



Chemical compositions, cytotoxicity and antioxidant activity of the endophytic fungus *Fusarium napiforme* isolated from *Psidium guajava*

Chutulo EC^{1,2,3}, Chalannavar RK^{4*} and Pramod Kumar P⁵

¹Department of Studies & Research in Microbiology, Mangalore University, Jnana Kaveri, P.G. Centre, Chikka Aluvara, Kodagu-571232, Karnataka, India

²Department of Biosciences, Mangalore University, Mangalagangothri-574199, Karnataka, India

³Department of Biology, Wolaita Sodo University, Wolaita Sodo, Ethiopia

⁴Department of Applied Botany, Mangalore University, Mangalagangothri-574199, Karnataka, India

⁵Department of Biochemistry, CSIR-Central Food Technological Research Institute, Mysuru-570020, Karnataka, India

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Abstract

The bioactive secondary metabolites from the endophytic fungus *Fusarium napiforme* was evaluated for the cytotoxic effect and antioxidant activity. The total antioxidant capacity (TAC) of the extract was determined by 2, 2-diphenyl-1-picrylhydrazyl (DPPH), phosphomolybdate, and reducing power assay methods. The cytotoxicity of the extract was evaluated against lung adenocarcinoma (A549) cells and mouse embryo fibroblast (NIH3T3) cells by methyl thiazolyl tetrazolium (MTT) method. The major composition of the crude extract was identified by the Gas Chromatography-Mass Spectrometry (GC-MS) analysis. Estimation of the endophyte crude extract revealed a high amount of the total flavonoid content (TFC) and total phenolic content (TPC). The extract showed high cytotoxic activity against the A549 cell line with the mean cytotoxicity of $69.74 \pm 0.49\%$. The extract did not show any cytotoxic effect against the NIH3T3 cell line. The extract exhibited high antioxidant activity as a function of the concentrations. At a test concentration of 1 mg ml^{-1} , the extract showed the highest inhibition against DPPH radical at $75.4607\% \pm 0.47688$, ferric ion reducing power at 0.882 ± 0.0120 , and 255.434 ± 21.404 AAE/g of the extract by phosphomolybdenum assay (PMA). There is a significant correlation between TPC and antioxidant activity at $p < 0.05$. The correlation between reducing power and DPPH is significant at $p < 0.01$. The major types of bioactive compounds identified by the GC-MS have shown the presence of nine major compounds. This result strongly exhibits that the endophyte *F. napiforme* can be a potential source for the formulation of natural anticancer drugs and protecting the body from oxidative damages.

Keywords – flavonoids – GC-MS analysis – lung adenocarcinoma cells – mouse embryo fibroblast cells – MTT assay – phenolics

Introduction

Fungal endophytes are fungi that inhabit internal plant tissues without harming their host (Schulz & Boyle 2005). The endophytic interaction of fungi and the host plant extends from mutualism to latent pathogens depending on plant immunity (Strobel 2002). The endophytic fungi

are promising sources of naturally occurring cytotoxic compounds (Danagoudar et al. 2018, Handayani et al. 2018). Endophytic microbes copy the biochemical mechanism of their host plants and produce the natural product(s) similar to the host (Venieraki et al. 2017, Fischer & Schüffler 2018, Naik et al. 2019). There are few reports on the bioactive endophytic fungi from *Psidium guajava* despite its wide ethnomedicinal uses. Bioactive compounds of an endophytic fungus *Phomopsis* sp. (Thu et al. 2019) and four endophytic *Aspergillus* species (Susilawati et al. 2018) isolated from *Psidium guajava* showed antimicrobial activity. The endophytic fungi KL-1.1 (Okoye et al. 2015), *B. ochroleuca* M21 (Li et al. 2016), and *Alternaria atrans* (Yang et al. 2019) associated to *Psidium guajava* showed antiproliferative activity against cancer cell lines.

Psidium guajava L. (Myrtaceae) is a food crop and medicinal plant that grows in tropical and subtropical countries (Lim 2012, Hiwale 2015). Different parts of the plant have been used in the ethnomedicinal preparation for the treatment of different human diseases (Shu et al. 2012, Paniagua-Zambrana et al. 2020) such as diarrhea, stomach ache, wounds, cold, cough, and fever; diabetes and anthelmintic (Khare 2007, Lim 2012). In India, the species of guajava has been used for the treatment of fever, muscle spasm, and rheumatism (Morton 1987), diarrhea, piles and vomiting (Debbarma et al. 2017), jaundice, infections, hypertension, and heat stroke (Raj et al. 2018). The leaves of *P. guajava* are well investigated for the isolation of different types of natural products with antioxidant activity and antimutagenic potentials (Zahin et al. 2017). The plant is a reservoir of diverse bioactive metabolites such as pentacyclic triterpenoid (Begum et al. 2007), the cytotoxic and antioxidant guavinoside (Feng et al. 2015), psiguanins (Shao et al. 2012), phenolics, flavonoids, carotenoids, essential oils and others (Gutiérrez et al. 2008).

Fusarium napiforme, an endophytic fungus isolated from the mangrove plant, *Rhizophora mucronata* produced two new naphthoquinone derivatives with moderate antibacterial activity (Supratman et al. 2019). The cytotoxic compound taxol was produced by the endophytic fungus *Taxomyces andreanae* isolated from Pacific yew (Stierle et al. 1993). The production of yew taxol and endophytic taxol from yews as well as other plant sources are reported (Strobel 2002). Cancer is one of the increasingly deadly diseases in the entire world (Torre et al. 2016). The morbidity and mortality rate of the human population now a day increases due to the cancerous growth of the cells, particularly in low-income countries. The global cancer burden is significant and increasing (Willans & Jankowski 2019, WHO 2020). Currently, the number of anticancer compounds isolated from endophytic fungi has been increasing (Fischer & Schüffler 2018) and 57 % of novel or analogs compounds were reported from endophytic fungi (Kumar et al. 2014). Most importantly, apart from its safety and reproducibility, the bioprospecting of natural products from the endophytic microorganisms prevent the extinction of the host plants (Aly et al. 2013).

Furthermore, the endophytic fungi isolated from medicinal plants produced important bioactive secondary metabolites in industry, agriculture, and medicine (Meena et al. 2019). Owing to this, the natural products from endophytic fungi receive increasing attention to prospecting the wide spectrum of biological potentials such as natural antioxidant activity, cytotoxic effects, antimicrobials, and anti-inflammatory activity (Gómez & Luiz 2018). Nevertheless, the endophytic fungi remain a vastly underexplored reservoir for natural product discovery (Hillman et al. 2017). Therefore, this work aimed to identify the major chemical composition of the extract (GC-MS), evaluate the phenolic compounds, flavonoids, and *in vitro* antioxidant activity of the bioactive secondary metabolite isolated from endophytic *F. napiforme* from *Psidium guajava*. The cell toxic effect of the extract was also determined against lung adenocarcinoma (A549) cells and mouse embryo fibroblast (NIH3T3) cells.

Materials & Methods

Fungus culture and extract preparation

Fungal endophyte, *F. napiforme*, isolated from the leaves of *Psidium guajava* in our previous report (Chutulo & Chalannavar 2020, in press) was selected for this study based on its preliminary biological activity. We used the extract of *F. napiforme* culture preserved in the refrigerator. The

crude extract was dissolved in methanol at a concentration of 1 mg ml⁻¹ and filtered by Millipore filter (0.45 µm) from which the working concentration was constituted (Barros et al. 2007).

Total phenolic content estimation

The total phenolic content of the crude extract was estimated with the Folin–Ciocalteu reagent method with modifications (Spanos & Wrolstad 1990, Zahin et al. 2010). Both the fungal extract and gallic acid standard were prepared in methanol at the concentration of 10, 20, 40, 60, 80, and 100 µg ml⁻¹ (Zahin et al. 2010). To 0.5 ml of each extract, 2.5 ml of 0.2 N Folin–Ciocalteu's reagent was added, mixed by gentle shaking, and kept for 5 minutes. Then, 2 ml of Na₂CO₃ (7.5%, w/v) was added to the mixtures and incubated at 30°C for 20 min. Three replicates were maintained per each experimental procedure. The absorbance of the sample was recorded at 765 nm (Zahin et al. 2017) using a UV/vis spectrophotometer. The phenolic content of the extract was estimated from the standard curve of Gallic acid and the result was expressed in Gallic acid equivalent per gram (GAE/g) of extract.

Total flavonoid content determination of the extract

The content of the total flavonoid of the endophytic fungal extract was evaluated by the AlCl₃ method (Qiu et al. 2010). Quercetin equivalent (QE) was used to prepare the standard curve in different concentrations (25, 50, 100, 200, and 400 µg ml⁻¹). Extract (1 ml) was mixed with 1 ml of 2% Aluminum Chloride (AlCl₃) methanolic solution (Quettier-Deleu et al. 2000). The mixtures were incubated at room temperature for 15 min (Qiu et al. 2010), and the absorbance was recorded at 430 nm in a spectrophotometer. The content of total flavonoid was reported in QE/g of extract.

Antioxidant activity evaluation of the extract

The antioxidant activity of the endophyte extract was evaluated by the methods of DPPH, reducing power assay, and phosphomolybdenum assay (Zahin et al. 2017).

Reducing power assay

The reducing capacity of *F. napiforme* extract was determined as stated by the method in Barros et al. (2007). The crude extract and standard ascorbic acid (AA) solutions were prepared into different working concentrations (25, 50, 100, 200, and 400 µg ml⁻¹). From this extract preparation, 2.5 ml was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture of extract and standard preparation was incubated at 50°C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v) was added, the mixture was centrifuged at 2000 rpm for 10 min (REMI R-8M centrifuge) (Barros et al. 2007). The upper layer (5 ml) was mixed with 5 ml of Milli-Q water and 1 ml of 0.1% ferric chloride, and the absorbance value was read at 700 nm using a spectrophotometer (ThermoFisher SCIENTIFIC MULTISKAN GO). The assay was performed in quadruplicate and the result was presented in mean values ± standard deviations. The regression analysis was used to determine the IC₅₀ values for the extract and the standard.

DPPH assay for free radical scavenging (FRS) activity

The free radical scavenging (FRS) activity of the endophyte extract was evaluated by DPPH antioxidant assay. The methanolic fungus extract was prepared in different dilutions (100, 200, 400, 600, and 800 µg ml⁻¹) and the DPPH solution was prepared in methanol according to Chan et al. (2007). To 1 ml extract preparations from each dilution, 2 ml DPPH was added in an aluminum foil covered taste tubes and incubated in dark at room temperature for 30 minutes. The experimental preparations were performed in triplicate and the absorbance was measured at 517 nm in a spectrophotometer (Merck Spectroquant® Prove 300). The percentage of free radical scavenging activity was calculated by the formula:

$$\% \text{ Free radical scavenging activity} = [(A_c - A_s)/A_c \times 100]$$

Where A_c is the absorbance of control; A_s is the absorbance of the sample (extract). The IC_{50} value required to reduce half the concentration of DPPH radical was calculated.

Antioxidant capacity determination by phosphomolybdenum method

The total antioxidant activity of the extract was determined by the method of phosphomolybdenum assay according to Prieto et al. (1999) with little modification. The fungus extract stock solution was prepared in 1mg ml^{-1} concentration in methanol and filtered by $0.45\mu\text{m}$ pore size Millipore filter paper from which the working solution was prepared by diluting in distilled water. The phosphomolybdate reagent was prepared by mixing 100 ml of each of (0.6 M H_2SO_4 , 28 mM Sodium phosphate, and 4 mM of Ammonium molybdate) in a brown bottle. The final volume was made 300 ml of reagent. Ascorbic acid was dissolved in distilled water and the standard solution was prepared in different concentrations (100, 200, 400, 600, and $800\ \mu\text{g ml}^{-1}$) at the time of recording the reading. From this standard solution, $400\ \mu\text{l}$ was pipetted into a borosilicate test tube (covered with Aluminum foil) to which 4 ml of phosphomolybdate reagent was added. The contents of the mixture covered in aluminum foil were incubated in a water bath at 95°C for 90 minutes. The blank was prepared by mixing 4 ml of the reagent with $400\ \mu\text{l}$ methanol and incubated in the same condition. After the incubation period, the mixture was allowed to cool to room temperature and the absorbance was measured at 695 nm (ThermoFisher SCIENTIFICMULTISKAN G0 spectrophotometer) against the blank. The antioxidant activity of the extract was reported in ascorbic acid equivalents per gram (AAE/g) of extract.

Cytotoxic effect of the extract

The cytotoxicity of the extract was assessed using Methyl Thiazolyl Tetrazolium (MTT) assay (Mosmann 1983). The crude extract was prepared at four series of threefold dilutions starting from $1000\ \mu\text{g ml}^{-1}$ at the upper limit (Monks et al. 1991). The cytotoxic effect of the extract was evaluated against the lung carcinoma (A549) cell line and Mouse embryo fibroblast (NIH3T3) cell line.

Cells and culture conditions

Lung adenocarcinoma (A549) cells and Mouse embryo fibroblast (NIH3T3) cells were purchased from National Center for Cell Sciences (NCCS), Pune. They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) amended with 10% FBS and 1% antibiotic-antimycotic solution. Cells were maintained at 37°C and in a 5 % CO_2 humid atmosphere (Monks et al. 1991) and were used for the experiments at least after three consecutive passages.

Assessment of cytotoxicity

Cells were seeded into 96 well microtiter plates at a seeding density of 5000 cells/well. After adherence, they were treated with different concentrations of the extract, viz., 37.037, 111.11, 333.33, and $1000\ \mu\text{g ml}^{-1}$ (Monks et al. 1991). After 48 hrs post-incubation with extract, MTT reagent was added to the wells and incubated at 37°C for 4 hrs. The formed Formazan crystals were solubilized using DMSO and absorbance was recorded at 570 nm using a multimode microplate reader (FluoSTAR Omega, BMG Labtech) (Sahana & Rekha 2019). The crude extract presenting cytotoxic activity against cancer cell lines was evaluated for the potent concentration that inhibited 50% of cancer cell growth (IC_{50}) in the average of a triplicate experiment. Percentage cytotoxicity of the extract was calculated considering untreated cell control (Kim et al. 2017).

Gas chromatography-mass spectrometry (GC-MS) analysis

The Perkin Elmer AUTOSYSTEM X (Perkin Elmer, USA) GC-MS was used for the analysis of the major components of the endophyte crude extract responsible for the biological activity of the extract. The GC-MS instrument was operated in the following conditions: An initial temperature set to 60°C . The oven temperature increases to 250°C with a rate of $3^\circ\text{C}/\text{min}$. The electron multiplier voltage was 1150 V (Pansanit & Pripdeevech 2018). The ion source temperature

was set at 230°C and the quadrupole temperature was set to 250°C. Helium was used as the carrier gas with a flow rate of 1 ml/min and with column 30.0 m × 250 µm, solvent delay 4.00 min. The ionization voltage was 70 eV. The sample was injected in split mode as 50:1. Mass spectral scan range was set at 29-300 (m/z) (Pansanit & Pripdeevech 2018). The metabolites were identified by comparing the mass spectra to the GC-MS library spectra using the NIST database.

Statistical data analysis

The experiments were performed independently and the results were presented as mean ± SD. The antioxidant activity of the extract was determined from the regression analysis of the standard curve plotted by using the Microsoft Excel spreadsheet. The IC₅₀ values for antioxidant activity and cytotoxicity were calculated by non-linear regression equation using GraphPad Prism 5 software. Pearson's correlation between the antioxidant activity and total phenolic and flavonoid content was analyzed by using SPSS 25 at a 95% confidence interval (Samaga et al. 2014).

Results

Total phenolic content determination of extract

The total phenolic content of the extract was determined spectrophotometrically from the standard curve of Gallic acid absorbance. The crude extract contains a significant amount of phenolic content. Based on calculation from a Gallic acid standard curve (Fig. 1), the total phenolic content of the extract was 23.5035443±0.10208786 GAE/g of extract.

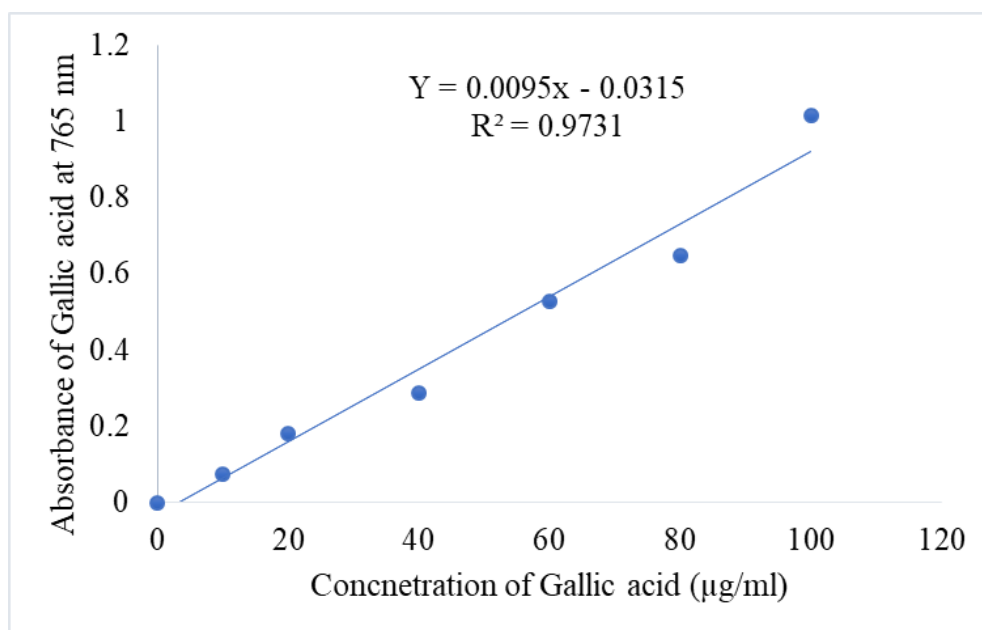


Fig. 1 – Gallic acid standard curve for the total phenolic content determination of the crude extract of the endophytic fungus.

Total flavonoid estimation of the extract

The flavonoid content of the crude extract was determined spectrophotometrically. The content of the total flavonoid of the extract was computed from the standard curve of quercetin (Fig. 2). The concentration of the total flavonoid content of the crude extract was 18.2591023±0.10434413 QE/g of extract.

Antioxidant activity of the endophyte extract

The antioxidant activity of the extract was evaluated by DPPH assay, phosphomolybdate assay, and ferric ion reducing power assay methods (Fig. 7).

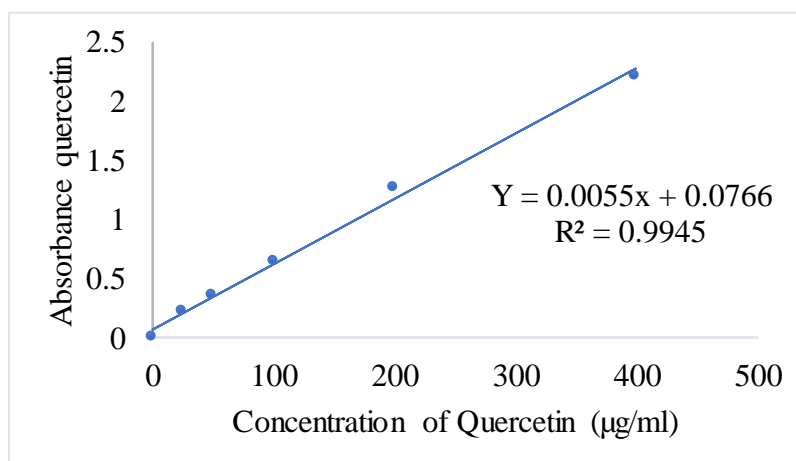


Fig. 2 – Quercetin standard curve for determination of total flavonoid content of the endophytic extract.

Reducing power activity

The reducing potential of the crude extract was evaluated spectrophotometrically from the standard curve of ascorbic acid absorbance (Fig. 3). The extract of the endophyte *F. napiforme* showed increased reducing capacity with the increase in the concentration of the fungal extract (Fig. 3).

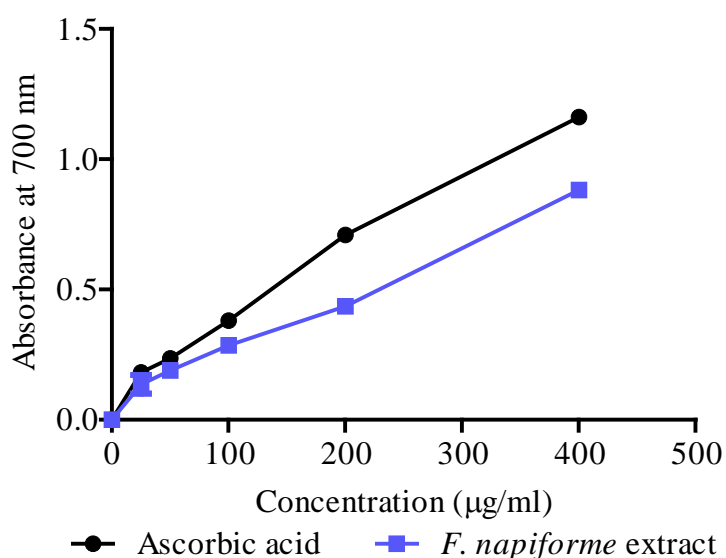


Fig. 3 – Reducing power activity of the endophyte extract and ascorbic acid standard. The blue line and the black line showing the increasing absorbance of the endophytic *F. napiforme* extract and standard ascorbic acid respectively.

The endophytic fungus extract showed a significant reducing power, which is very much closer to the standard (Fig. 3). At a high concentration of 400 µg ml⁻¹, the extract and the standard ascorbic acid had a reducing capacity of 0.882±0.0120 and 1.162±0.00974 respectively. At low concentration (25 µg ml⁻¹), the ferric ion reducing capacity of the crude extract was 0.136±0.0357 and the ascorbic acid was 0.182±0.00765 respectively (Table 1). At low concentration, the extract showed lower reducing power activity in comparison to the standard antioxidant compound (Fig. 7).

Table 1 Antioxidant activity evaluation of the extract by reducing power assay (n=4)

Concentration ($\mu\text{g ml}^{-1}$)	Reducing power absorbance (mean \pm SD) *	
	<i>F. napiforme</i> extract	Ascorbic acid
25	0.136 \pm 0.0357	0.182 \pm 0.00765
50	0.190 \pm 0.0118	0.236 \pm 0.00829
100	0.286 \pm 0.00746	0.380 \pm 0.0079
200	0.436 \pm 0.00874	0.710 \pm 0.0183
400	0.882 \pm 0.0120	1.162 \pm 0.00974
IC ₅₀ ($\mu\text{g ml}^{-1}$)	204.6	176.2
R ²	0.9457	0.9812

*Mean ferric reducing absorbance of the fungal extract and standard in quadruplicate experiments.

DPPH radical scavenging capacity of the extract

The free radical scavenging activity of the methanol fraction of the crude extract is shown in Figs 4 & 7. The endophyte crude extract exhibited high free radical scavenging activity (75.4607 \pm 0.47688%) (Table 2). The crude extract of the endophyte showed strong DPPH radical scavenging activity. At 1 mg ml⁻¹ concentration, the extract and ascorbic acid (AA) have exhibited the free radical scavenging activity with an IC₅₀ values of 299.4 $\mu\text{g/ml}$ (R² = 0.9627) and 384.1 $\mu\text{g ml}^{-1}$ (R²= 0.9705) respectively. The extract has demonstrated greater antioxidant activity than that of the standard antioxidant vitamin C, particularly at lower concentrations (Fig. 4).

Table 2 The free radical scavenging potential of the endophyte extract (n=3)

Concentration ($\mu\text{g ml}^{-1}$)	The mean free radical scavenging activity (%)*	
	Ascorbic acid	<i>F. napiforme</i> extract
100	8.99687 \pm 3.903429	46.6765 \pm 1.92949
200	24.84552 \pm 2.341544	57.6048 \pm 1.34830
400	55.11759 \pm 1.243202	64.1053 \pm 1.40160
600	62.33139 \pm 0.540178	69.0450 \pm 0.73659
800	78.43973 \pm 0.374230	72.5955 \pm 0.64701
1000	91.04513 \pm 1.383641	75.4607 \pm 0.47688

*The percent inhibition capacity of endophyte extract by DPPH (Mean \pm SD)

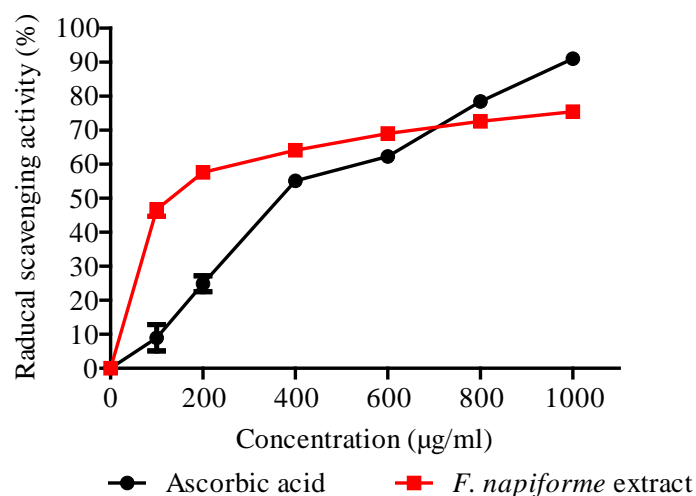


Fig. 4 – The total antioxidant activity of the endophytic extract and the standard ascorbic acid by DPPH free radical scavenging assay. Redline and the blackline showing the increasing trend of the antioxidant activity of the endophytic fungal extract and the standard ascorbic acid respectively.

Phosphomolybdenum assay

The total antioxidant capacity of the endophyte crude extract determined by spectrophotometer from the standard curve of ascorbic acid (Fig. 5) and presented as ascorbic acid equivalent/gram (AAE/g) of the extract (Table 3).

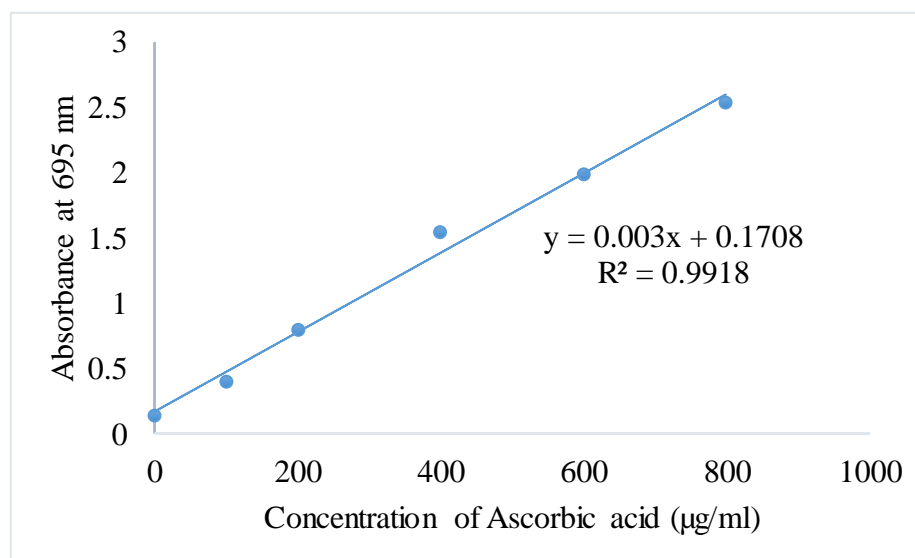


Fig. 5 – Ascorbic acid standard curve for total antioxidant evaluation by phosphomolybdenum method.

Table 3 Estimation of total antioxidant capacity (TAC) by phosphomolybdate (n=3)

Concentration (µg ml ⁻¹)	Total antioxidant capacity (AAE/g)*
100	16.217±2.484
200	43.869±2.511
400	85.853±3.916
600	173.405±16.904
800	255.434±21.404
IC ₅₀ (µg ml ⁻¹)	492.8
R ²	0.9573

* The amount of TAC reported in Mean±SD of triplicate measurements.

The extract showed increasing antioxidant activity in a concentration-dependent function (Fig. 6). At low concentration, the extract had the TAC of 16.217±2.484 (AAE/g) whereas the amount of TAC of the extract at higher concentration, at 800 µg ml⁻¹, was 255.434±21.404 (AAE/g). The crude extract of *F. napiforme* and ascorbic acid revealed antioxidant capacity with IC₅₀ values of 492.8 µg ml⁻¹ (R²=0.9573) and 371.4 µg ml⁻¹ (R²=0.9766) respectively. At 0.8 mg ml⁻¹ concentration, the extract showed moderate total antioxidant activity (Table 3). The extract showed a concentration-dependent increase in the TAC against the damaging free radical.

Correlation between total phenolic and flavonoid content, and antioxidant activity

There was a significant correlation between TPC and antioxidant activity (DPPH & RPA) at $p < 0.05$. The correlation between RPA and DPPH is significant at $p < 0.01$ (Table 5). The correlation between TFC and antioxidant activity (DPPH & RPA) was not significant whereas the observed correlation between TFC and PMA was weak.

Cytotoxicity of the extract

The extract of *F. napiforme* presented a potent toxic activity against the lung cancer cell line (A549). The extract inhibited the proliferation of A549 cells in a concentration-dependent manner. The extract showed the cytotoxic activity to the A549 cell line with an IC_{50} value of $249.8 \mu\text{g ml}^{-1}$ ($R^2 = 0.9943$) (Fig. 8). The mean cytotoxicity of the crude extract was $69.74 \pm 0.49\%$ at 1.0 mg ml^{-1} concentration of the extract (Table 4). Most importantly, this extract has shown no toxicity to the mouse embryo fibroblast (NIH3T3) cell line (Table 4). Only at higher concentration ($1000 \mu\text{g ml}^{-1}$), negligible cell toxicity was observed ($6.06 \pm 0.96\%$) against the normal cell line (NIH3T3).

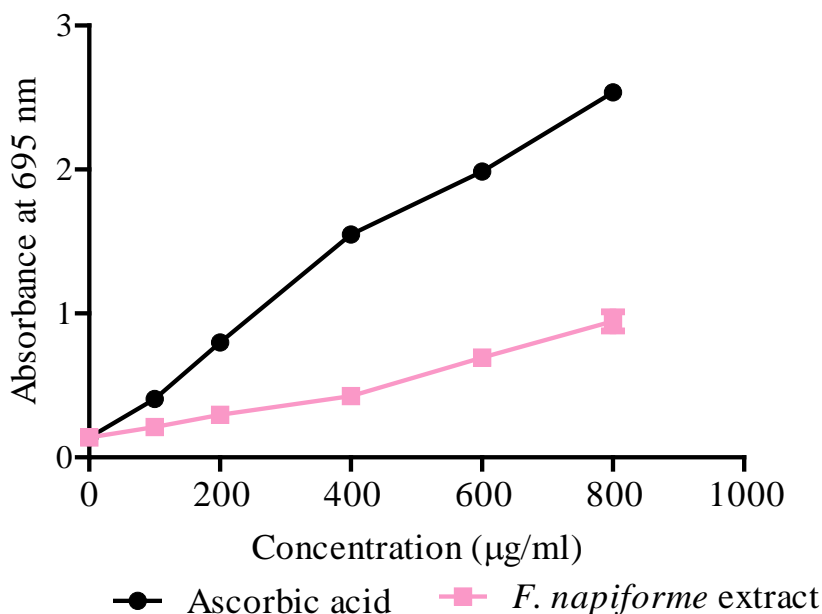


Fig. 6 – Total antioxidant activity of the extract by phosphomolybdate assay. Pink Line and the black line showing the increasing trend of the antioxidant activity of the endophytic fungal extract and the standard ascorbic acid respectively.

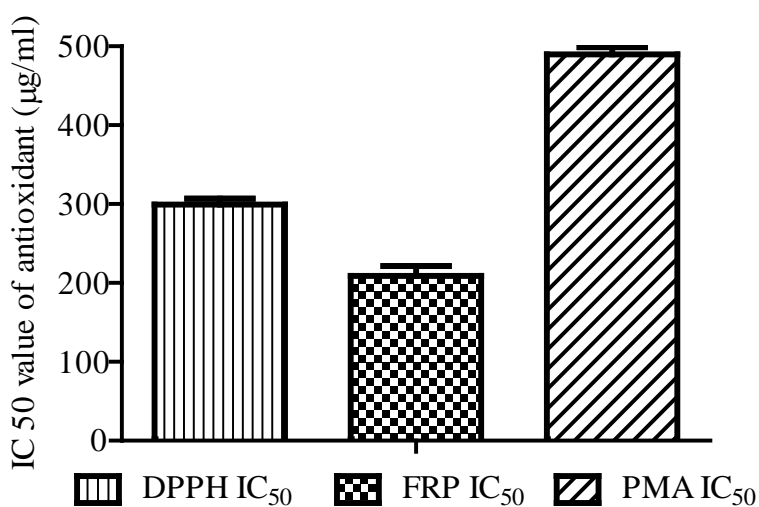


Fig. 7 – The IC_{50} values of antioxidant activity of the endophytic extract by DPPH, phosphomolybdenum, and reducing power assays. Vertical parallel line bar represents DPPH, square box bar represents reducing power and diagonal line bar represents the phosphomolybdenum assays.

Table 4 Cytotoxicity test of the endophytic fungus extract assessed using MTT assay (n=3).

Mean cytotoxicity of the endophyte extract ^{ab}		
Concentration ($\mu\text{g ml}^{-1}$)	^a Cytotoxicity (Mean % \pm SD)	^b Cytotoxicity (Mean % \pm SD)
37.037	6.32 \pm 0.51	0.32 \pm 0.13
111.11	16.82 \pm 0.56	0.91 \pm 0.19
333.33	46.51 \pm 1.37	1.13 \pm 0.33
1000	69.74 \pm 0.49	6.06 \pm 0.96
IC ₅₀ ($\mu\text{g ml}^{-1}$)	249.8 (R ² =0.9943)	431.5 (R ² =0.9504)

^aCytotoxicity of the extract against A549 cells; ^bCytotoxicity of the extract against NIH3T3 cells.

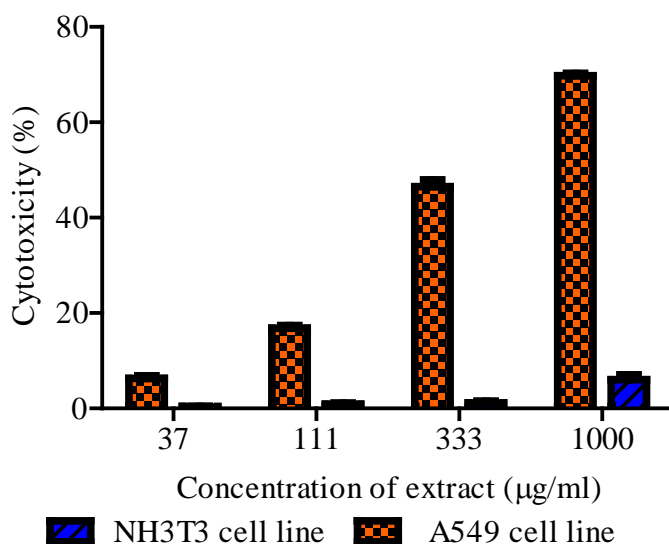
Table 5 Pearson's correlation for quantitative determinations of antioxidant activity^c

Correlations between phenolic compounds and antioxidant activity					
Variables	DPPH	RPA	PMA	TFC	TPC
DPPH	1				
RPA	1.000**	1			
PMA	0.882	0.880	1		
TFC	-0.829	-0.831	-0.467	1	
TPC	-0.998*	-0.998*	-0.846	0.866	1

RPA: Reducing power activity; PMA: Phosphomolybdate assay; DPPH; TFC: Total flavonoid content; and TPC: Total phenolic content.

^c95% confidence interval; ** Correlation is significant at $p < 0.01$; *Correlation is significant at $p < 0.05$.

The crude extract was not cytotoxic to the mouse embryo fibroblast (NIH3T3) cell line at the concentration tested (Table 4). The IC₅₀ value of the extract against the NIH3T3 cell line was 431.5 $\mu\text{g ml}^{-1}$ (R² =0.9504).

**Fig. 8** – The IC₅₀ value for cytotoxicity of the endophytic extract against the lung adenocarcinoma (A549) cell line and mouse embryo fibroblast (NIH3T3) cell line.

The major types of bioactive compounds identified from the extract

The GC/MS analysis of the crude extract showed the presence of 9 major compounds (Fig. 9a-j). The metabolites were identified by the mass fragmentation pattern of each compound compared with the authentic compounds in the National Institute Standard and Technology (NIST) database. The major types of bioactive compounds, volatile and aliphatic constituents, identified showed the presence of aliphatic compounds and their derivatives (methyl esters). Some of the

major representative endophytic fungal volatile metabolites identified are the aliphatic compounds such as Dichloroacetic acid, 6-ethyl-3-octyl ester; 3,4,4, Trimethyl-1-pentyn-3-ol; Benzaldehyde, 3,4- dimethyl; Phenol, 3,5-bis(1,1-dimethylethyl); Hexadecanoic acid, methyl ester; Methyl stearate; trans-13-Octadecenoic acid, methyl ester; 9,12-Octadecadienoic acid, methyl ester; 2,6-Diaminoanthraquinone, and other derivatives (Table 6).

Table 6 The major bioactive compounds identified from the crude extract

Peak No.	RT (min)	Area %	m/z	Chemical formula	Name of compounds identified
1	5.679	1.925	268	C ₁₂ H ₂₂ Cl ₂ O ₂	Dichloroacetic acid, 6-ethyl-3-octyl ester
2	5.759	1.443	126	C ₈ H ₁₄ O	3,4,4-Trimethyl-1-pentyn-3-ol
3	8.515	1.404	134	C ₉ H ₁₀ O	Benzaldehyde, 3,4-dimethyl-
4	11.652	5.214	206	C ₁₄ H ₂₂ O	Phenol, 3,5-bis(1,1-dimethylethyl)
5	11.747	16.027	270	C ₁₇ H ₃₄ O ₂	Hexadecanoic acid, methyl ester
6	14.668	5.365	298	C ₁₉ H ₃₈ O ₂	Methyl stearate
7	15.133	15.306	296	C ₁₉ H ₃₆ O ₂	trans-13-Octadecenoic acid, methyl ester
8	15.918	9.815	294	C ₁₉ H ₃₄ O ₂	9,12-Octadecadienoic acid, methyl ester
9	20.545	2.469	238	C ₁₄ H ₁₀ N ₂ O ₂	2,6-Diaminoanthraquinone

Discussion

The plant *Psidium guajava* is known for its high antioxidant properties due to the presence of a high amount of phenolic compounds (Naseer et al. 2018). Thus, the plant was chosen for the investigation of the endophytic fungi with cytotoxic and antioxidant activity.

The natural product is a dependable source of therapeutic and nutritional compounds (Akpotu et al. 2017). The natural secondary metabolites are produced from plants and microorganisms. The endophyte from medicinal plants has of particular interest owing to their potential production of therapeutic compounds similar to the host plant (Venieraki et al. 2017, Naik et al. 2019). Endophytic microorganisms are inexhaustible sources for the bioprospecting of biologically active natural products with agricultural, medical, and industrial applications (Aly et al. 2013, Meena et al. 2019). Fungi, especially endophytes, are one of the endophytic microorganisms that received great attention, recently, in the search of natural pharmaceutical compounds (Gond et al. 2010).

Qiu et al. (2010) have isolated endophytic fungi *Aspergillus* sp. which produce flavonoids and phenolic compounds. In this report, the endophyte *F. napiforme* produced an appreciable amount of flavonoids, which could be promising sources for therapeutic and dietary antioxidants. The antioxidant potential of the natural product is generally associated with the presence of phenolic compounds in the extract. The amount of total phenol and flavonoid found in the extract were 23.5035±0.102 GAE/g of extract and 18.259±0.104 QE/g of extract respectively. Similarly, the amount of total phenol and flavonoid estimated from *Phyllosticta* sp. were of 18.33 ± 0.68 GAE/g and 6.44 ± 1.24 µg/mg of QE respectively (Srinivasan et al. 2010). Theantana et al. (2012) have isolated 39 endophytic fungi from Thai Medicinal Plants of which *Eupenicillium shearii* CMU 18 showed the highest amount of phenolic compounds (18.25 ± 0.138 mg GAE/ 100 ml). This isolate, *E. shearii* CMU 18, has shown high reducing power (0.293 ± 0.004). In this study, the secondary metabolite extract of *F. napiforme* contains more phenolic and flavonoids contents than *E. shearii* CMU 18 and *Phyllosticta* sp.

In this study, the endophytic fungal extract from *F. napiforme* was tested for the antioxidant capacity by DPPH, Reducing power assay, and Phosphomolybdenum assay methods. The endophyte *F. napiforme* has shown a potent antioxidant potential with IC₅₀ of 299.4 µg ml⁻¹ against DPPH radical, 492.8 µg ml⁻¹ against Phosphomolybdate ion, and 204.6 µg ml⁻¹ against Ferric reducing power. The ferric ion reducing capacity of the endophyte extract increased as a function of the extract concentration (Barros et al. 2007). The extract showed a very good reducing power activity that proves it can be a good source of reductant molecules. The finding of this study is in

agreement with a similar study on *Phyllosticta* species, endophytic fungus, isolated from *Guazuma tomentosa*. *Phyllosticta* sp. that showed the antioxidant activity with an IC₅₀ value of 2030.25 ± 0.81 µg/ml against DPPH radicals (Srinivasan et al. 2010). Our endophytic fungus showed stronger DPPH radical scavenging activity than the *Phyllosticta* species. Further separation of the extract into its active components would enhance the antioxidant activity of the extract. Therefore, the *F. napiforme* endophyte can be a reliable resource for the discovery of therapeutic compounds.

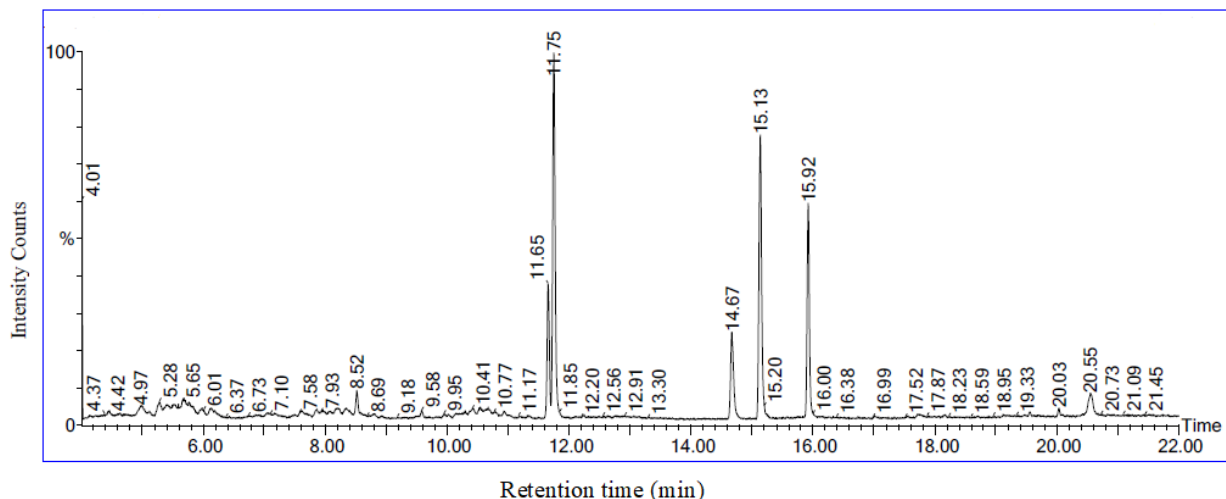


Fig. 9a – The GC-MS analysis of the crude extract of *F. napiforme* isolated from *P. guajava* leaves. The peaks in the figure show the retention time and the intensity count of the crude extract.

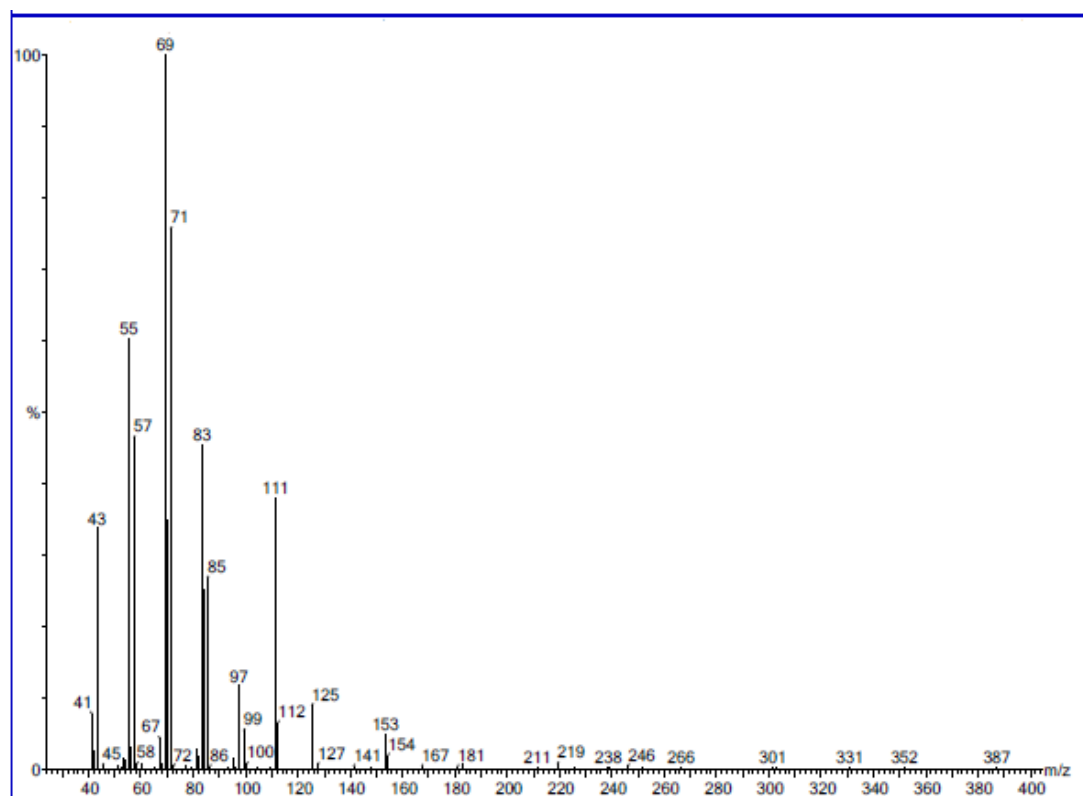


Fig. 9b – GCMS-Chromatogram of Dichloroacetic acid, 6-ethyl-3-octyl ester. The peak in the figure shows the m/z of the crude extract at retention time 5.679.

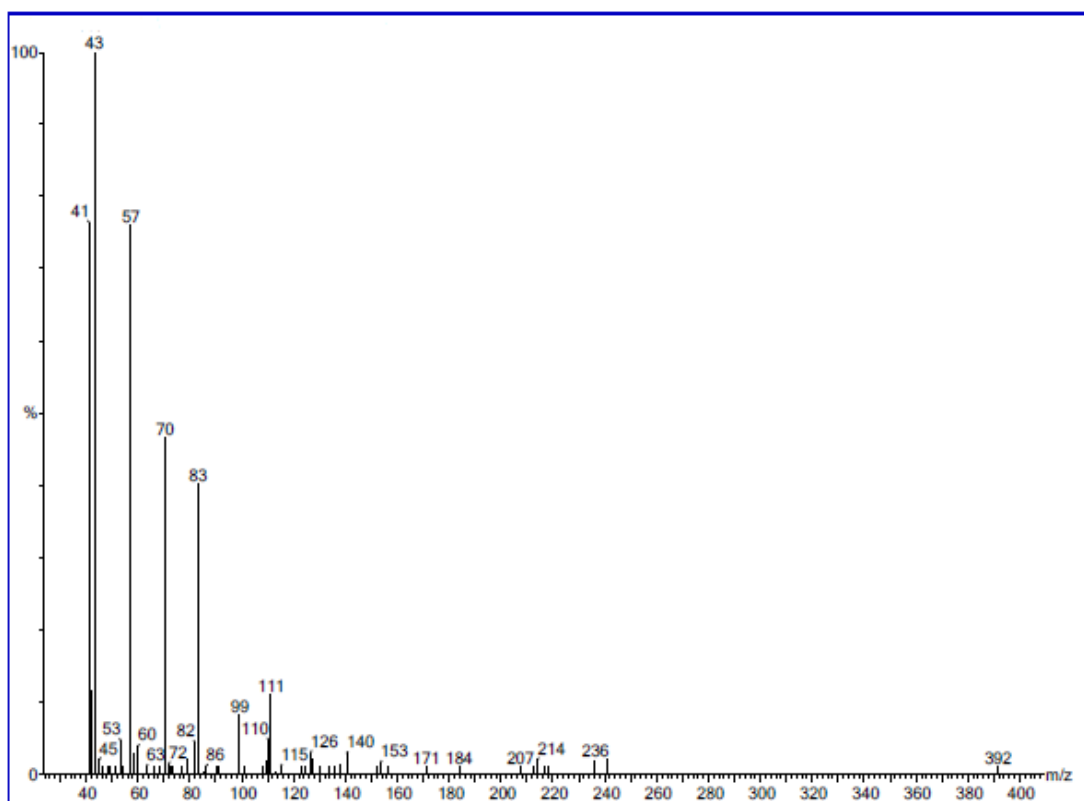


Fig. 9c – GCMS spectrum of 3, 4, 4-Trimethyl-1-pentyn-3-ol. The peak in the figure shows the m/z of the crude extract at retention time 5.759.

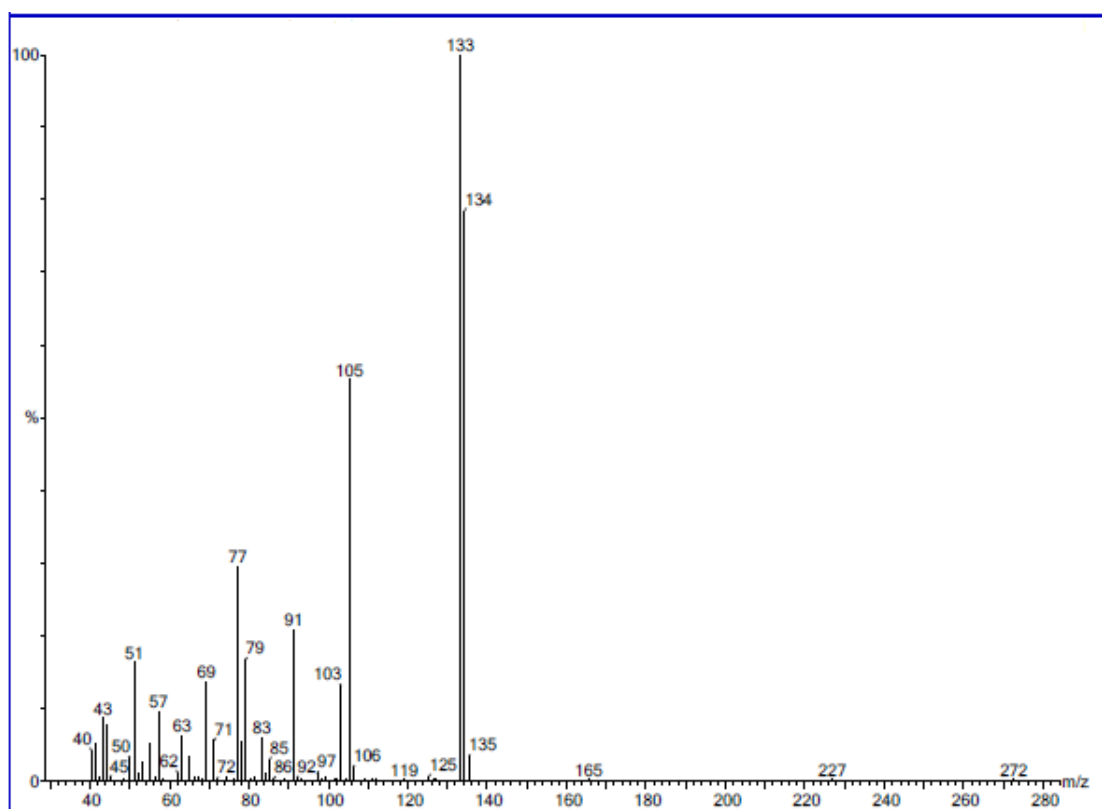


Fig. 9d – GCMS-Chromatogram of Benzaldehyde, 3, 4-dimethyl-. The peak in the figure shows the m/z of the crude extract at retention time 8.515.

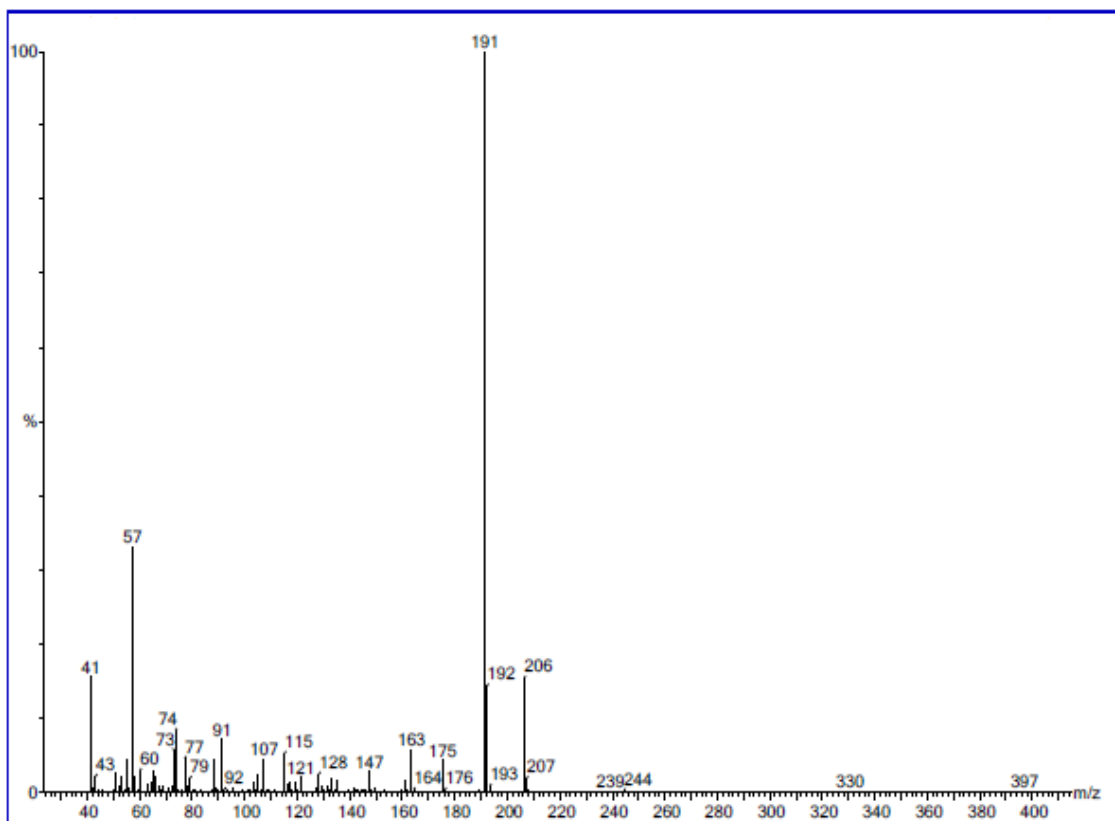


Fig. 9e – GCMS spectrum of Phenol, 3, 5-bis (1, 1-dimethylethyl). The peak in the figure shows the m/z of the crude extract at retention time 11.652.

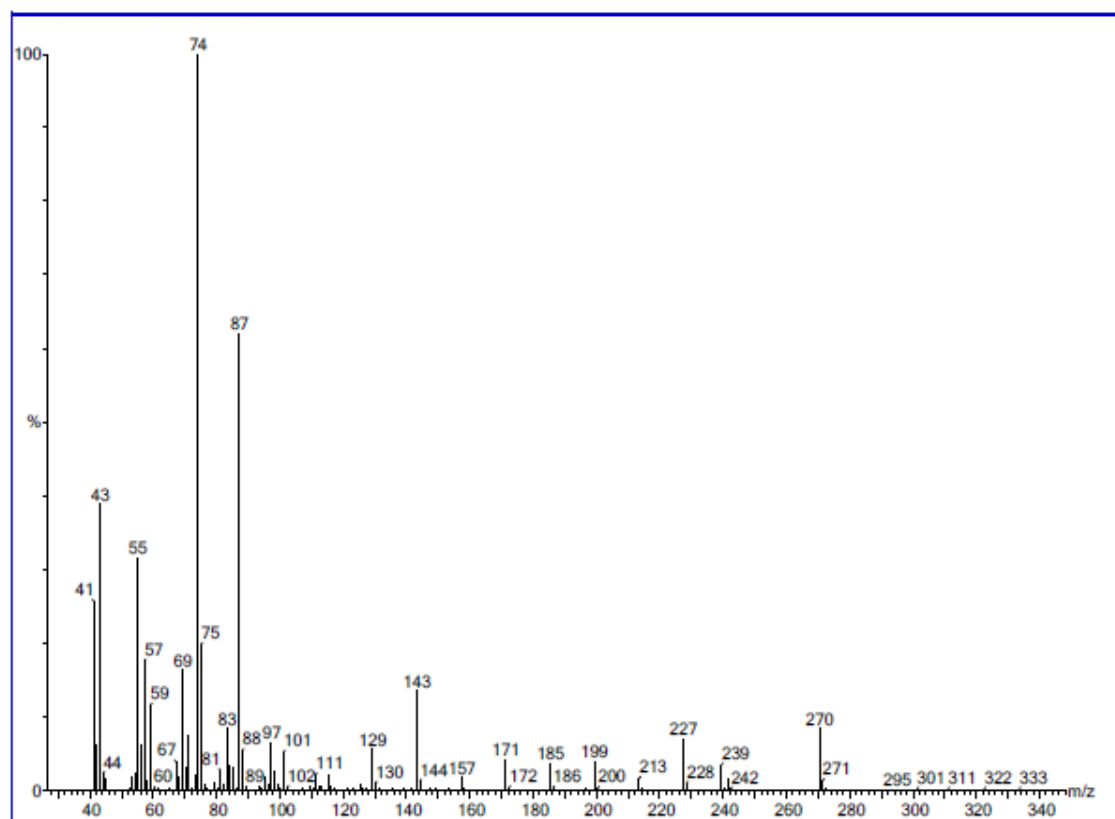


Fig. 9f – GCMS-Chromatogram of Hexadecanoic acid, methyl ester. The peak in the figure shows the m/z of the crude extract at retention time 11.747.

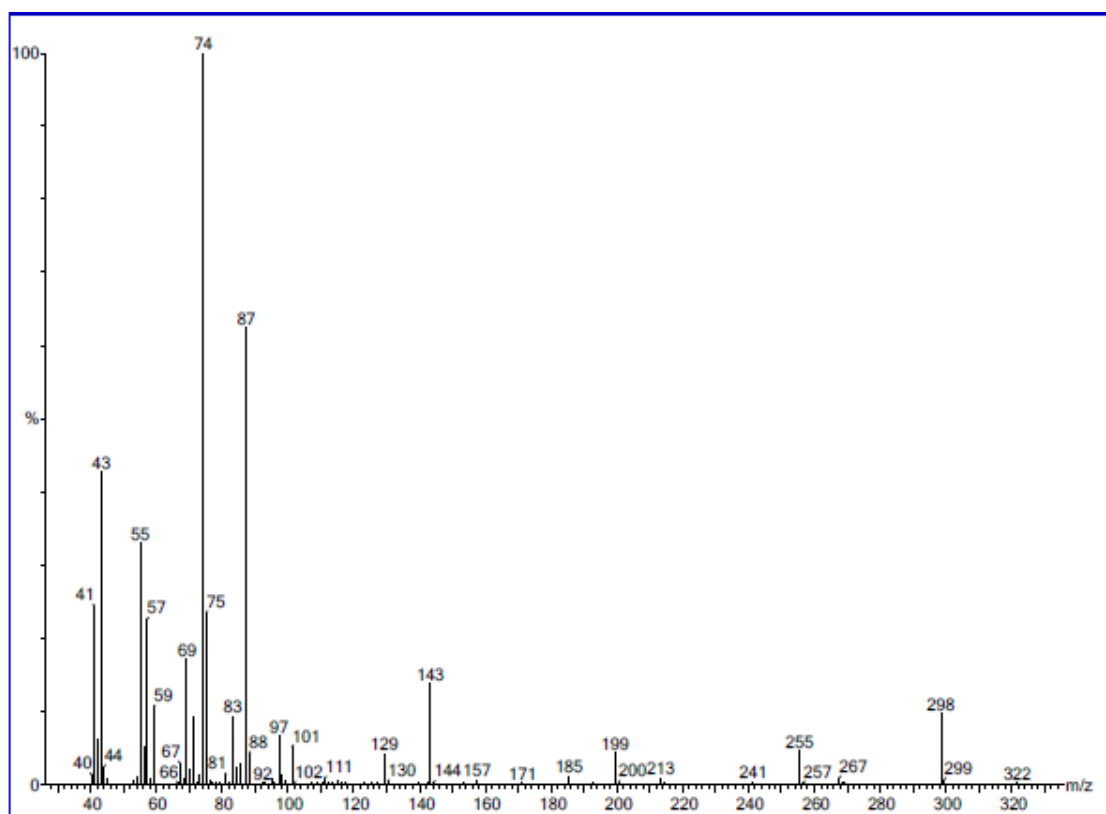


Fig. 9g – GCMS spectrum of Methyl stearate. The peak in the figure shows the m/z of the crude extract at retention time 14.668.

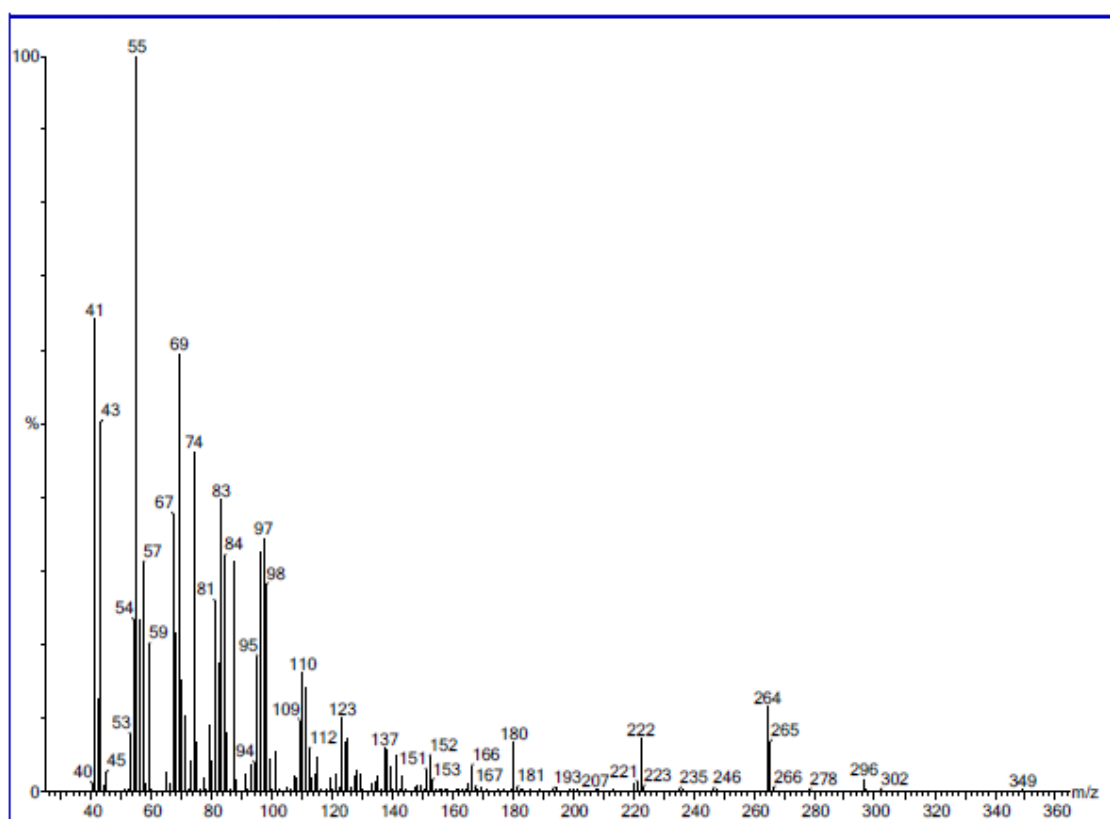


Fig. 9h – GCMS-Chromatogram of trans-13-Octadecenoic acid, methyl ester. The peak in the figure shows the m/z of the crude extract at retention time 15.133.

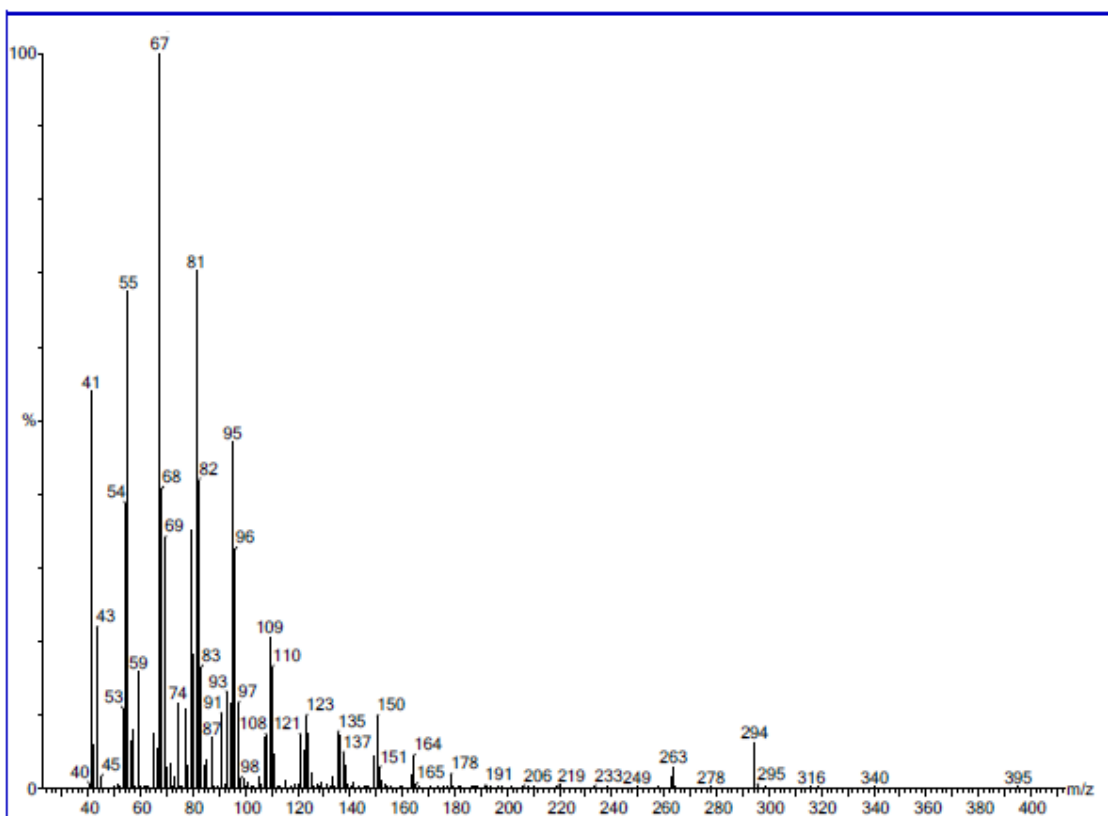


Fig. 9i – GCMS spectrum of 9, 12-Octadecadienoic acid, methyl ester. The peak in the figure shows the m/z of the crude extract at retention time 15.918.

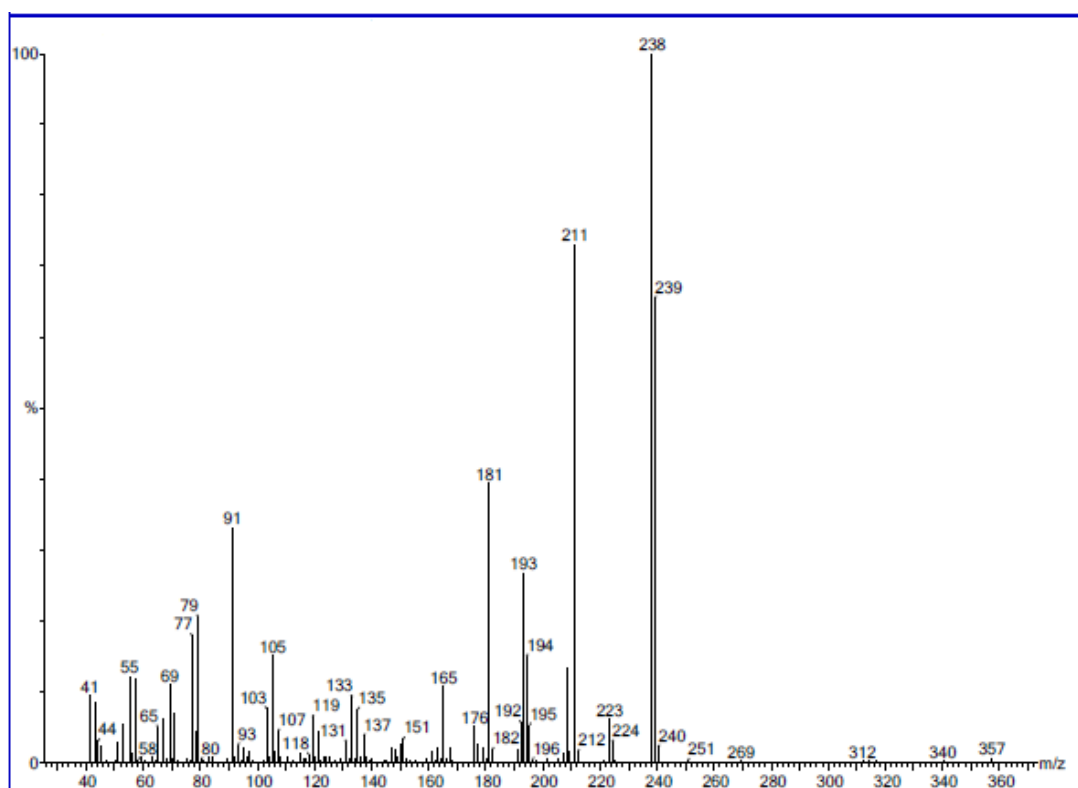


Fig. 9j – GCMS-Chromatogram of 2, 6-Diaminoanthraquinone. The peak in the figure shows the m/z of the crude extract at retention time 20.545.

The natural antioxidant activity of secondary metabolites from the natural sources; microorganisms and plants, is correlated to the amounts of antioxidant compounds found in the extract (Zhou et al. 2019). In our study, we observed a significant correlation between total phenolic content and antioxidant capacity (DPPH & RPA) at $p < 0.05$. The correlation between RPA and DPPH is significant at $p < 0.01$ (Table 7) whereas there was a negative and insignificant correlation between TFC and antioxidant potential (DPPH & RPA). This may be an indication of the presence of other constituents of the compounds contributing to the antioxidant capacity of the extract besides flavonoids and phenols. Unlike the synthetic antioxidants, which can accumulate in the body and carcinogenic, antioxidants extracted from natural sources are safe and have nutritional and therapeutic values (Deng et al. 2011). The reductant present in the extract reacts with free radical ions, stabilize, and stops radical chain reactions thereby prevent the body from oxidative damage (Barros et al. 2007). Therefore, the natural antioxidant compound investigation is highly needed to prospect its nutritional and medicinal benefits from endophytic sources.

The natural products from fungi have received an increasing attraction in biotechnology to the development of novel drugs (Hillman et al. 2017). Bioprospecting the plant-associated bioactive molecules from endophytic microorganisms have of greatest importance to maintain a healthy ecology and sustainable economy (Aly et al. 2013, Vyas & Bansal 2018). The use of plants to bioprospecting novel metabolites are limited in productivity and vulnerability to plant extinction due to cutting of tree to metabolite extraction (Gómez & Luiz 2018, Tejesvi & Pirttilä 2018). On the contrary, microorganisms, particularly endophytic fungi, are renewable sources for producing novel pharmaceutical molecules that are a crucial strategy to circumvent the plant extinction (Chandra 2012). This is because a metabolite originated from microbes would avoid dependence on the plant, which would reduce the cost of drugs to patients and save the environment as well (Stierle et al. 1995).

The discovery of cancer therapeutic agents from endophytic fungal extract has been prominently screened (Lopaczynski & Zeisel 2001). *Taxomyces andreanae*, endophytic fungus, from Pacific Yew produced the anticancer natural compound similar to the host (Stierle et al. 1993). Uzma et al. (2018) have isolated cytotoxic compounds from endophytic *Fusarium* sp. In India, the same compound, taxol, was produced from Himalayan Yew associated endophytic fungus *Fusarium redolen* (Garyali et al. 2013) and the mangrove endophytic fungus *Fusarium oxysporum* (Elavarasi et al. 2012). The natural, anticancer compound, paclitaxel is produced from the pacific yew plant (Stierle et al. 1993) and their endophytic fungus associated (Strobel 2002). Above all, as to current knowledge, the endophytic fungi inside the plant have a vital role in the synthesis of bioactive metabolites. The endophyte *Aspergillus terreus* exhibited anticancer activity on the A549 cell line within pure and crude complexes (Goutam et al. 2017). Some endophytic *Fusarium* species are known producers of anticancer compounds (Aly et al. 2013). Similarly, the exopolysaccharide (EPS) from the endophytic fungus *B. ochroleuca* M21 showed anti-proliferative activity against liver cancer (HepG2), gastric cancer (SGC-7901) and colon cancer (HT29) cell lines (Li et al. 2016). The *F. napiforme* endophyte in this report shown a very strong cytotoxic activity against human lung cancer (A549) cell line and it can be a potential resource to the development of effective drug molecules targeting cancer therapies. Further activity assay on different cancer cell lines in pure compounds could give more cytotoxic effect. Li et al. 2016 examined the cytotoxicity of the EPS from the endophytic fungus *B. ochroleuca* M21 on human liver HL-7702 cells and did not have shown cytotoxic activity. In the present study, the crude extract from endophytic *F. napiforme* shown potent cytotoxic effect against the A549 cell line. More importantly, the endophyte extract tested for the anti-proliferative activity on mouse embryo fibroblast (NIH3T3) cell line showed no cytotoxic activity. The absence of toxicity to the normal cell line is an implication of the suitability of the extract to the chemotherapeutic purpose and the safety of the metabolites to the cell. The crude extract from the endophyte fungus, *F. napiforme*, needs to be further evaluated in the animal model. Further purification of the crude extract is suggested for prospecting a better anti-cancer effect.

The major bioactive compounds identified by GC-MS has shown the presence of volatile and aliphatic constituents responsible for the antioxidant activity of the crude extract (Samaga et al. 2014). Huang et al. (2007) have identified aliphatic compounds such as hexadecanoic acid methyl ester; octadecanoic acid methyl esters; 7-octadecenoic acid methyl ester) and 9, 12-Octadecadienoic acid, methyl ester in the crude extracts of the endophytic fungi isolated from *Nerium oleander*. Similarly, heptadecanoic acid and methyl hexadecanoic acid identified from marine *Bacillus* showed anti-proliferative activity against HT-29 (Human colorectal adenocarcinoma) and A549 with IC₅₀ value 93.4 µg/ml and 50.04 µg/ml (Syed et al. 2019). The major group of compounds identified in this study may be responsible for the antioxidant capacity and cytotoxicity of the extract. As a result, the *F. napiforme* endophyte can be a promising resource for bioprospecting anticancer therapeutics. The purification of the crude extract and further biological activity evaluation is being carried out.

Conclusion

The crude extract of the endophytic *F. napiforme* isolated from the leaf of guajava showed significant antioxidant activity. The fungus produces a high content of total flavonoids and phenolic compounds. Apart from this, the crude extract from the fungus has a high cytotoxic effect against lung carcinoma cell line (A549) and non-toxic to normal cell line (NITHI3). Further, the GC-MS analysis of the crude extract of *F. napiforme* indicated the presence of phenols, quinone, Benzenamine, aliphatic compounds, and their derivatives. Therefore, the endophyte *F. napiforme* is a potential source for antioxidant and anticancer therapeutic formulation. More studies of the extract in different model cells are recommended to further prospect the antioxidant and cytotoxic effect in pure compounds.

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