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Antioxidant and hepatoprotective potentials of novel endophytic fungus *Achaetomium* sp., from *Euphorbia hirta*

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ABSTRACT

Objective: To isolate, identify and evaluate the antioxidant, antimicrobial, hepatoprotective potentials, total phenolic content, flavonoid content, tannin content of ethyl acetate extract of endophytic fungus *Achaetomium* sp., isolated from *Euphorbia hirta*.

Methods: Hepatoprotectivity of ethyl acetate extract of *Achaetomium* sp., was evaluated by CCl_4 induced toxicity in HepG2 cells and subsequently analysed for cell viability using MTT assay. It also demonstrates antioxidant and antimicrobial potentials by DPPH radical scavenging assay and well diffusion assay respectively. Quantification of total phenolic content, tannin content and flavonoid content were assessed by spectroscopic methods.

Results: Phenols, flavonoids and tannins were the phytochemicals present in ethyl acetate extract of *Achaetomium* sp., with rich phenolic content exhibited potent hepatoprotective, antimicrobial and antioxidant activities. The hepatoprotective activity was recorded as of 72.13% ± 2.948% of cell viability at a concentration of 150 μg/mL, whereas the standard silymarin showed 93.260% ± 0.784%. It was observed to be dose dependent, when CCl_4 exposed HepG2 cells were treated with different concentrations of ethyl acetate extract. Antibacterial activity showed significant inhibition against *Staphylococcus aureus, Pseudomonas aeroginosa*, and *Klebsiella pneumoniae*. The antioxidant activity ranged from 66.890% ± 1.385% to 87.340% ± 0.289% with (44.02 ± 1.57) μg of total phenolics, (54.54 ± 1.82) μg of flavonoid content and (18.790 ± 1.018) μg of tannin content. Ascorbic acid, BHT (butylated hydroxyl toluene) Gallic acid and Pyrogallol were used as standards which showed 98.370% ± 0.763%; 97.080% ± 0.636%; 94.890% ± 1.103% and 96.980% ± 0.098% reducing potential respectively.

Conclusions: The results reveal that the metabolites produced by endophytic fungi isolated from *Euphorbia hirta* could be novel natural products that could lead to new drug discovery.

1. Introduction

Endophytes are microorganisms that reside within the plant without causing any symptomatic changes in the plant. It is not understood that how these microbial communities impact plant physiology and function [1]. Secondary metabolites produced by the microorganisms are well enquired and investigated due to the many antibiotics produced by fungi and bacteria [2]. Metabolites biosynthesized by the plant endophytes have been tested for their antimicrobial activities [3].

Natural products from plants had always been of great resources for therapeutics [4]. Endophytic fungus has been described as store house of various novel pharmaceutical compounds which have various applications [5]. Host plants and their endophytes live in a mutualistic or in neutral relationships [6].

Euphorbia hirta L. (*E. hirta*), Euphorbiaceae family, is plant distributed throughout India and also found as a pantropical weed [7]. The plant has been reported to be used as diuretic, and has an anti-inflammatory, antispasmodic and antidiarrheals actions [8]. It is used in treating respiratory tract inflammations and asthma in Africa. In Malagasy it is used in treating cough,

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pulmonary disorders, and chronic bronchitis, and in treating diarrhoea and particularly amoebic dysentery [9].

While there is more information available about the medicinal uses of *E. hirta*, not many reports are available on medicinal property of endophytes associated with it. Hence, this study on endophytic fungus *Achaetomium* sp., isolated from *E. hirta* was conducted in isolation, identification and evaluation of quantitative analysis of phytochemical, antioxidant, antimicrobial and hepatoprotective potentials.

2. Materials and methods

2.1. Isolation of endophytic fungus

Healthy plants of *E. hirta* were carefully uprooted and brought to the lab. The roots of the plant were washed in flowing tap water to eliminate the soil and dust particles, other microbes and the samples were sterilized [10]. The root samples were cut into 2 mm segments and were surface sterilized by sequentially immersing in 70% ethanol for 1 min in 4% sodium hypochlorite for 2 min and then finally rinsed with sterilized distilled water for 1 min. The moisture was removed by pressing in the sterilized tissue paper. Then efficiency of the sterilization was confirmed with the method [11]. The root explants were transferred and impregnated to the petriplates containing PDA (Potato Dextrose Agar) supplemented with chloramphenicol 150 mg/L. The petridishes with the explants were incubated at 27 °C and were regularly observed for any fungal growth. The emerging fungus from the sample explants was carefully transferred to new petriplates with PDA free from antibiotics.

2.2. Microscopic and molecular identification of fungal isolate

Fungal endophyte was identified by microscopic and molecular [12]. Fungal DNA was isolated using kit (Xcelgen, Xcelris laboratories, Ahmedabad, India). The partial 28S rRNA gene, was amplified using the forward primer (5′ ACCCGCTGAACTTAAGC 3′) and reverse primer (5′GTCCGTGTTTCAAGACGG 3′). The PCR amplified products were then subjected to automated DNA sequencing on ABI 3730xl Genetic Analyzer (Applied Biosystems, USA). Consensus sequence of 600 bp of 28S rRNA was generated from forward and reverse sequence data using aligner software. Consensus sequences were submitted in the GenBank. Multiple BLASTN searches against the sequence were made at the National Center for Biotechnology Information (NCBI). Primarily, Multiple Sequence Alignment was first carried out by Clustal Omega [13] and further, alignment file was later aligned for trimming using trimAI tool [14]. Phylogenetic analysis was carried out with the neighbor-joining method using MEGA 7.0 software program [15].

2.3. Cultivation and extraction

A small piece of agar plug from fungal colony was cut and inoculated in 300 mL of PDB in 1000 mL flask and incubated for 21 d. The *Achaetomium* sp., has turned the Potato Dextrose Broth, red due to the production of red pigmented metabolite. The fungal broth was filtered to separate the broth and mycelium. The filtered broth was centrifuged at 3600 rpm for 10 min and the supernatant was extracted with equal volumes of ethyl acetate. The organic ethyl acetate extract was evaporated. The crude extract, a red shiny powder was dissolved in DMSO to determine total phenol, tannin, total flavonoid, antioxidant, antibacterial, and hepatoprotective potentials.

2.4. Determination of total phenolic, flavonoid tannins contents

Total phenolic, flavonoid and tannin content of ethyl acetate extract was evaluated as described by Siddhuraju and Manian [16] with a small modifications. About 100 μL ethyl acetate extract of *Achaetomium* sp., was pipetted into a test tube and 900 μL of distilled water, 1 mL of 1 N Folin-ciocalteu phenol reagent and 2.5 mL sodium carbonate solution (20%) were added sequentially and incubated in dark for 40 min, the readings of the absorbance were recorded at 725 nm against the reagent blank.

The estimation of tannins was performed for the same extract by addition of polyvinylpolypyrrolidone (PVPP), with the capacity to bind with tannin. PVPP (100 mg) was added into a test tube and 1 mL of dist. water was added followed by addition of 1.0 mL of tannin with phenolic extract. The test tubes were vortexed and incubated at 4 °C for about 4 h. After incubation the samples were centrifuged at 3000 rpm for 10 min and the supernatant was collected. PVPP has precipitated the tannins and the supernatant has only simple phenols without tannins. The supernatant with the phenolic content was measured as mentioned above. The total tannin content was evaluated from results by calculating the difference between non tannin phenols and total phenols [17].

The flavonoid content was evaluated by following the method of Zhishen *et al.* [18]. The ethyl acetate extract of 0.5 mL (1 mg/mL) was taken and 2 mL dist. water was added followed by subsequent addition of 0.15 mL of 5% $NaNO_2$ solutions. About 0.15 mL of 10% $AlCl_3$ was added after 6 min. The mixture was set to stand for about 6 min, followed by addition of 2 mL of 4% NaOH solution to the mixture. The final volume of the mixture was made to 5 mL by readily adding distilled water. The mixture was mixed well, and allowed to stand for another 15 min. Absorbance was recorded at 510 nm and dist. water was used as blank. Rutin was used as the standard in the quantification of total flavonoids and the results were revealed as rutin equivalents (RE).

2.5. Antioxidant assay

DPPH assay has been used to evaluate the antioxidant potentials of the ethyl acetate extract of the strain *Achaetomium* sp., and the experiments were performed in triplicates and the mean value was taken.

2.5.1. DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging activity

Antioxidant activity was determined through free radical scavenging of ethyl acetate extract from *Achaetomium* sp. Change in the optical density of the DPPH radical was monitored by following the method described by Blois [19]. Crude

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extract at various concentrations 50, 100, 125, 150, 400 μg/mL were measured and the volume was made up to 1 mL with methanol followed by addition of 5 mL of 0.1 mM DPPH solution. The test tubes were incubated in dark for 20 min at room temperature. The absorbance was read at 517 nm. IC_{50} values of the ethyl acetate extract were calculated. A lower IC_{50} value reveals higher activity.

2.6. Determination of antibacterial activity

Five pathogenic test bacteria such as *Klebsiella pneumoniae* (*K. pneumoniae*), *Bacillus subtilis* (*B. subtilis*), *Escherichia coli* (*E. coli*), *Staphylococcus aureus (S. aureus), and Pseudomonas aeruginosa* (*P. aeruginosa*) were used for the antibacterial activity using agar well diffusion assay [20]. One hundred microlitre of 24 h inoculum of the test bacteria were spread on Muller Hinton agar plates. Wells about 8 mm were made aseptically and the ethyl acetate extract dissolved in DMSO at different concentrations of 25 μg/mL, 50 μg/mL, and 75 μg/mL, were dropped into the 8 mm diameter well made in petriplates containing Muller Hinton Agar. Antibacterial activity was evaluated by measuring the diameter of the zone of inhibition in mm for the pathogenic bacteria and compared to the standard drug ampicillin.

2.7. In vitro hepatoprotective potentials of ethyl acetate extract of Achaetomium sp.

HepG2 cell lines were cultured in standard conditions. The cells were supplemented with DMEM and 10% foetal bovine serum in a 5% humidified CO_2 atmosphere at 37 °C. The cells were trypsinized and as the cells become 90% confluence were plated in a 96-well microplate, at 1.2×10^4 cells/well. The cells are left to attach. The hepatoprotective potentials on HepG2 cells of ethyl acetate extract were as follows. Normal HepG2 cells were incubated with DMEM and supplemented with (10%) foetal bovine serum for 12 h. For normal control the cells are treated only with DMEM and DMSO v/v. The cells were incubated in DMEM with ethyl acetate extract at 12.5, 25, 50, 75, 100, 150 μg/mL for 12 h and treated with CCl_4 (1%) for 1.5 h. For standard, the cells were incubated in media and silymarin at 12.5, 25, 50, 75, 100, 150 μg/mL for 12 h and later treated with CCL_4 (1%) for 1.5 h. Each assay was performed in triplicate.

Cell viability was evaluated using the MTT assay with slight modifications [21]. The colourimetric assay involves mitochondrial succinate dehydrogenase which exists only in live cell where it acts in converting MTT to a purple formazan derivative. The cells were treated with ethyl acetate extract as sample, silymarin as a standard and both the sample and the standard were exposed to CCl_4 as a toxic agent. The medium was then removed and 100 μL of fresh media with MTT 10 μL (0.5 mg/mL) were added and the cells were incubated for 2 h and 100 μL DMSO was added to dissolve the formazan crystals and the absorbance was read at 570 nm.

Percentage of cell survival was calculated by the following formula:

$$\text{Viability}\% = (\text{Test OD}/\text{Control OD}) \times 100$$

$$\text{Cytotoxicity}\% = 100 - \text{Viability}\%$$

2.8. Statistical analysis

Values are expressed as mean of triplicate determinations ± standard deviation and statistical analysis was carried out by analysis of variance (ANOVA) followed by Duncan's test. $P < 0.05$ was considered as indicative of significance, as compared to the control group using the SPSS (version 13.0).

3. Results

3.1. Microscopic and molecular identification of fungal isolate

Fungal colony was isolated from *E. hirta.* On the basis of microscopic examination and molecular characterization, it was confirmed that the isolate belonged to the genus *Achaetomium*. It is evident from the phylogenetic tree that the isolate reveals maximum homology with *Achaetomium strumarium* (*A. strumarium*) (JX863914). Best BLAST matches for endophytic fungus *Achaetomium* sp., with maximum homology. On the basis of 28S rRNA gene homology the fungal isolate is designated as *Achaetomium* sp., the 28S rRNA gene sequences submitted to GenBank with the accession number KY231923 (*Achaetomium* sp.,) shown in Figure 1.

3.2. Determination of total phenolic, tannin and flavonoid contents of ethyl acetate extract of Achaetomium sp.

The total phenolic and tannin contents were expressed in gallic acid equivalence and the total flavonoid content was expressed in rutin equivalence. The 250 μg/mL of ethyl acetate extract of the strain *Achaetomium* sp. was estimated to have (44.02 ± 1.57) μg of total phenolic content; total flavonoid content was found to be (54.540 ± 1.820) μg and the total tannin content was found to be (18.790 ± 1.018) μg. The assay was performed in triplicates and the mean values were taken and the results were exhibited in gallic acid equivalents (GAE). Both the phenolic and the flavonoid contents had contributed for the antioxidant potentials.

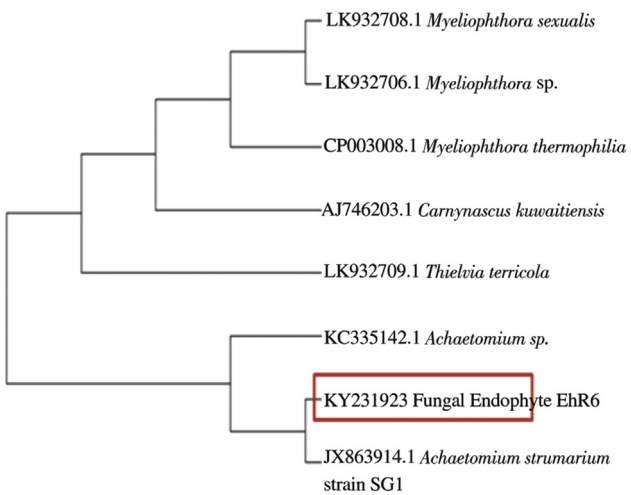

Figure 1. Phylogenetic tree inferred using Neighbour-Joining method for query and control ITS sequence (organism indicated within box).

3.3. Antioxidant assay (DPPH method)

The antioxidant activity was seen as a change of colour from purple to yellow. The ethyl acetate extract of *Achaetomium* sp., shows significant antioxidant activity (Table 1). Inhibition values of the ethyl acetate extract concentrations 50, 100, 150 and 500 μg/mL were found to be 66.89% ± 1.385%; 71.43% ± 1.414%; 79.10% ± 1.081% and 87.34% ± 0.289% respectively. The antioxidant capacity of ethyl acetate extract of *Achaetomium* sp., was performed with references butylated hydroxyl toluene (BHT), ascorbic acid (AA), gallic acid (GA), and Pyrogallol (PG). In DPPH scavenging assay the IC_{50} value of the *Achaetomium* sp., extract was found to be 32.26 μg/mL.

3.4. Antimicrobial activity

The *in vitro* antibacterial activity of the ethyl acetate extract of *Achaetomium* sp., was evaluated against five bacterial strains *S. aureus*, *K. pneumoniae*, *E. coli*, *B. subtilis*, and *P. aeruginosa*, with three different concentration 25 μg/mL, 50 μg/mL, and 75 μg/mL. Ethyl acetate extract shows remarkable antibacterial activity with significant inhibition of 22 mm and a minimum inhibition 17 mm. There was significant antibacterial action against *S. aureus, P. aeruginosa* and *K. pneumoniae* and a moderate action against *B. subtilis*, and *P. aeruginosa.* Broad spectrum activity of the ethyl acetate extract against the five pathogenic bacteria was significant. There was no activity against any of the bacterial pathogens at low concentrations 25 μg/mL. But at a dose of 50 μg/mL there was a significant inhibitory activity against *K. pneumoniae* (18 mm), *B. subtilis* (18 mm), and *P. aeruginosa* (18 mm) and a moderate activity of (14 mm) against *E. coli* and (13 mm) against *S. aureus*. But at a concentration of 75 μg/mL there was a greater inhibitory action against *S. aureus* (22 mm), *K. pneumoniae* (22 mm), *P. aeruginosa* (22 mm) and against *E. coli* (18 mm) and *B. subtilis* (18 mm). The results were comparable with ampicillin as standard drug.

3.5. Hepatoprotective activity

The HepG2 cell lines were pre-treated with hepatoprotective agents and subsequently treated with CCl_4 which causes damage in the cells. The CCl_4 (1%) exposed HepG2 cell lines exhibited cell viabilities in range from 30% to 72% compared with the standard silymarin from 42% to 93% at concentrations ranging from 12.5 μg/mL to 150.0 μg/mL respectively in Table 2. CCl_4 treated cells exhibited viability in a dose dependent manner when pre-treated with silymarin and ethyl acetate. The untreated cells were 100% viable. The extract at a low concentration 12.5 μg/mL showed 30.920% ± 2.036% whereas silymarin showed 42.0200% ± 0.1414% viability. Silymarin found to exhibit significant viability of 93.260% ± 0.784% and extract showed 72.17% ± 2.94% at high concentration 150 μg/mL. The ethyl acetate extract + CCl_4 exhibit significant percentage viability but not as much as standard silymarin + CCl_4.

Table 2
Hepatoprotectivity potential of ethyl acetate extract of *Achaetomium* sp., isolated from *E. hirta.*

Concentration (μg/mL)	Ethyl acetate extract	Silymarin
12.5	30.92 ± 2.0364	42.02 ± 0.141
25	35.80 ± 1.718	50.38 ± 1.046
50	44.26 ± 0.919	63.28 ± 0.855
75	53.26 ± 1.491	77.11 ± 1.230
100	63.39 ± 0.714	87.88 ± 0.912
150	72.17 ± 2.948	93.26 ± 0.784

4. Discussion

The medicinal herb *E. hirta* is a medicinal herb with various phytochemicals and medicinal properties [8,9]. The fungal isolate *Achaetomium* sp., was isolated from the roots of the plant. *Achaetomium* sp., belongs to *Ascomycetes* and family Chaetomiaceae. Thus on microscopic, morphological and molecular characterization was confirmed that the endophytic fungal isolate belongs to the genus *Achaetomium*. It is also evident from the phylogenetic tree and the 28S rRNA gene homology with the reference strain the fungal isolate isolate more homologous with *A. strumarium*, and so it is confirmed as *Achaetomium* sp. The genus *Achaetomium* was described with only three species *A. strumarium, Achaetomium globosum* and *Achaetomium luteum.* Probably there are only twenty species of *Achaetomium* reported till now and over the past half of the century not much research has been carried out on the bioactive compounds from *Achaetomium* sp [22]. Molecular techniques are accurate tools, widely used in the identification of the fungal endophytes. Sequencing of the amplified PCR product has enabled to identify the endophytic fungi at genus level. In this study the endophytic fungus has more homologous to *A. strumarium*, but there are no previous reports on pigment producing *A. strumarium*. But the endophytic fungal isolate *Achaetomium* sp., from *E. hirta* produces a red pigmented bioactive compound.

There are previous studies of phenols in extracts of endophytic fungus *Chaetomium* sp., *Aspergillus niger, Fusarium* sp., *Curvularia lunata* isolated from *Eugenia jambolana* [23]. The redox properties of phenols mainly contribute to their antioxidant potentials which play a vital role as reducing agents, singlet oxygen quenchers and hydrogen donor [24]. Ethyl acetate selectively extracts high phenolic and selectively

Table 1
DPPH radical scavenging activity of ethyl acetate extract of *Achaetomium* sp., isolated from *E. hirta.*

Concentration (μg/mL)	Concentrations (μg/mL)			
	50	100	150	500
Ethyl acetate extract	66.890 ± 1.385	71.430 ± 1.414	79.100 ± 1.081	87.340 ± 0.289
Ascorbic acid	83.030 ± 0.685	90.240 ± 1.173	91.210 ± 1.414	98.370 ± 0.763
Gallic acid	85.510 ± 1.145	88.920 ± 0.692	92.10 ± 0.48	94.890 ± 1.103
BHT	91.260 ± 0.707	93.250 ± 1.286	95.540 ± 0.855	97.080 ± 0.636
Pyrogallol	89.090 ± 0.452	90.840 ± 0.374	92.39 ± 0.89	96.980 ± 0.098

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removes non phenolic compounds [25]. On the other hand, ethyl acetate allows the highest phenolic content and allows selective removal of non phenolic compounds [26]. It is evident that the ethyl acetate extract from *Achaetomium* sp., could be phenols. The fermentation broth was extracted with various solvents in increasing polarity with petroleum ether, dichloromethane, butanol, and ethyl acetate. But there were only traces of the extract from the other solvents except ethyl acetate. With respect to this fungus ethyl acetate was the only solvent which could effectively extract the pigmented culture media. The pigment from the cultivation media was only soluble in ethyl acetate and so the choice of solvent for extraction was ethyl acetate.

Previously, bioactive flavonoids 2′, 4′-Dihydroxy-6′-methoxy-3′ 5′-dimethylchalcone was produced by an endophytic fungus *Ceriporia lacerate* isolated from *Cleistocalyx operculatus* [27]. Flavonoids also play a role in chelation of trace elements involved in free-radical production, protection of antioxidant defences, scavenging reactive species and suppressing reactive oxygen species formation [28]. Previous tannin from the endophytic fungus *Chaetomium* sp., and *Tricoderma* sp. from the leaves of *Azadirachta indica Juss* (Neem) [29] are reported. This is the first time that a pigment producing endophytic fungus *Achaetomium* sp., is being isolated from *E. hirta*.

Radical scavenging activity of the ethyl acetate extract was evaluated by DPPH assay and it was observed that, there was a notably reduction in DPPH radical and it also clearly shows, that as the concentration of ethyl acetate extract increases there is an increase in free radical scavenging activity. DPPH free radical scavenging assay is a basic, most widely and regularly used method in the evaluation of antioxidant potentials of sample [30]. Four standards Gallic acid, BHT, ascorbic acid and Pyrogallol have been used in the assay. There is almost an equipotent effect of the ethyl acetate extract of *Achaetomium* sp., in the inhibition of free radical production with that of the standard antioxidant ascorbic acid at 500 µg/mL. Pestacin and isopestacin, the two antioxidants, were produced by *Pestalotiopsis microspora* an endophytic fungus isolated from *Terminalia morobensis* in [31]. There are previous reports on 21 endophytic fungus with antioxidant potentials from five Sudanese medicinal plants, where the *Aspergillus* sp., from *Trigonella foenum* showed significant antioxidant potential [32]. *Chaetomium* sp., from *Eugenia jambolina* shows 80% radical scavenging activity with phenolic content (60.13 ± 0.41) mg/GAE. In this study the *Achaetomium* sp., shows 87% radical scavenging activity with phenolic and flavonoid content of 44.02 ± 1.57 and 54.540 ± 1.820, respectively.

The antimicrobial activity of the ethyl acetate extract showed to inhibition at a lower concentration of but showed a significant inhibition at a concentration of 75 µg/mL. It is more prominent that the ethyl acetate extract inhibits the gram negative bacterial pathogens which are the causative factors for most of the infectious diseases. There are previous reports on antibacterial activity from endophytes *Colletotrichum gloeosporiodes and Fusarium oxysporum from Pinguicula acuminata and Peperomia obtusofolia* have significant antibacterial activity [33]. The flavonoids are produced by many endophytes and the flavonoids as well as phenols play an important role as antimicrobial agents [34]. So phenols and the flavonoids could have contributed to the antibacterial activity.

Hepatoprotective effect of the ethyl acetate extract was evaluated using HepG2 cell lines. *In vitro* cytotoxic and hepatoprotective potentials of the bioassay guided fractions has gained more importance for screening at the preliminary levels. CCl_4 is very often used toxic agent to evaluate the hepatoprotective effects of drugs and plant extracts in *in vitro* or *in vivo* models and CCl_4 toxicity is concentration and time dependent [35]. HepG2 cell line is the most commonly used and an efficacious *in vitro* model for evaluating hepatoprotective potential of many bioactive compounds [36]. The (3-(4,5 dimethylthiazole-2 yl)-2,5diphenyltetrazolium bromide) tetrazolium salt is taken up into the cells and reduced to a blue coloured formazan derivative in a mitochondria. This derivative accumulates in the cells, as it is not permeated through the plasma membrane. On solubilisation, the accumulated derivative is liberated which can be readily detected and quantified by colourimetric method. The ability of cells to reduce MTT reveals the mitochondrial integrity and the functions of the live cells which can be interpreted as a measure of viability of the cells [37].

Result showed as the concentration of the sample is increased there is an increase in the percentage viability of the cells and it also indicates a positive correlation between the phenolic, flavonoid and tannin content. The hepatotoxicity of CCl_4 is caused by the cleavage of bond between carbon and chlorine to generate a trichloromethyl free radical (CCl_3) which is oxidised to form a trichloromethyl peroxyl radical (CCl_3O_2). This oxidized metabolite may react with membrane polyunsaturated fatty acids and causes lipid peroxidation. Another study on hepatoprotective activity from the extracts of endophytic fungus from *Ocimum sanctum* Linn has been reported [38]. This is the first report on endophytic fungal extract on hepatoprotective activity from *E. hirta.*

The fungal isolate from *E. hirta* was identified as *Achaetomium* sp., and the result reveals that it produces the secondary metabolite with high content of phenolics including flavonoids and tannins which contributed for the significant biological potentials. The phenolic rich ethyl acetate extract of *Achaetomium* sp., exhibited remarkable antibacterial, antioxidant, hepatoprotective potentials and also can serve as a substantially sustainable resource for novel secondary metabolites.

Conflict of interest statement

We declare that we have no conflict of interest.

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