaves (Table 2). Carotenoids are essential for photoprotection in photosynthetic plant tissues and their levels are influenced by several factors, including plant species and organs, production practice, maturity as well as environmental growth factors, such as light, temperature and soil properties (Van den Berg *et al.*, 2000). For instance, it has been reported that the carotenoid content in tomato fruits was higher than leaves (Perrin *et al.*, 2016). They also reported carrot roots showed higher carotenoid content than leaves.

Table 2 Chlorophyll, total carotenoid and pigment contents (mg/g dry weight) of field-grown and *in vitro A. comosus* var. MD2 leaves.

Sample	Pigment content (mg/g dry weight)						
	C_a	C_b	$C_a + C_b$	$C_{(x+c)}$	C_a/C_b ratio	$C_a + C_b / C_{(x+c)}$ ratio	Total
Field-grown	0.03 ± 0.00^a	0.16 ± 0.00^a	0.19 ± 0.00^a	0.07 ± 0.00^a	0.18 ± 0.00^a	2.74 ± 0.00^b	0.26 ± 0.00^a
In vitro	0.38 ± 0.00^b	1.83 ± 0.00^b	2.21 ± 0.00^b	0.83 ± 0.00^b	0.21 ± 0.00^b	2.66 ± 0.00^a	3.04 ± 0.00^b

Means with different letters within the same column are significantly different at *p*<0.05 according to Duncan's multiple range test (DMRT).
*C_a chlorophyll *a*, C_b chlorophyll *b*, $C_a + C_b$ total chlorophyll *a* and *b*, $C_{(x+c)}$ total carotenoid (xanthophyll and carotene).

Distribution of anthocyanin content

Similar to Chl content, the content of anthocyanin for *in vitro* leaf extracts (3.16 mg/g) was also significantly (*p*<0.05) higher than field-grown (1.35 mg/g) (Table 3). Anthocyanins are common flavonoids which are found ubiquitously in many plants. They show bright red-orange to blue-violet color in various fruits and vegetable-based food products (Wallace, 2011). Anthocyanins reversibly change color with pH which limits their effectiveness as food colorants for many applications, but they provide an easy and convenient way for measuring total pigment concentration (Giusti and Wrolstad, 2001).

Anthocyanin content has been known to be correlated with Chl content (Gitelson *et al.*, 2001). Its content is often affected by pH, temperature, light, presence of co pigments, self-association, metallic ions, enzymes, oxygen, ascorbic acid, sugar and their degradation products, protein and sulfur dioxide (Sahari *et al.*, 2004). Low amount of anthocyanins in field-grown samples might be due to the degradation of these compounds by natural environmental conditions since anthocyanin is an unstable compound (Jiang, 2000). Previous study also showed that anthocyanins in pineapple was low and only found on fruits (Pavan *et al.*, 2012). Other plant species, such as banana, asparagus, pea, fennel, pear and potato, also exhibited less amount of anthocyanins and probably absent in some species such as green gooseberries (Wu *et al.*, 2004).

Table 3 Anthocyanin content of methanolic leaf extracts of field-grown and *in vitro A. comosus* var. MD2.

Sample	Absorbance maximum at pH 1, A_{max}	Monomeric anthocyanin pigment content (mg/g)
Field-grown	0.036	1.35^a
In vitro	0.084	3.16^b

Means with different letters within the same column are significantly different at *p*<0.05 according to ANOVA and Duncan's multiple range test (DMRT).

Measurement of total phenolic content

The total phenolic content for *in vitro* leaves (0.296 mg/g GAE) were significantly (*p*<0.05) lower than field-grown plants (0.433 mg/g GAE) (Table 4). One of the possibilities for this might be due to the influence of light intensity. The field-grown plants may receive higher

light intensity (30,000 to 100,000 lux) compared to *in vitro* plants (approximately 1,000 lux) which may induce higher amount of phenolic contents (Colonna *et al.*, 2016). Phenolic compounds are very important plant constituents due to their hydroxyl groups that can confer scavenging activity on free radicals constituents and reactive oxygen species (Hatano *et al.*, 1989, Prior and Cao, 2000). In plants, different organs exhibit different amount of phenolic compounds. For instance, total phenolic content of pineapple fruits (40.4 mg/g) was found to be higher than field-grown (1.3 mg/g) leaves (Upadhyay *et al.*, 2013). Other parts, such a pulp, seeds and peels, recorded 10 mg/g of GAE, suggesting that the sequence order of phenolic contents for these organs are fruits>pulp/seeds/peels>leaves.

Table 4 Total phenolic content of methanolic leaf extracts of field-grown and *in vitro A. comosus* var. MD2.

Sample	Total phenolic content (mg GAE per g of extract)
Field-grown	0.433^b
In vitro	0.296^a

Means with different letters within the same column are significantly different at *p*<0.05 according to ANOVA and Duncan's multiple range test (DMRT).

Analysis of DPPH free radical scavenging activity

Both field-grown and *in vitro* leaf samples had lower DPPH radical scavenging activity than ascorbic acid (Figure 2). The DPPH radical scavenging activity was dose-dependent, whereby the DPPH scavenging activity increased proportionally with the increased concentration of the extracts. Field-grown leaf extracts had significantly lower IC50 value than *in vitro* samples (Table 5). It has been reported that there are a strong correlation between antioxidant activities and total phenolic compounds (de Oliveira *et al.*, 2009, Bertoncelj *et al.*, 2007). Since pineapple leaves contained less phenolic compounds, the DPPH scavenging efficiency was also low. Furthermore, numerous vital phenolic compounds, such as gallic acid, ferulic acid, rutin, quercetin, and apigenin, which contribute to high DPPH scavenging activity (Can *et al.*, 2015) were not detected in our study.
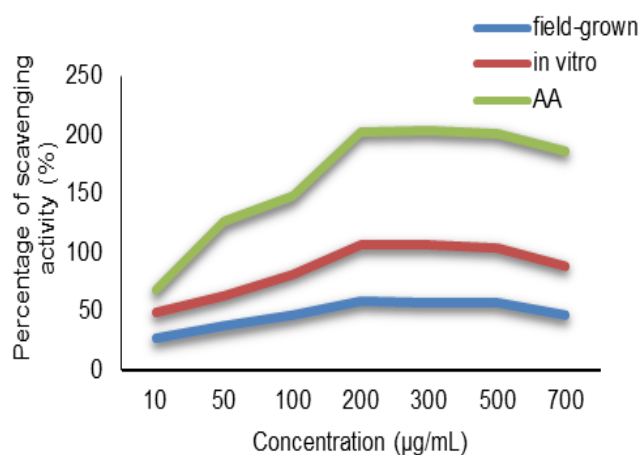
Fig. 2 DPPH free radical scavenging activity of field-grown and *in vitro A. comosus* var. MD2 leaves.
*AA ascorbic acid.

Table 5 DPPH Free radical scavenging activity and IC$_{50}$ of field-grown and *in vitro* grown *A. comosus* var. MD2 leaves.

Sample	Percentage of inhibition at 200 µg/mL	IC$_{50}$ values (µg/mL)
Ascorbic acid	71.07	17.07
Field-grown	45. 06	38.15
in vitro	36. 22	41.30

Means with different letters within the same column are significantly different at *p*<0.05 according to Duncan's multiple range test (DMRT).

Analysis of ferric reducing capacity

The ferric reducing potential of a compound indicates its "antioxidant power". The dose response curve for the reducing power of field-grown and *in vitro* leaf samples was compared to the standard (ascorbic acid). Both field-grown and *in vitro* leaf extracts were found to have lower FRAP activity than ascorbic acid (Table 6). Similar to DPPH activity, the total ferric reducing activities is also correlated with total phenolic contents and DPPH radical scavenging activities (Can *et al.*, 2015) With low amount of phenolic compounds in the samples, DPPH radical scavenging and FRAP activities were also found to be low (Borges *et al.*, 2009).

Table 6 FRAP (mM Fe^{2+}/g) values of field-grown and *in vitro A. comosus* var. MD2 leaves.

Sample	FRAP (mM Fe^{2+}/g)
Ascorbic acid (standard)	0.2014[c]
Field-grown	0.0012[a]
in vitro	0.0083[b]

Means with different letters within the same column are significantly different at *p*<0.05 according to ANOVA and Duncan's multiple range test (DMRT).

Analysis of phytochemical compounds

The presence of various phytochemical constituents in *A. comosus* var. MD2 leaves was analyzed. The phytochemical analysis showed that flavonoids, tannins, sterols and alkaloids were present in both field-grown and *in vitro* leaf extracts (Table 7). Similar to the study carried out by (Upadhyay *et al.*, 2013) the present study also detected sterol in the pineapple leaves but no other important organic compounds, such as phlobatannins, terpenoids, sterols, glycosides, saponins and xanthoproteins. Other organic compounds, namely saponins, diterpenes, triterpenes and antraquinones, have also not been detected in pineapple leaves (Subramanian *et al.*, 2014).

Table 7 Analysis of phytochemical constituents of field-grown and *in vitro A. comosus* var. MD2 leaves.

Phytoconstituents	Field-grown	*In vitro*
Phenols	-	-
Flavonoids (I)	+	+
Flavonoids (II)	+	+
Tannins	+	+
Phlobatannins	-	-
Alkaloids I	+	+
Alkaloids II	+	+
Alkaloids III	+	+
Terpenoids	-	-
Saponins	-	-
Sterols	+	+
Protein – xanthoprotein	-	-
Cardiac glycosides	-	-

*+ present, - absent

Determination of cytotoxicity effect on SKOV-3 and MCF-7 cells

In this study, the methanolic extract of field-grown *and in vitro A. comosus* var. MD2 leaves were investigated for their cytotoxic effects on human ovarian SKOV-3 and breast MCF-7 cancer cell lines. In general, both field-grown and *in vitro* leaf extracts showed low cytotoxic activity (< 50 % inhibition) against both SKOV-3 and MCF-7 cancer cells (Figure 3), probably due to the low amount of bioactive compounds in pineapple leaves.
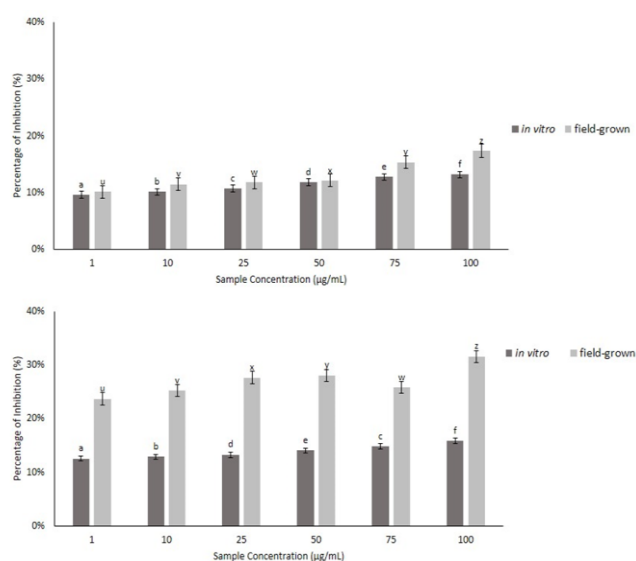

Fig. 3 Cytotoxic effect of field-grown and *in vitro* grown *A. comosus* var. MD2 leaf samples towards human breast MCF-7 cancer cell line (top); Cytotoxic effect of field-grown and *in vitro* gro wn *A. comosus* var. MD2 leaf samples towards human ovarian SKOV-3 cancer cell line (bottom).

CONCLUSION

The extracts of pineapple leaves (field-grown and *in vitro*) displayed weak radical scavenging activities, low reducing power and were less effective to inhibit human breast cancer MCF-7 and human ovarian cancer SKOV-3 cell proliferations. Our findings suggested that the pineapple leaves might not be suitable to serve as chemotherapeutic agent and further exploitation on other secondary metabolites is required.

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