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ORIGINAL ARTICLE

In Vitro Cytotoxicity and Antioxidant Activities of *Pestalotiopsis microspora* Culture Filtrate

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Abstract

Endophytic fungi have been studied to provide protection and survival conditions to their host plant by producing a plethora of substances which, once isolated and characterized, may also have potential for use in industry, agriculture, and medicine. In this study, the culture filtrate of an endophytic fungus (*Pestalotiopsis microspora* (PM)) was evaluated for its cytotoxic and antioxidant activities in vitro. The cytotoxic activity of PM was determined using brine shrimp lethality assay (BSLA), while its antioxidant effect was investigated against DPPH, reducing power and hydroxyl radicals. Judging by the LC_{50} value of 2.71 $mg \cdot mL^{-1}$ for the BSLA, the culture filtrate could be considered highly potent. The PM also significantly scavenged free radicals and the effects elicited could be attributed to its phenolics and other phytoconstituents as revealed by the GC-MS results. It is thereby evident from the data presented that PM is endowed with chemotherapeutic constituents that could be potentially useful for the development of new lead anticancer agents.

Keywords: Bioactive metabolites; Cytotoxicity; Endophytic fungi; *Pestalotiopsis microspora*

Introduction

Endophytes are organisms, usually fungi and bacteria, that inhabit plant tissues without causing any manifestation of physiological abnormality in the plant (Bacon and White 2000; Harper *et al*., 2003). They live in aerial parts (leaves, stems, barks, petiole, reproductive structures and roots) of the plant and as such makes them distinct from the mycorrhizal symbionts (Faeth and Fagan, 2002; Firakova *et al*., 2007). Endophytes are promising source of natural bioactive compounds that are pharmaceutically important with ability to fight against pathogens and cancerous cells in humans (Agrawal *et al*., 2014; Kusari *et al*., 2014).

Fungi are achlorophyllous and hence heterotrophic. They establish different relationship with autotrophs, living in mutualistic as well as antagonistic co-existence (Dayle *et al*., 2001). These class of organism have been identified to contain enormous bioactive compounds with potential antimicrobial properties and one of such was penicillin from *Penicillium notatum* as discovered by Alexander Fleming in 1928 (Agrawal *et al*., 2014). Also,

the immunosuppressive drug known as cyclosporine was isolated from *Tricoderm polysporum* and *Cylindrocarpon lucidum* (Webber, 1981). An anticancer agent, taxol, was obtained from the fungus *Taxomyces andereanae* (Sterile *et al.,* 1993). Endophytic fungi, especially those that are associated with medicinal plants, are a cheap source of antioxidant and cytotoxic compounds (Agrawal *et al.,* 2014). *Fusarium pseudonygamai, Colletotrichum dematium, Acremonium strictum, Botryodiplodia theobromae, Myrothecium verrucaria, Nigrospora oryzae* and *Pestalotiopsis* spp. are common endophytic fungi (Nalini *et al*., 2014). *Pestalotiopsis microspora* is one of the endophytic fungi that is most widely distributed and produce bioactive organic substances including taxol which is an anticancer drug (Strobel *et al*., 1996). This fungus is known to produce certain natural metabolites such as isopestacin and pestacin (Zhang and Demain, 2007).

Despite the discovery of ability of endophytic fungi to produce bioactive secondary metabolites that are pharmaceutically and industrially useful, exploration of these organisms is still in its infancy. Hence, studies on the bioactivities of the active compounds from these endophytic fungi like the *P. microspora* have become imperative and have prompted the present research. This work evaluated the *in vitro* cytotoxicity and antioxidant potential of the culture filtrate of *P. microspora.* The GC-MS analysis of its culture isolate was also performed.

Materials and Methods

Preparation of Fungal Filtrate of Pestalotiopsis microspora

The pure cultures of *P. microspora* (Figure 1) was collected from Mycology Laboratory, Department of Plant Biology, University of Ilorin, Ilorin, Kwara State, Nigeria. The fungus was grown in 250 mL sterile conical flask containing 100 mL Potato Dextrose Broth and incubated at 25±2 °C for 14 days (Garuba *et al*., 2014). After incubation, the culture filtrates were filtered into pre-sterilized conical flasks using Whatman no. 1 filter paper. The filtrates were stored in a refrigerator at 4±2 °C.

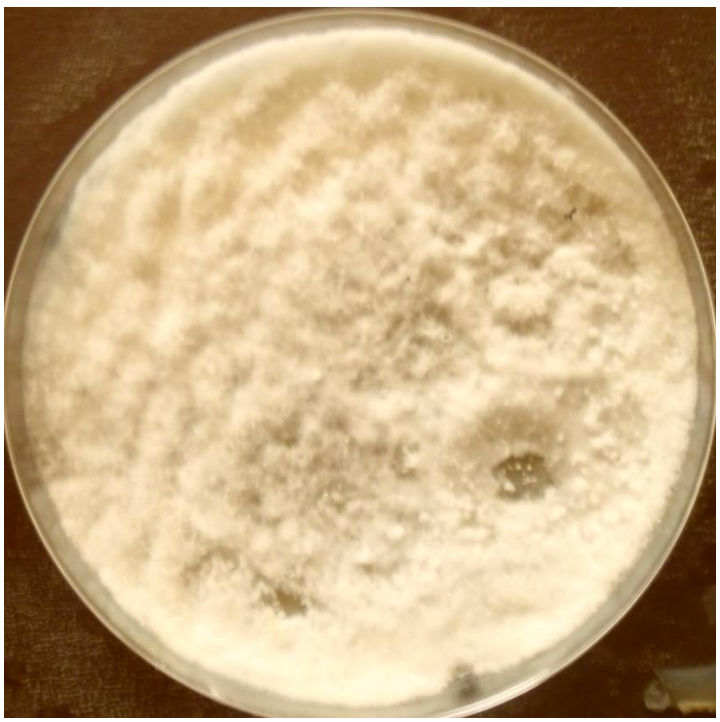

Figure 1. Pure culture of *Pestalotipsis microspora*

Cytotoxic Activity of *Pestalotiopsis microspora* Culture Filtrate

Adopting the method of Meyer *et al.* (1982), the Brine shrimp lethality test (BST) for the isolate was performed. Briefly, filtered seawater (500 mL) was put in a small hatching chamber with a partition for dark (covered) and light areas. Shrimp eggs (30 mg) were added into the dark side of the chamber while the lamp above the other side (light) was positioned to attract hatched shrimps. The set up was left for 48 h to allow shrimps to hatch and mature as nauplii (larvae). After incubation, when the larvae were ready, different concentrations (10 - 100 $mg \cdot mL^{-1}$) of the isolate were prepared from the stock solution and ten nauplii were transferred into each vial using Pasteur pipettes. The control vials were prepared using seawater only. Following 24 h of incubation under lamp at room temperature, the experiment was terminated and larvae were considered dead if they did not exhibit any observable movement during several seconds of observation. The number of survivors in each vial was counted and percentages of mortality calculated for each concentration. Using probit analysis, the lethality concentration (the concentration of the filtrate causing 50% mortality of nauplii (LC_{50}) was evaluated at 95% confidence intervals. LC_{50} values of less and greater than 100 $mg \cdot mL^{-1}$ were respectively considered toxic and non-toxic (Moshi *et al*., 2010).

Antioxidant Activities of Pestalotiopsis microspora Culture Filtrate

DPPH Radical Scavenging Assay

Using the method described by Turkoglu *et al*. (2007), the antioxidant activity of the culture filtrate was determined by measuring its capacity of bleaching the purple- coloured ethanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). Exactly 200 µL of varying concentrations (10 – 100%) of the culture filtrate in methanol was added to 500 µL of 0.2 $mmol \cdot mL^{-1}$ sample of DPPH in methanol. After 30 min incubation period at room temperature, the absorbance was read against blank at 517 nm. Ascorbic acid was used for the control. Inhibition rate (1%) of the filtrate on the DPPH radical was calculated using the expression:

$$Percentage\ Inhibition\ (I\%) = \left(\frac{A_{control} - A_{culture\ filtrate}}{A_{control}}\right) \times 100 \quad \ldots (1)$$

Where, $A_{control}$ is the absorbance of the control, $A_{culture\ filtrate}$ is the absorbance of the filtrate.

Reducing Power Potential of *Pestalotiopsis microspora* Culture Filtrate

The reducing power of the culture filtrate was evaluated by adding 200 µL of the culture filtrate (10 – 100%) in 1 mL of distilled water and mixed with 500 µL of 0.2M phosphate buffer (pH 6.6) and 500 µL of 1% potassium ferricyanide ($K_3Fe(CN)_6$). The mixture was incubated at 37°C for 30 min prior to addition of 500 µL of trichloroacetic acid (TCA). Following centrifugation at 3000 rpm for 10 min, 2.5 mL of the supernatant was mixed with an equal amount of distilled water and 0.5 mL of 0.1% $FeCl_3$. Ascorbic acid was used for the control. The absorbance of the resulting solution was then read at 700 nm (Oyaizu, 1986).

Hydroxyl Radical Scavenging Assay

The reported procedure of Smirnoff and Cumbes (1996) was used for this assay. Briefly, 200 µL of varying concentrations (10 – 100%) of the culture filtrate was addede to 600 µL of $FeSO_4$ (8 mM), 500 µL of H_2O_2 (20 mM) and 2 mL of salicylic acid (3 mM) in a test tube. Following a 10 min incubation period at 37°C, distilled water (2.9 mL) was added and the resulting mixtures were shaken and allowed to settle for 15 min. The absorbance was subsequently read at 510

nm and percentage OH radical scavenging capacity of the filtrate was estimated as per the expression:

$$Percent\ hydroxyl\ radical\ scavenged\ (\%) = \left(\frac{A_{control} - (A_{sample} - A_{culture\ filtrate})}{A_{control}}\right) \times 100 \quad \ldots (2)$$

Where, $A_{control}$, A_{sample} and $A_{culture\ filtrate}$ represent the absorbance of the mixture without filtrate, mixture with the filtrate and that of the filtrate alone, respectively.

Quantification of Phenol

Following the reported method of Wolfe *et al*. (2003), the total phenol contents in the culture filtrate was determined. Briefly, an aliquot of the filtrate was mixed with 1.58 µL of distilled water and 100 µL of Folin ciocalteu reagent. After 5 min at room temperature, 300 µL of Na_2CO_3 (75 g·L^{-1}) was added. The tubes were vortexed for 15 sec and allowed to stand for 30 minutes at 40 ºC for colour development. Absorbance was read at 765 nm using a spectrophotometer. The filtrate was evaluated at a final concentration of 1 mg·mL^{-1}. Total phenolic content was expressed as mg/g gallic acid equivalent using the equation obtained from a calibration curve of gallic acid.

Detection of Bioactive Compounds by GC-MS Analysis

The isolate was subjected to GC-MS analysis using a Shimadzu GC-MS-QP 2010 Plus fitted with a RTX-5 (60 m × 0.25 mm × 0.25 µm) capillary column. The instrument was set to an initial temperature of 70 ºC, and maintained at this temperature for 2 min. At the end of this period the oven temperature was rose up to 280 ºC, at the rate of an increase of 5 °C·min^{-1}, and maintained for 9 min. Injection port temperature was ensured as 260 ºC and Helium flow rate as 1 mL·min^{-1}. The ionization voltage was 70 eV. The sample was injected in split mode as 10:1. Mass spectral scan range was set at 45–450 (m/z). Subsequently, the identification of bioactive compounds present in the isolate was performed by comparing its mass spectra with data from National Institute of Standards and Technology (NIST05, US), Wiley 8, and Flavour and Fragrance Natural and Synthetic Compounds (FFNSC1.3) libraries. The name, molecular weight and structure of the identified constituents of the sample were established.

Results

The degree of lethality was directly proportional to the concentration of the culture filtrate. Maximum mortality (100%) was observed at the highest investigated concentration. Hence, the brine shrimp lethality of the *P. microspora* culture filtrate was concentration-dependent (Table 1).

Table 1. Cytotoxicity of *Pestalotipsis microspora* culture filtrate on brine shrimp nauplii

Conc. (mg·mL^{-1})	Log of Conc. (mg·mL^{-1})	Total no. of larvae	Number of dead nauplii / Test		Average mortality	% mortality
			T_1	T_2	$T_{1/2}$	
10	1.00	10	3	2	2.50 ± 0.71	25.00
20	1.30	10	8	7	7.50 ± 0.71	75.00
30	1.47	10	8	8	8.00 ± 0.00	80.00
40	1.60	10	9	7	8.00 ± 1.41	80.00
100	2.00	10	10	10	10.00 ± 0.00	100.00
LC_{50}						2.71

$T_{1/2}$values are means ± standard deviation of two determinations.

The *in vitro* antioxidant effects of the culture filtrate of *P. microspora* are shown in Figures 2-3. The filtrate dose dependently inhibited or scavenged the generated radicals in all the assays investigated. The capability of the filtrate to scavenge DPPH and hydroxyl radicals revealed significant effects when compared with ascorbic acid (Figures 2a and 2b). Furthermore, at the highest tested concentration (100%), the reducing power effect of the filtrate on ferric ion competed well with that of ascorbic acid (Figure 3).

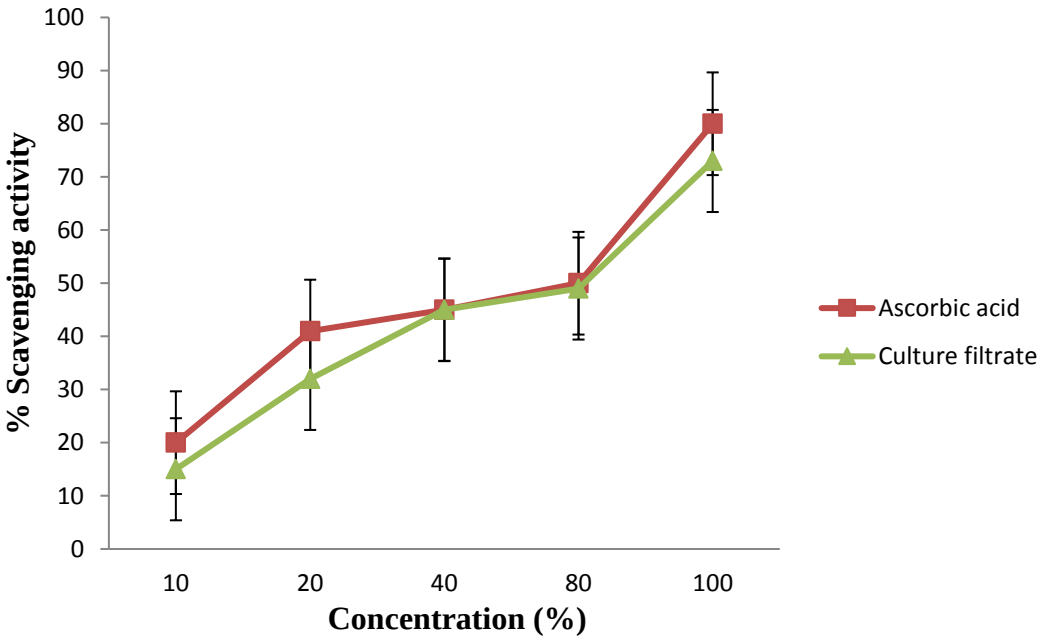

Figure 2a. DPPH radical scavenging effect of *Pestalotiopsis microspora* culture filtrate

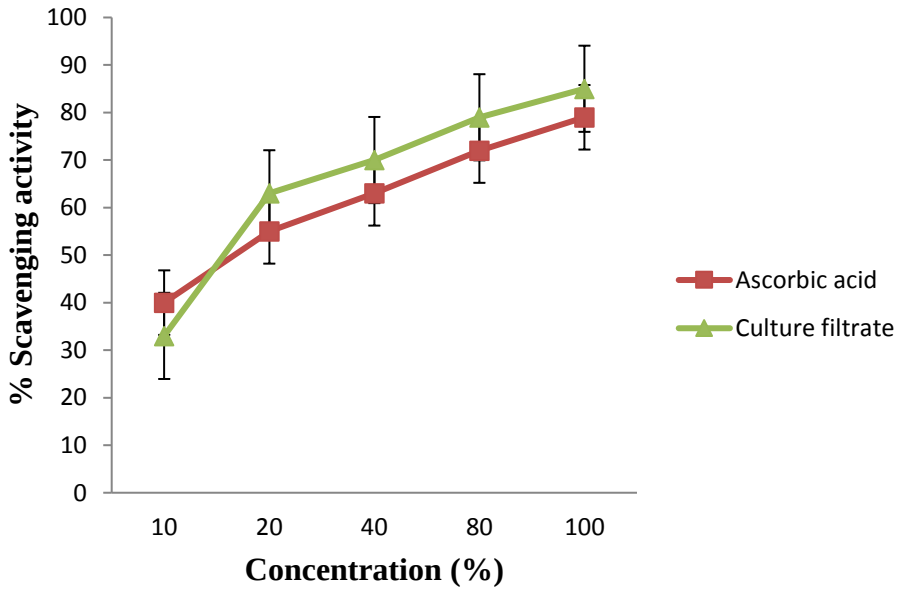

Figure 2b. Hydroxyl radical scavenging effect of *Pestalotiopsis microspora* culture filtrate

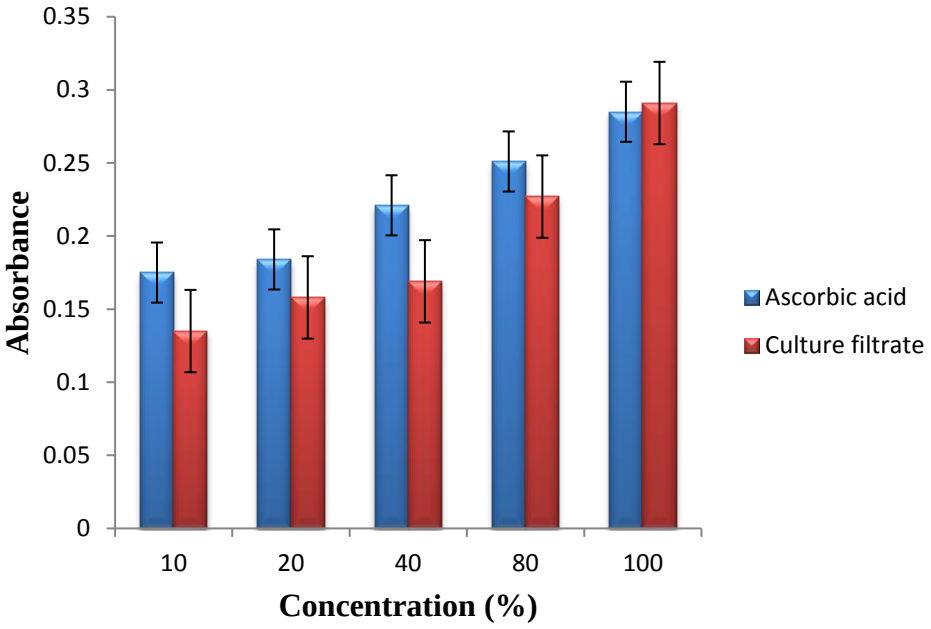

Figure 3. Reducing power potential of *Pestalotiopsis microspora* culture filtrate

The standard calibration curve for the estimation of the phenolic content of the *P. microspora* filtrate is presented in Figure 4. The estimated phenolic content of the filtrate was 45.85 ± 1.25 mg gallic acid g^{-1}.

The GC-MS results of the fungal filtrate revealed the presence of 1-adamantyl fluoroformate (47.92%), 6-nitro-8-methoxy-2H-chromene (15.08%), 1-acetyl-3-methoxycarbonyl-β-carbolin (8.21%), isoxazolidine (6.72%) and indolizine, 2-(4-methylphenyl)- as its major bioactive metabolites (Figure 5 and Table 2).

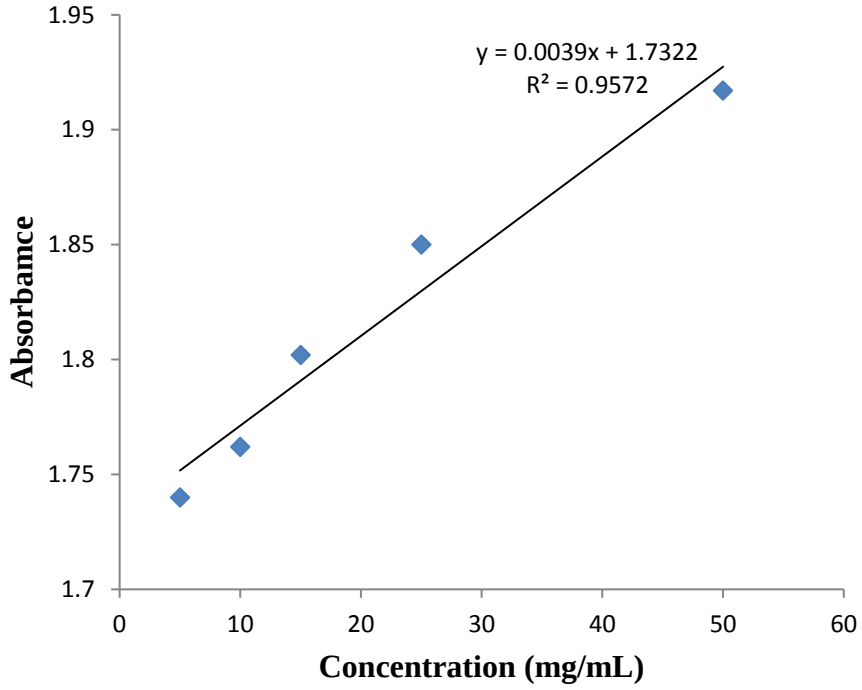

Figure 4. Gallic acid standard curve for the estimation of phenolic contents of culture filtrate.

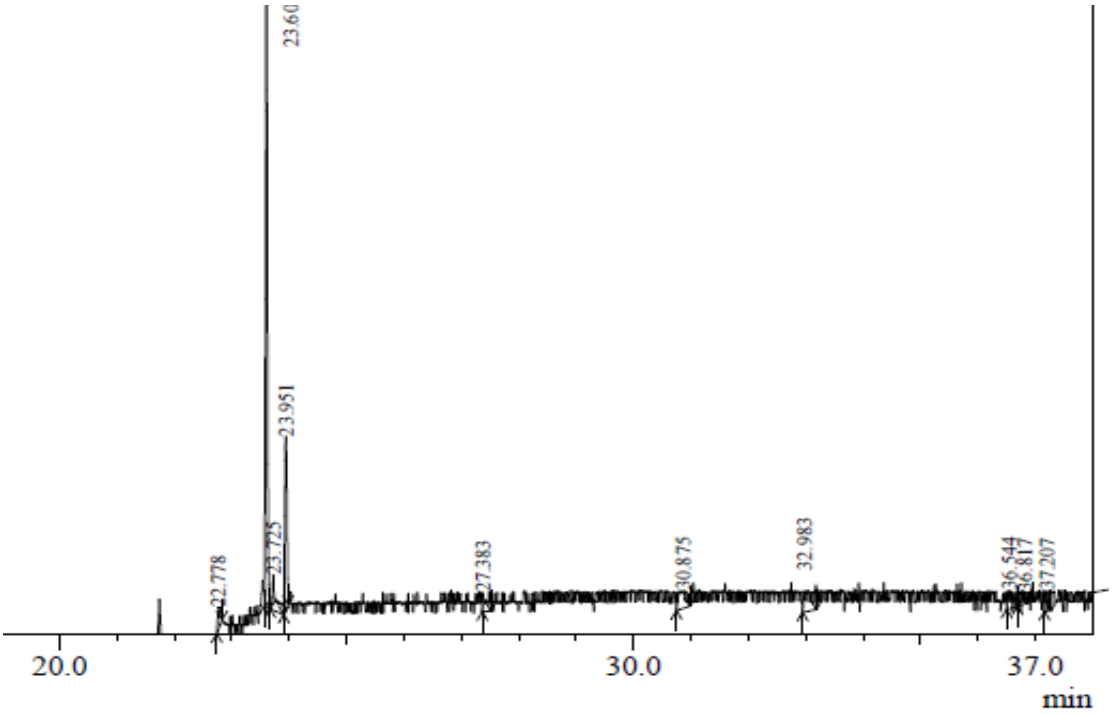

Figure 5. GC-MS chromatogram of *Pestalotiopsis microspora* culture filtrate

Table 2. Bioactive metabolites of *P. microspora* culture filtrate as revealed by GC-MS chromatogram.

Peak	RT	Area (%)	Compound	MF	Activity	Reference
1	22.78	2.54	Yadanzioside D	$C_{22}H_{28}O_{11}$	Antimalarial, antidysentary	Weici and Gerhard (1992)
2	23.61	47.92	1-adamantyl fluoroformate	$C_{11}H_{15}FO_2$	Antioxidant, antiviral	Maugh (1979)
3	23.73	5.90	Indolizine, 2-(4-methylpheny)-	$C_{15}H_{13}N$	Antitumor	Sathyaprabha *et al.* (2011)
4	23.95	15.08	6-nitro-8-methoxy-2H-chromene	$C_{10}H_9NO_4$	Antioxidant, anti-inflammatory,immunomodulatory, anticancer	Zachariah and Thomas (2013)
5	27.38	3.50	1,2,2-triphenyl-3,3-bis(trimethylsilyl)phosphiran	$C_{26}H_{33}PS_{12}$	-	-
6	30.88	6.72	Isoxazoline	C_3H_7NO	Anticancer, antiviral	Kokosza *et al.* (2015)
7	32.98	8.21	1-acetyl-3-methoxycarbonyl-β-carbolin	$C_{15}H_{12}N_2O_3$	Anticancer	Figueiredo *et al.* (2011)
8	36.54	3.59	Heptanol	$C_7H_{14}O$	Anti-Arrhythmic	Tse *et al.* (2016)
9	36.82	3.55	N-N-diethyl-2-formyl-3-methoxybenzamide	$C_{13}H_{17}NO_3$	-	-
10	37.21	3.00	1-chloro-1,2,2,2-tetrafluoroethane sulfinylchloride	$C_2Cl_2F_4OS$	Bronchodilator	Shulman and Sadove (1967)

RT= retention time (min.), MF= molecular formula

The lethality of a test sample against brine shrimps has been previously utilized and it is a very useful tool to screen a wide range of chemical compounds for various bioactivities (Meyer *et al*., 1982). It has been demonstrated that BST correlates reasonably well with cytotoxic and other biological properties (Mclaughlin *et al.,* 1991) and has been established as a safe, practical and economic method for determination of bioactivities of synthetic compound (Almeida *et al.,* 2002) as well as plant and microbial products of fungal origin (Meyer *et al.,* 1982; Sathyaprabha *et al.,* 2011; Sabiu and Ashafa, 2016). The significant correlation between the BST and *in vitro* growth inhibition of human solid tumor cell lines demonstrated by the National Cancer Institute, USA is significant because it shows the importance of this bioassay as a pre-screening tool for antitumor drug research (Anderson *et al.,* 1991). Many studies showing cytotoxic properties of either plant or fungal isolates in BST have also implicated their active metabolites in a diverse number of activities ranging from antifungal, pesticidal, teratogenic, antiviral, immunomodulatory, anti-inflammatory and antioxidant (Vanhaecke *et al.,* 1981; Sathyaprabha *et al.,* 2011; Zachariah and Thomas*,* 2013; Alayande *et al.*, 2017). In this study, the *P. microspora* culture filtrate exhibited good cytotoxic activity against the brine shrimps and this may be considered as significant. Such effect could be ascribed to the filtrate containing active or potent components as shown from its GC-MS chromatogram. A significant number of the identified constituents in the filtrate have been reported to potentiate either antitumor or anticancer properties (Table 2) and as such supporting the candidature of the *P. microspora* culture filtrate as a probable new lead in the pursuit of new anticancer drug. Previous studies on microbial cultures have also demonstrated similar cytotoxic activity and the effects were attributed to their bioactive metabolites (Sathyaprabha *et al.,* 2011; Sharma *et al.,* 2016).

Phenolic compounds have been studied to either inhibit cancer cells through induction of xenobiotic metabolizing enzymes that alter metabolic activation of potential carcinogens or alter hormone production that subsequently inhibit aromatase to prevent the development of cancer cells (Prasad *et al*., 2009). They could also reduce the amount of cellular protein, mitotic index and colony formation during cell proliferation (Gu and Sivam, 2006). Interestingly, the *P. microspora* culture filtrate investigated in this study was found to be rich in phenolics and this is another probable fact supporting its anticancer activity. This assertion also agrees with the report of Uddin *et al*. (2011), where antioxidant and anticancer effects were directly associated with a good number of secondary metabolites including phenolics. Furthermore, the relationship between generation of free radicals and the pathogenesis of cancer has been demonstrated (Jose and Janardhanan, 2000). In the present study, the culture isolate of *P. microspora* exhibited significant anti-radical activities when compared with the reference antioxidant (ascorbic acid) used. This observation is suggestive of the capability of the isolate to stall free radicals chain reactions associated with carcinogenesis. This effect may also be attributed to either the phenolic constituents of the isolate or its other phytonutrients as evidently identified in it chromatogram.

Conclusion

This study indicated that *P. microspora* culture filtrate is rich in phenolics with significant antioxidant and cytotoxic properties. The observed effect of the culture filtrate on the brine shrimps also suggested the presence of potent cytotoxic and probably antitumor constituents. Overall, these results have supported the antioxidant potential of *P. microspora* culture filtrate and presenting it as viable candidate in the development of new anticancer agent.

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