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Acetone extract of *Flammulina velutipes* caps: A promising source of antioxidant and anticancer agents

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ABSTRACT

The high death rate associated with cancer has fuelled the search for complementary cancer treatment methods in recent times. Among the leading alternative methods is natural products which have attracted much attention due to their high body tolerance. In this study, the caps of *Flammulina velutipes* (commonly called Enoki), an edible mushroom, were extracted with different solvents and characterized for *in vitro* antioxidant and anticancer activities. The anticancer activity of the extract was studied against breast cancer cells (MCF-7 and MDA-MD-231) and normal or Vero breast cells (MCF-10a). The radical scavenging, metal reducing power, and anticancer properties of the extracts were investigated via *in vitro* chemical and cell-based methods. Furthermore, a phytochemical profiling process was performed on the extract fraction with the highest level of biological activities using Liquid chromatography-mass spectrometry coupled with quadrupole time of flight (LC-MS-QTof). The results showed acetone fraction of Enoki caps to exhibit more radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radicals (IC_{50} = 0.840 mg/mL) and hydrogen peroxide (H_2O_2) radicals (IC_{50} = 0.890 mg/mL) compared to the ethyl acetate fraction (IC_{50} = 0.97 mg/mL against DPPH and 1.180 mg/mL against H_2O_2). Additionally, acetone fraction showed a better metal reducing property in a concentration-dependent manner. The *in vitro* cytotoxicity study showed a preferential level of toxicity of the acetone extract to the studied cancer cell lines compared to the Vero cells. This extract showed more cytotoxicity against MCF-7 (IC_{50} value ranged from 17.7 μg/mL to 38.36 μg/mL) and MDA-MB-231 (IC_{50} value ranged from 114.5 μg/mL to 184.2 μg/mL) compared to MCF-10a (> 250 μg/mL). These findings present *F. velutipes* caps as a potential natural source of antioxidant and anticancer agents.

1. Introduction

Cancer is a globally recognized deadly disease that is associated with a high mortality rate. It results from an uncontrolled growth of cells or a defect in the genes that control normal cell division and growth (National Cancer Institute, 2015). According to the US breast cancer statistics report of 2018, breast cancer is a leading cause of death globally. About 266,120 new cases of invasive breast cancer have been forecasted to occur in the USA alone by 2018, while about 63,960 new non-invasive cases will be diagnosed. As per the number of deaths expected from breast cancer-related cases, approximately 40,920 deaths have been predicted in the USA in 2018 alone. The January 2018 report showed more than 3.1 million US women having a history of breast cancer (US Breast Cancer Statistics, 2018). These are challenging findings that require expedient action towards diversifying the sources of anticancer agents. Although chemotherapy and irradiation are the commonest forms of treatment for cancer, early detection and prevention remain the best approach (National Cancer Institute, 2015).

Mushrooms have served as a major source of dietary nutrients and wholesome body benefits due to their low fat and caloric contents, as well as their high essential amino acids, minerals, and vitamins content (Ramesh and Manohar, 2010). However, more effort has recently been dedicated to mushrooms as a source of phytochemicals with medicinal effects (Madhanraj et al., 2017). Mushrooms have been found to contain some phytochemicals with both anti-hypertensive, cholesterol-lowering, and anti-cancer activities (Bobek and Galbavy, 1999). The health benefits associated with mushroom consumption are attributable to their richness in phenolic compounds (Esmaeili et al., 2011). In China, Japan, and Korea, several mushroom extracts are widely used as potential adjuvants to chemotherapy and radiation treatments in cancer management (Sullivan et al., 2006; Borchers et al., 2008). However, most reports on the major medicinal properties of mushrooms have

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been on their antioxidant, immune modulation, anticancer, antiviral, and anticholesterolemic effects (Bobek and Galbavy, 2008; Nowacka et al., 2015). Different parts of mushrooms contain different phytochemicals that have different biological/pharmacological effects. Mushroom cap extracts have been reported to be more biologically active due to the higher concentration of phytochemicals in the caps compared to the stem (Ukaegbu and Shah, 2017; Akyuz and Kirbag, 2009; Breene, 1990; Sun et al., 2014). Phytochemicals can be extracted from mushrooms only when suitable solvents with a suitable level of polarity are used during the extraction process (Alnoumani et al., 2017). It is often difficult to identify a single solvent that can efficiently extract all the phytochemicals in any biological material (Akowuah et al., 2009). Because of this challenge, researchers have adopted the method of using solvents with varying levels of polarity when targeting different components of biological materials. Thus, this study aims to evaluate the effect of solvent (acetone and ethyl acetate) polarity on the biological activities of extracts from the dried caps of *Flammulina velutipes* (common name – Enoki) This mushroom was selected for this study due to the lack of supportive evidence on the anticancer activity of its extracts against human breast cancer despite its abundance in the study area.

2. Material and methods

2.1. Mushroom sample

The mushroom (*F. velutipes,* henceforth referred to as Enoki), was first identified based on literature evidence and supplied by a local biological materials supplier in Kuantan, Pahang, Malaysia.

2.2. Chemicals used

Folin reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), anhydrous sodium carbonate, monobasic and dibasic potassium phosphate, 50% hydrogen peroxide solution, gallic acid, potassium ferricyanide, aluminum chloride, trichloroacetic acid (TCA), quercetin, ascorbic acid, and ferric chloride were bought from Merck- Louis, MO, USA. MCF-7, MDA-MB-231, and MCF-10a were bought from ATCC (USA). The cell culture media (Dulbecco's Modified Eagles Medium) and the supplements (Fetal Bovine Serum and Penicillin/Streptomycin solution) were purchased from Merck- Darmstadt, Germany. Phosphate Buffered Saline, and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) cytotoxicity assay kit were bought from Solarbio, China.

2.3. Preparation and extraction

The mushrooms were first separated into caps and stems; the caps were dried to a constant weight in an incubator at 70 °C. A drying temperature of 70 °C was preferred to ensure a complete drying of the extracts within a short time since the water content of the mushroom was about 95% total weight. This drying temperature of 70 °C has been previously reported to have little or no significant effect on the phytochemical content of biological materials (Hazrulrizawati et al., 2016). The dried mushroom caps were pulverized and preserved in a dark plastic bag prior to use. For the extraction, 180 g of the pulverized mushroom caps was weighed and added into a 500-mL flat bottom conical flask, followed by the addition of 500 mL of acetone or ethyl acetate. The flask was covered with aluminum foils, mounted on a rotary shaker at 250 rpm, and shaked for 24 h at ambient temperature (25 ± 2.4 °C). After the extraction process, the suspension was filtered through several changes of Whatman no. 1 filter paper and later concentrated in a rotary evaporator (EYELA rotary system). The concentrated extract was further allowed to dry under a hood chamber. The dried extract was stored in sterile universal bottles and preserved at − 20 °C. The extraction yields of the acetone and ethyl acetate processes were 17.4% and 12.5%, respectively

2.4. Total phenolic content (TPC) evaluation

The TPC of the extracts was evaluated using the guideline provided by Slinkard and Slingleton (1997) and later modified by Seonwook et al. (2017). In this process, the extract was first solubilized in distilled water at 1 mg/mL concentration. Further, 1 mL of the extract solution was added to a conical flask (100 mL volume), followed by the addition of 45 mL of distilled water. Then, Folin-phenol reagent (1 mL) was added to the solution and mixed. The flask was incubated at ambient temperature for 3 min before adding 3 mL of 2% Na_2CO_3 solution. The flask was further allowed for 2 h at ambient temperature with occasional shaking. After 2 h, the absorbance of the solution was read at 760 nm. Gallic acid was used as the standard phenol source (for developing the standard curve). All the experiments were carried out in triplicate while the findings were presented as the mean ± SD of triplicate measurements. The TPC of the extract was determined as micrograms of gallic acid equivalent (μgGAE/mg) from the prepared standard curve ($R^2 = 0.99$).

2.5. Total flavonoids content (TFC) evaluation

The TFC of the extracts was evaluated using a standard guideline provided by Meda et al. (2005) and Muhammad et al. (2017). Briefly, equal volumes of 2% $AlCl_3$ (prepared in absolute methanol) and the extracts (1 mg/mL in absolute methanol) were incubated in a glass tube for 10 min at ambient temperature. After 10 min, the absorbance of the mixture at 415 nm was determined in a spectrophotometer. Quercetin was used at different concentrations to prepare the standard curve ($R^2 = 0.98$). The TFC of the extract was determined from the quercetin curve in micrograms of quercetin equivalent (QE)/mg and presented as the mean ± SD of triplicate measurements.

2.6. Antioxidant activity

In vitro-based chemical methods were used to evaluate the antioxidant activities of the extracts. These methods are based on the ability of the extracts at different concentrations to scavenge free radicals and to reduce metals.

2.6.1. DPPH free radical scavenging

Different concentrations of the extracts were studied for DPPH radicals scavenging using a standard method (Dorman et al., 2004; Hajdari et al., 2018). First, a methanol solution of DPPH (126 μM) was prepared. Furthermore, different concentrations of the extracts (0.2–1.0 mg/mL) were prepared in distilled water. For the assay, 1 mL of the extract solutions (different concentrations) was incubated with 2 mL of DPPH solution in capped glass tubes in a dark cupboard for 30 min at ambient temperature. A control set-up was prepared in a similar way but without the extract. After the incubation time, the fading of the purple color of the DPPH solution which indicates radical stabilization was monitored in a spectrophotometer at 517 nm. A standard protocol was set up using ascorbic acid at the equivalent concentrations. The DPPH radical scavenging activity of the extract was determined (using Eq. (1) and both as IC_{50} values and as percentages with respect to the initial DPPH concentration.

$$DPPH\ scavenging\ effect\,(\%) = \left[\frac{A_0 - A_1}{A_0}\right] * 100 \quad (1)$$

where

A_0 = control absorbance
A_1 = test/standard absorbance.

2.6.2. Hydrogen peroxide (H_2O_2) radical scavenging

Likewise, the extracts were evaluated for H_2O_2 radicals scavenging using a standard method (Odeja et al., 2017). Briefly, a 40 mM H_2O_2 solution was first prepared; then, 50-mM phosphate buffer solution (pH 7.4) was prepared as well. The initial concentration of radicals in the prepared 40 mM H_2O_2 solution was determined against a phosphate buffer blank at 230 nm and the absorbance was recorded as A_0. For the assay, 3.4 mL of the H_2O_2 solution was incubated with different concentrations (0.2–1.0 mg/mL) of the extracts (0.6 mL) for 10 min at ambient temperature. After the incubation, the absorbance of the solutions was read spectrophotometrically at 230 nm and recorded as A_1. The activity of the extracts was determined by estimating the percentage of the initial free H_2O_2 radicals scavenged after 10 min of incubation with different concentrations of the extracts using Eq. (2). Ascorbic acid was used as a standard during the investigation. The activity of the extracts was expressed both as percentages (with respect to the initial concentration of H_2O_2) and as IC_{50} values, where higher percentages and lower IC_{50} values indicate stronger antioxidant activities.

$$\textit{Hydrogen peroxide scavenged}\,(\%) = \left[\frac{A_0 - A_1}{A_0}\right] * 100 \tag{2}$$

2.6.3. Ferric reducing antioxidant power (FRAP)

The metal reducing capability of the extracts was evaluated using a procedure presented by Maruthamuthu and Ruckmani (2016). The extracts were first reconstituted in distilled water at different concentrations (0.2–1.0 mg/mL). Then, 1 mL of the extracts (different concentrations) was added into glass tubes containing 2.5 mL of 0.2 M sodium phosphate (pH 6.6) buffer. Furthermore, 2.5 mL of 1% potassium ferricyanide solution was added into the tubes. The tubes were capped, shaken, and allowed to incubate for 20 min at 50 °C. After the incubation, 10% TCA solution (2.5 mL) was added to each tube. At this stage, the tubes were vigorously shaken before being centrifuged at 3000 *g* for 10 min. After centrifugation, 2.5 mL of the supernatants were carefully transferred to a new set of tubes, followed by the addition of 2.5 mL of deionized water. Finally, 0.1% $FeCl_3$ solution (0.5 mL) was added to the tubes to initiate a redox reaction between the antioxidants in the extracts and the introduced unstable ferric ions. This reaction manifests in the formation of a colored complex with TCA. The absorbance of this complex, which reflects the strength of the antioxidant present in the sample, was read at 700 nm in a spectrophotometer. During this process, a standard protocol was equally set up with ascorbic acid. The observed FRAP of the extracts was reported as the mean ± SD of triplicate absorbance readings.

2.7. Cytotoxicity assay

2.7.1. MTT assay

The cytotoxicity of the acetone extract (selected fraction because of its higher antioxidant activity) was determined *in vitro* when the confluence the of the cultured cells was up to 80% (Mftah et al., 2015). A 50-mg stock extract solution was first made and further diluted to the required working concentrations of 3.91–250 μg/mL. This concentration range was used to ensure that the cytotoxicity of the extract could be monitored at high and low concentrations. The cells were seeded into 96-well transparent microtiter plates at the concentration of 5×10^3 cells/mL (counted using a hemocytometer method) and incubated for 24 h at 37 °C with 5% CO_2 concentration. After 24 h, the media in the wells were carefully replaced with fresh media supplemented with different extract concentrations to final concentrations of 250, 125, 62.5, 31.25, 15.63, 7.81, and 3.91 μg/mL. The media in the positive control wells were replaced with fresh media supplemented with doxorubicin at the same concentration as the extracts, while the media in the negative control cells were replaced with just fresh media. All the treatment groups were prepared in three 96-well transparent microtiter plates. One set of the plates was incubated for 24 h, while the second and third sets were incubated for 48 and 72 h, respectively. At the end of each incubation period, MTT solution (10 μL) was added into the wells and allowed to incubate for 4 h at 37 °C. After 4 h, 10% DMSO (100 μL) was added to the wells to facilitate the solubilization of the formed formazan crystals by the mitochondrial dehydrogenase of the viable cells. The absorbance of the formed purple color which is equivalent to the viable cells population in each well was determined at 570 nm in a plate reader. The cytotoxicity of the extracts was determined and expressed as the percentage of viable cells relative to the untreated control using Eq. (3). The concentration of extract needed to reduce the population of the viable cells by 50% (IC_{50} values) was determined from a prepared concentration-cell death plot of the extract.

$$\textit{Cytotoxicity}\,(\%) = \left[\frac{\textit{Absorbance of Treated cells}}{\textit{Absorbance of Control cells}}\right] * 100\% \tag{3}$$

2.7.2. Cell morphology

Having determined the IC_{50} value of the extract, a fresh population of cells was cultured in a 6-well transparent culture plate at the concentration of 5×10^3 cells/mL. The cells were incubated overnight to attach to the plates at 37 °C. After attachment, the media in the wells were replaced with fresh media containing the IC_{50} concentration of the extract. The flask was further incubated for 72 h with a 24-hourly morphological observation of the cells for signs of stress or induction of apoptosis. The morphology of the cells after 48 and 72 h was directly captured using an OLYMPUS camera mounted on an inverted Olympus light microscope (Hazrulrizawati et al., 2018).

2.8. LC-MS-QTof analysis

The phenolic content of the extracts was identified using an AGILENT 6560 UHPLC-IM-QTOF-MS/MS system. This instrument is the first commercially-available uniform field ion mobility coupled with Agilent 1290 UHPLC which can combine both LC, IM and MS selectivity and separation power. It can perform both quantitative and qualitative analysis with high-speed analysis and sensitivity. It is a hybrid quadrupole system to the mass analyzer of Ion Mobility Time-of-Flight for high-resolution accurate mass and accurate collision cross section (CCS) measurement with the added dimension of separation for the most complex samples. It uses a diode array (DA) detector in the wavelength range of 190–640 nm for the real-time preliminary analysis of separated sample mixtures during connection to a mass analyzer. For the analysis, the extract (5 mg) was solubilized in methanol (1 mL). The separation of the constituents was achieved using a mobile phase made up of 0.1% formic acid in water and acetonitrile. The sample was injected into the machine at a pre-set volume of 5 μL, maintaining a flow rate of 0.2 mL/min. The identification of the compounds was achieved by comparing the determined molecular weights to the theoretical molecular weights (MS/MS) from a standard database (NIST Mass Spectrometry Data Center) and calculating the mass error (ppm). This mass comparison and mass error calculation were automatically performed by the software inbuilt in the machine (Hazrulrizawati et al., 2018).

2.9. Data analyses

The differences in the activity of different fractions of the extracts were evaluated using non-paired Student *t*-test at a significance level of $p < 0.05$, while the correlation of the antioxidant activity of the extracts with the TPC was determined using Cronbach alpha at the same level of significance in the Microsoft Excel® (Microsoft, Redmond, WA, USA). The graphs were prepared with Origin 7.0 (Origin Labs, Massachusetts, USA).

Table 1
Total phenolic and total flavonoid contents of acetone and ethyl acetate extracts of Enoki caps.

Acetone fraction		Ethyl acetate fraction
TPC (μg GAE/mg)		
Enoki caps	62.46 ± 0.14[a]	48.41 ± 0.06[a]
TFC (μg QE/mg)		
Enoki caps	56.52 ± 0.02[b]	41.22 ± 0.20[b]

Note: TPC = Total phenolic content. TFC = Total flavonoid content. GAE = gallic acid equivalent, QE = quercetin equivalent. Different superscripts within rows represent statistically significant difference at $p < 0.05$; n = 3.

3. Results and discussion

3.1. Total phenolics and flavonoids contents (TPC and TFC)

The TPC and TFC of the acetone and ethyl acetate extracts of Enoki caps are shown in Table 1. From the results, acetone extract contained more TPC and TFC compared to the ethyl acetate fractions ($p < 0.05$). This observation could be attributed to several factors, such as the degree of polarity of the extraction solvent (acetone is more polar than ethyl acetate), the presence of side chains, glycosidic bond formation, or degree of aromatic rings conjugation. These factors interfere with the solubility of several phenolic compounds (Hazrulrizawati et al., 2016). Notably, the solubility of phenolic compounds in different solvents depends on two factors, first is the number of OH^- groups contained in the compound, and second is the degree of polarity of the solvent. Solubility largely increases with both an increasing number of OH^- groups and the solvent polarity (Liu et al., 2017). Visht and Chaturvedi (2012) reported the existence of a dipole interactive force in polar solvents and suggested that this force can increase the solubility of molecules. Different species of mushrooms contain different phytochemicals, but the biological activity of these mushroom extracts largely depend on the type of phytochemical they contain (such as simple phenolics, flavonoids, phenolic acids, or hydroxycinnamic acid derivatives) (Liu et al., 2017). The observed solvent polarity-dependent differences in the TPC and TFC of the studied fractions have also been previously reported (Addai et al., 2013; Dar et al., 2016).

3.2. Antioxidant activity

The largely reported antioxidant activity of mushroom extracts has often been attributed to the hydrogen-donating and singlet oxygen-quenching attributes of their phenolic phytochemical content (Khan et al., 2016). In this study, the extracts were tested for antioxidant activities using 3 chemical guided assays (DPPH radical scavenging, H_2O_2 radical scavenging, and FRAP (Alam et al., 2013). In determining the antioxidant activity of an extract using any free radical-based method (such as DPPH and H_2O_2), the presence of proton donors is manifested in color formation indicating the acceptance of hydrogen radicals and diamagnetic molecules formation (Baratzadeh et al., 2013). The reducing power-based assays (such as FRAP) assess the ability of the extract to reduce the oxidation state of a metal (such as the reduction of Fe^{3+} to Fe^{2+}).

The antioxidant activities of the extracts were compared to that of ascorbic acid (Figs. 1 and 2, and Table 2). Acetone extract was found to show more antioxidant activity against DPPH (IC_{50} = 0.84 mg/mL) and H_2O_2 (IC_{50} = 0.89 mg/mL) compared to ethyl acetate fraction (DPPH IC_{50} = 0.99 mg/mL and H_2O_2 IC_{50} = 1.35 mg/mL). A lower activity was noted with the ethyl acetate extract likely due to the type of phytochemicals extracted with ethyl acetate (highly non-polar) compared to those extracted with acetone. This could be due to the presence of certain groups (such as glycosides) extracted by acetone which can participate in antioxidant activities via proton transfer to radicals,

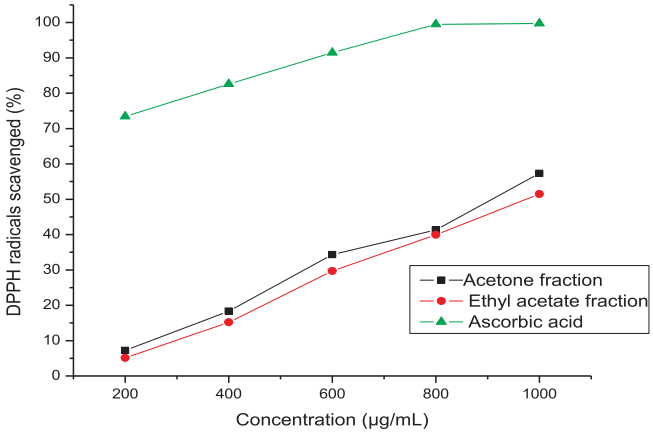

Fig. 1. DPPH radical scavenging activity of acetone and ethyl acetate fractions of Enoki.

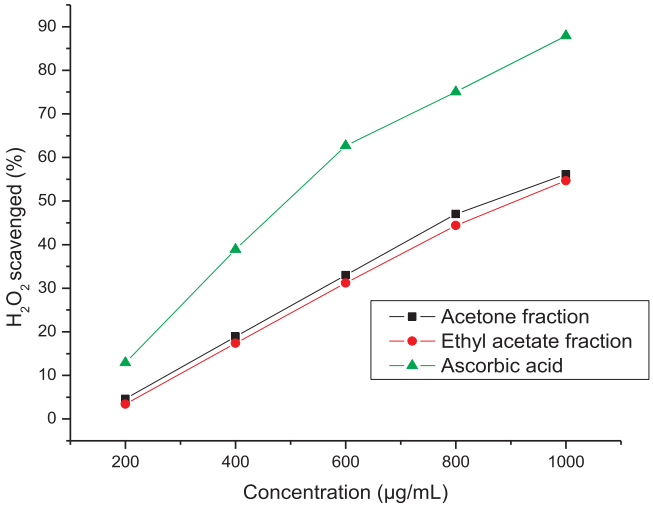

Fig. 2. H_2O_2 radical scavenging activity of acetone and ethyl acetate fractions of Enoki caps.

leading to the formation of phenoxide radicals and a consequent stabilization of unstable radicals (Hazrulrizawati et al., 2016; Siddhuraju and Becker, 2003; Ukaegbu and Shah, 2017). These results agreed with previous reports where higher extracts' antioxidant activities were reported against DPPH (Maskam et al., 2014) and H_2O_2 (Sroka and Cisowski, 2003) using solvents of varying polarities.

The observed FRAP of the extracts is presented in Table 2. The FRAP showed a similar trend of antioxidant activity to those against DPPH and H_2O_2. The table showed that a better concentration-dependent FRAP of the acetone extract compared to the ethyl acetate extract. The reason for the notable differences in the FRAP of the different fractions is not far from those earlier discussed (solvent polarity and phytochemical content). The extracts showed a significantly lower FRAP ($p < 0.05$) compared to ascorbic (Table 2). FRAP has been recommended by Hodzic et al. (2009) as a simple way of evaluating the antioxidant activity of an extract, while Schafer and Buettner (2001) suggested FRAP as a reliable way of assessing the antioxidant activity of extracts. The FRAP of mushroom extracts has previously been correlated to the ability of their inherent phytochemicals to donate hydrogen ions (Moure et al., 2001) even though there are other factors (such as solvent type) which can affect the biological activity of extracts. From the correlation studies, there was a positive relationship between the extracts' TPC and their antioxidant activities. The observed Pearson coefficients r of the relation between the TPC of acetone extract and its antioxidant activities was $r = 0.974$, while that of the ethyl acetate

Table 2
FRAP of acetone and ethyl acetate extracts of Enoki caps (Absorbance readings @ 700 nm).

Conc. (mg/mL)	0.2	0.4	0.6	0.8	1.0
Acetone fraction					
Enoki caps	0.050 ± 0.001[a]	0.098 ± 0.003[a]	0.192 ± 0.002[a]	0.275 ± 0.001[a]	0.339 ± 0.001[a]
Ethyl acetate fraction					
Enoki caps	0.029 ± 0.002[b]	0.081 ± 0.001[b]	0.174 ± 0.001[b]	0.231 ± 0.001[b]	0.291 ± 0.001[b]
Ascorbic acid	0.202 ± 0.002	0.796 ± 0.005	1.262 ± 0.001	1.824 ± 0.002	2.130 ± 0.002

Note: Different superscripts within the same column represent statistically significant difference compared to ascorbic acid at $p < 0.05$; $n = 3$.

Table 3
Anticancer activities of acetone extract of Enoki caps on MDA-MB-231, MCF-7, and MCF-10a.

	After 24 h	After 48 h	After 72 h
MDA-MB-231	184.20	133.62	114.50
MCF-7	38.36	26.34	17.70
MCF-10a	> 250	> 250	> 250
**Doxorubicin*			
MDA-MB-231	9.44	7.38	7.33
MCF-7	4.34	3.72	3.25
MCF-10a	1.46	1.03	0.94

Note: Values are presented as the concentration of extracts (μg/mL) needed to inhibit the cell line growth by 50% (IC_{50}). MCF-10a = Vero or normal breast cells. All experiments were performed in triplicate. * implies standard chemotherapeutic agent.

fraction was $r = 0.743$. This is in agreement with the report of Bazzaz et al. (2011) who reported a positive correlation between extracts' TPC and their antioxidant activities.

3.3. Cytotoxicity assay

Having shown a better antioxidant activity, the cytotoxicity of the acetone extract was studied on three cell lines using an *in vitro* MTT assay method. The extract concentration required to achieve a 50% cell death (IC_{50} value) was determined and presented in Table 3. From the table, the extract was found to be more antiproliferative against MCF-7 cell line (IC_{50} value ranged from 17.7 to 38.36 μg/mL) compared to MDA-MB-231 cell line (IC_{50} value ranged from 114.5 μg/mL–184.2 μg/mL) after 72 h. The Vero cells (MCF-10a) were highly insensitive to the extracts even at higher concentrations, showing the lower toxicity of the extracts to normal body cells compared to cancerous cells. The Vero cells were about 2.19 times less sensitive to the extract compared to MDA-MB-231 cells, and about 14.12 times less sensitive to the extract compared to MCF-7 cells. This less sensitivity of the Vero cells to the extract can be attributed to the absence of specific proteins expressed by the cancerous cells which facilitate the binding of the active phytochemicals in the extracts on the cells for the induction of apoptosis (Hazrulrizawati et al., 2016). Doxorubicin, a standard chemotherapeutic agent used as a positive control had more anticancer activity

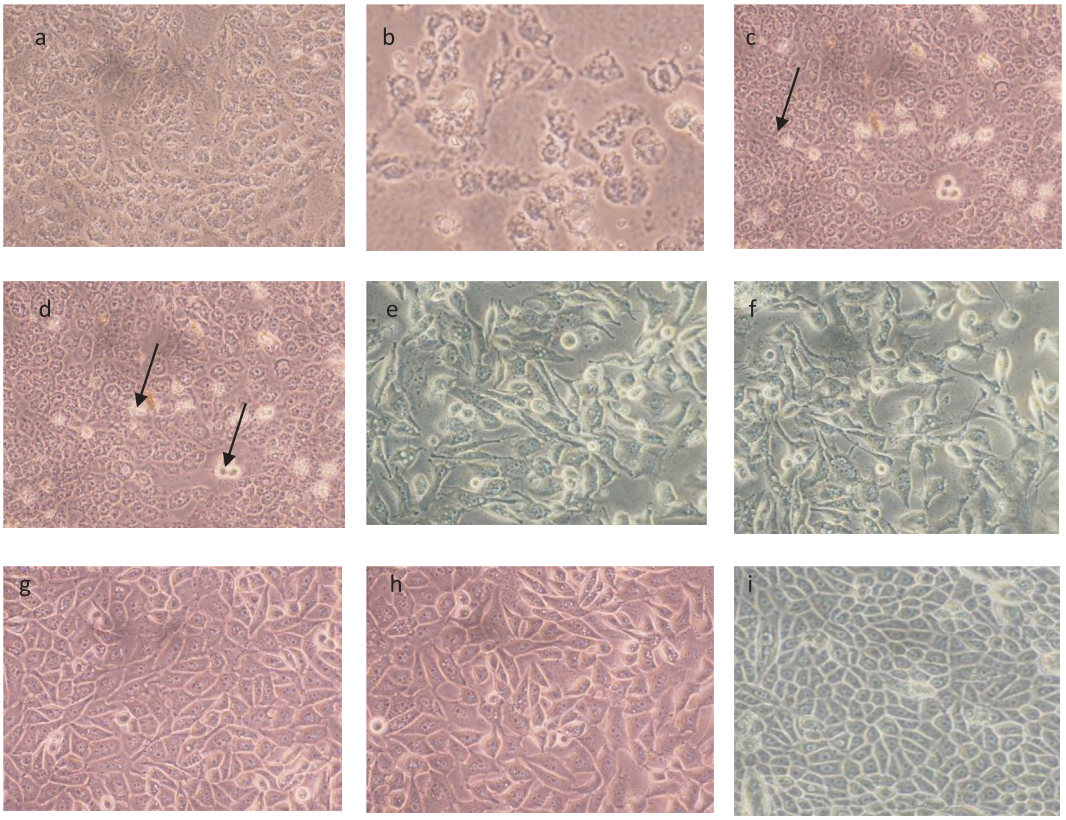

Fig. 3. Morphology of cell lines: (a) and (b) = MCF-7 after 48 and 72 h of treatment with the mushroom extract (×40 magnification). (c) and (d) = MDA-MB-231 after 48 and 72 h of treatment with the extract (×40 magnification). (e) and (f) MCF-10a after 48 and 72 h of treatment with the extract (×40 magnification). (g), (h), and (i) = Non-treated MCF-7, MDA-MB-231, and MCF-10a (×40 magnification). Black headed arrows pointing at the apoptotic (dead) cancer cells after treatment with the extract.

Table 4
List of identified compounds in the acetone fraction of Enoki caps using LC-MS-QTof analysis.

S/N	Component name	Formula	Identification status	Observed neutral mass (Da)	Observed m/z	Mass error (mDa)	Mass error (ppm)	Observed RT (min)	Response	Adducts	Observed CCS (Å^2)	Total Fragments Found
1	Norbergenin	C13H14O9	Identified	314.0623	359.0605	−1.5	−4.0	0.35	167	+HCOO	169.64	2
2	p-Tolualdehyde	C7H6O2	Identified	122.0371	167.0354	0.4	2.2	0.44	214	+HCOO	132.27	0
3	Campneoside I	C30H38O16	Identified	654.2173	699.2155	1.3	1.8	0.45	137	+HCOO	243.06	30
4	Forsythoside D	C20H30O13	Identified	478.1697	523.1679	1.0	2.0	0.45	649	+HCOO	200.75	26
5	Polygoacetophenoside	C14H18O10	Identified	346.0882	345.0810	−1.8	−5.1	0.45	420	−H	168.51	25
6	Pyrogallic acid	C6H6O3	Identified	126.0320	171.0302	0.3	1.9	0.46	290	+HCOO	155.45	0
7	Moracin G	C19H16O4	Identified	308.1030	353.1012	−1.8	−5.2	0.46	280	+HCOO	173.61	8
8	3,5-O-Dicaffeoylquinic acid	C25H24O12	Identified	516.1268	515.1196	0.1	0.1	0.46	1015	−H	201.59	32
9	3′-O-Methylbrazilin	C17H16O5	Identified	300.1009	299.0937	1.2	3.9	0.46	609	−H	159.06	10
10	Renifolin	C18H24O7	Identified	352.1534	397.1516	1.2	3.1	0.46	981	+HCOO	180.95	43
11	Renifolin	C18H24O7	Identified	352.1538	397.1520	1.6	4.1	0.46	186	+HCOO	202.85	9
12	3,4-Dihydroxyphenothyl-3-O-β-D-glucopyranoside	C12H16O8	Identified	288.0859	333.0841	1.4	4.2	0.46	398	HCOO, −H	168.72	23
13	Norbergenin	C13H14O9	Identified	314.0635	313.0562	−0.3	−0.8	0.46	474	−H, +HCOO	181.92	52
14	Polygoacetophenoside	C14H18O10	Identified	346.0900	391.0882	0.0	0.1	0.46	1055	+HCOO, −H	175.04	63
15	2′-Hydroxy-3′,4′-dimethoxy-isoflavan-7-O-β-D-glucoside	C23H28O10	Identified	464.1676	463.1603	−0.7	−1.4	0.46	156	−H	207.86	25
16	Ehretioside B	C14H17NO7	Identified	311.1013	356.0995	0.8	2.2	0.46	1038	+HCOO	173.62	40
17	Isotachioside	C13H18O8	Identified	302.0994	347.0976	−0.8	−2.3	0.47	175	+HCOO	168.08	20
18	3,4-Dihydroxybenzamide	C7H7NO3	Identified	153.0428	198.0410	0.2	0.9	0.47	615	+HCOO	153.04	0
19	1,5-Bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one	C19H18O5	Identified	326.1141	325.1068	−1.4	−4.2	0.47	2689	−H	167.23	6
20	Vanillin	C8H8O3	Identified	152.0471	197.0453	−0.2	−1.1	0.47	147	+HCOO	164.25	0
21	Norbergenin	C13H14O9	Identified	314.0641	359.0623	0.3	1.0	0.47	1298	+HCOO, −H	171.40	39
22	1-Galloyl-β-D-glucose	C13H16O10	Identified	332.0741	377.0723	−0.2	−0.6	0.47	1482	+HCOO	172.30	44
23	2,4,5-Trihydeoxy benzaldehyd	C7H6O4	Identified	154.0262	199.0244	−0.4	−2.2	0.47	234	+HCOO	129.29	0
24	Polygoacetophenoside	C14H18O10	Identified	346.0901	345.0828	0.1	0.2	0.47	178	−H	201.82	14
25	Syringaldehyde	C9H10O4	Identified	182.0574	227.0557	−0.5	−2.0	0.47	120	+HCOO	177.63	8
26	2,4,5-Trihydeoxy benzaldehyd	C7H6O4	Identified	154.0263	199.0245	−0.3	−1.6	0.47	524	+HCOO	155.56	0
27	Catechol	C6H6O2	Identified	110.0368	155.0350	0.0	0.2	0.47	117	+HCOO	163.78	0
28	Syringaldehyde	C9H10O4	Identified	182.0567	227.0549	−1.2	−5.5	0.47	217	+HCOO	186.23	7
29	2′-Hydroxy-3′,4′-dimethoxy-isoflavan-7-O-β-D-glucoside	C23H28O10	Identified	464.1665	463.1592	−1.7	−3.7	0.49	193	−H	192.54	27
30	p-Hydroxyacetanilide	C8H9NO2	Identified	151.0625	196.0607	−0.8	−4.3	0.50	159	+HCOO	139.51	0
31	3,4-Dihydroxyphene thylamine	C8H11NO2	Identified	153.0790	198.0772	0.0	0.2	0.72	293	+HCOO	138.76	0
32	Osmanthuside H	C19H28O11	Identified	432.1628	477.1610	−0.4	−0.8	3.69	2675	+HCOO	199.73	0
33	Forsythoside D	C20H30O13	Identified	478.1690	477.1617	0.4	0.8	4.20	4402	−H	199.74	1
34	Forsythoside D	C20H30O13	Identified	478.1678	523.1660	−0.8	−1.6	5.74	2609	+HCOO	205.36	3
35	Forsythoside D	C20H30O13	Identified	478.1672	523.1654	−1.5	−2.8	6.57	1652	+HCOO	206.35	4
36	Xanthoxylin	C10H12O4	Identified	196.0749	241.0731	1.3	5.5	9.30	331	+HCOO	220.44	0
37	Renifolin	C18H24O7	Identified	352.1519	351.1446	−0.3	−0.8	10.53	665	−H	179.19	0
38	6-Gingerol	C17H26O4	Identified	294.1824	339.1806	−0.8	−2.2	11.57	1953	+HCOO	178.85	0
39	Brazilein	C16H12O5	Identified	284.0688	329.0670	0.3	0.9	14.20	3897	+HCOO	175.23	0

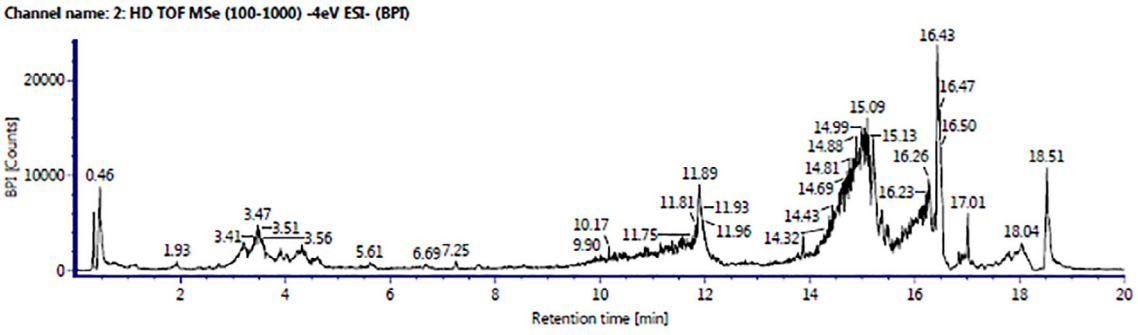

Fig 4. Base Peak Intensity plot of LC-MS-QTOF-identified phytochemicals in acetone fraction of Enoki caps showing the retention times of the phytochemicals.

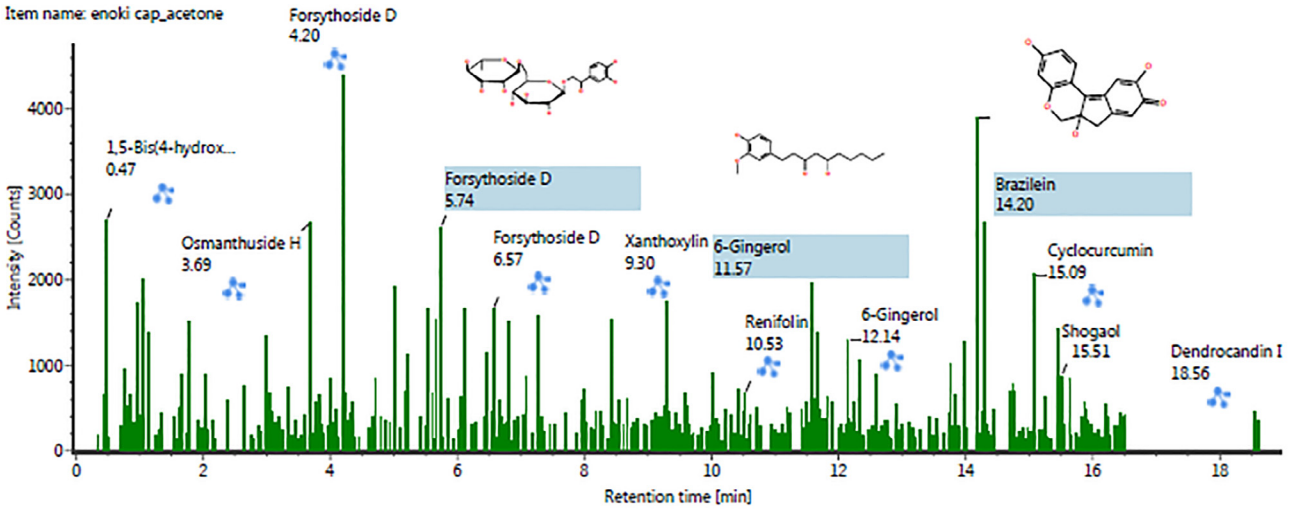

Fig. 5. Identities and retention times of some LC-MS-QTOF-identified phytochemicals in the acetone fraction of Enoki caps.

against both cells (IC_{50} value ranged from 12.3 to 17.4 μg/mL against MDA-MB-231, and 5.4–7.7 μg/mL against MCF-7) compared to MCF-10a. This higher cytotoxicity of doxorubicin on the Vero cells can be attributed to the non-selective side effects of the chemotherapy agent.

Studies have previously reported several mushrooms as a source of medicinal agents (Liu et al., 2017; Marijana et al., 2016). Various mushrooms have historically been used to treat various diseases, including cancer owing to their abundant steroids, polysaccharides, proteoglycans and other bioactive content (Ding et al., 2012). Anticancer agents are known to act as reactive oxygen species inducers, angiogenesis inhibitors, mitotic kinase inhibitors, topoisomerase inhibitors, as well as inducers of apoptosis (Patel and Goyal, 2012). Reports on the anticancer activity of edible mushrooms exist in the literature. Kim et al. (2010) reported the *in vivo* cytotoxicity of *L. deliciosus*-derived hetero-polysaccharides. Similarly, Xu et al. (2012) reported the capability of *M. procera* extract to inhibit the metastasis of 26- M3.1 cells.

3.3.1. Cell morphology

The morphology of the cell lines after 48 and 72 h of treatment with the extract are shown in Fig. 3. The figures showed marked alterations and deformations on the surface of the cells which could be attributed to the extract-induced stress. A comparison of the treated and non-treated cells showed marked morphological differences. The non-treated cells were observed to have a better confluency compared to the distorted proliferation observed in the treated cells. Dead or apoptotic cells were also observed in the cells treated with the extracts (black headed arrows in the figures), showing the ability of the extract to induce cell death.

3.4. LC-MS-QT of analysis

Being that phytochemicals are responsible for the observed anticancer activity of the extract, a mass spectrometry analysis of the acetone fraction of Enoki caps was carried out to identify its phytochemical content. A complete list of the phytochemicals identified in the extract is shown in Table 4, while the base peak intensity (BPI) chromatograph (with retention times) and the structure of some of the identified compounds are presented in Figs. 4 and 5, respectively. Some of the identified compounds in the extract are xanthoxylin, 6-gingerol, cyclocurcumin, dendrocandin 1, osmanthuside H, and forsythoside D. A previous study has reported the ability of xanthoxylin in combination with a novel ruthenium complex to mediate ERK1/2 apoptosis and induce S-phase arrest in HepG2 cells via a p53-independent pathway (de Carvalho et al., 2018). The concentration-dependent cytotoxicity of 6-gingerol against HCT15, L929, and Raw 264.7 has been reported as well (Kumara et al., 2017). The antioxidant, anticancer, anti-inflammatory, antimicrobial, cardioprotective, neuroprotective, and radioprotective effects of cyclocurcumin have been reported (Ataie et al., 2010). These compounds were identified by comparing their respective molecular weights to the theoretical molecular weights (MS/MS) from a standard database (NIST Mass Spectrometry Data Center) and calculating the mass error (ppm).

4. Conclusion

In this study, both acetone and ethyl acetate extracts of Enoki caps were biologically characterized for antioxidant activities, but only acetone extract was evaluated for *in vitro* anticancer activity against MCF-7 and MDA-MB-231 (breast cancer cell lines) and MCF-10a (Vero cells) due to its higher antioxidant activity compared to ethyl acetate extract ($p < 0.05$). Furthermore, the anticancer activity evaluation showed a selective activity of the acetone extract against the studied cancer cells compared to the Vero cells. The proliferation of MCF-7 cell line was highly inhibited compared to that of MDA-MB-231, while the Vero cells almost showed no sensitivity to the extract. Therefore, it is concluded that Enoki caps contain phytochemicals which can help prevent the building up of free radicals in the body, and also curbed the development of certain types of cancer.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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