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Original Article

Assessment of the Impact of Wild Stinkhorn Mushroom Extracts on Different Cancer Cell Proliferation and Study of Primary Metabolites

Ribhu Ray, Amrita Pal, Santanu Paul*

ABSTRACT

Objective: Present study aims to evaluate the efficacy of methanolic and ethyl acetate extracts of wild mushroom *Phallus* sp. on cell proliferation of both normal and cancer cells. This study also looked at anti-oxidant potentiality of methanolic extract and also unravels the phytochemical profiling of both extracts. **Methods:** Anti-proliferative activity was assessed by MTT assay on different human cancer cell lines such as MCF-7, MOLT-4, REH and Peripheral Blood Mononuclear Cells or PBMC isolated from a healthy donor. Gas Chromatography-Mass Spectrometry (GC-MS) analysis was used for comparative assessment of phytochemical constituents of both extracts. The anti-oxidant profile of methanolic extract was also evaluated by DPPH and ABTS•+ assays. **Results:** Results indicated that the both methanolic and ethyl acetate extracts of *Phallus* sp. showed appreciable anti-proliferative activity against breast cancer cell line MCF-7 with IC_{50} of 8.544±2.812 µg/mL and 35.279±2.863 µg/mL respectively. Both of the extracts also showed its moderate impact on human B cell precursor leukemia cell line (REH) with IC_{50} of 25.987±2.696 µg/mL for methanol and 51.484±1.480 µg/mL for ethyl acetate extract respectively. No effect was observed in MOLT-4 cell line. Methanolic extract was selected as better anti cancer extract over ethyl acetate extract. No significant anti-proliferative activity was observed in normal PBMC by both extracts. GC-MS analysis indicated that 43 and 114 compounds were identified from methanolic and ethyl acetate extracts respectively. Among them nine compounds shared its existence in both of the extracts. Different derivatives of ergosterol and several fatty acid esters ware identified as major components from both of the extracts. Methanolic extracts of the *Phallus* sp. showed its effectiveness on both of DPPH and ABTS•+ free radical, and result indicated that it contain more flavonoid content than phenol. **Conclusion:** The methanolic extract of *Phallus* sp. show very specific anti-proliferative effect on MCF-7 with moderate anti-oxidant activity and holds a great promise for isolation of bio molecules for treating Breast Cancer. Several derivatives of ergosterol identified as probable anti-cancer compound.

Key Words: MTT assay, GC-MS, *Phallus*, MCF-7, ABTS•+.

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INTRODUCTION

Cancer is identified as the first or second leading cause of death in 91 to 172 countries and it is also ranked as third and fourth in an additional 22 countries[1,2]. Cancer is one of the major public health problem and the second leading cause of death in the United States[3]. It has been estimated that in 2020, 1,806,590 new cancer cases may occur in United States and among them 606,520 cancer death has been projected[3]. In India cancer is ranked as second leading cause of death in the urban area and placed at fourth in rural areas[4]. In India, 1.15 million new cancer patients were recorded in 2018, and it is predicted to almost double by 2040[4]. Cancer is characterized by uncontrolled cell proliferation and it may appear in different forms such as lung, breast, colon, skin, blood cancer etc.[5]. In India, it is now cause of catastrophic health spending, calamity financing and rising expenditure before death[4-8]. The invasiveness and mortality rate of the cancer vary among different forms, lung cancer is highly fatal with a very high death rate of 70%, followed by colon cancer and breast cancer[5].

The most commonly used potential strategy for treating cancer is the use of various chemotherapeutic drugs along with radiotherapy[9]. Chemotherapeutic drug should be target specific to cancer cells, and should not affect the normal cells. But most of the chemotherapeutic drugs available in the market, show various adverse effects on the normal cells[10,11]. There is an urgent need for the further development of safe anticancer drugs to counteract these negative consequences of the existing drugs. This study aims at the identification of alternative anti-cancer drugs preferably from natural sources with minimal collateral damages to normal cells[12]. Mushroom contain wide range of bioactive compound such as different variety of phenols and flavonoids[13], polysaccharides, glycosides, alkaloids, volatile oils, tocopherols, carotenoids, organic acids[14] and may act as a potential alternative substitute for naturally occurring anti-oxidants, anticancer,

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immunomodulating, anti-inflammatory, cardiovascular, anti-microbial and anti-diabetic drug[15-19].

The stinkhorn-- (genus *Phallus* Junius ex L.) is classified under the family of Phallaceae of order Phallales, which are easily identified by its foetid odour, saccate rhizomorph containing volva, unbranched sponge like pseudostipe with extremely attached olive brown to dark brown slime containing the spore mass (gliba) and occasionally a skirt-like indusium[20]. All species under the Phallaceae are short-lived[21], begin their development as an oval or round shaped structure termed as "eggs" (early stage of basidiocarp) but mature basidiocarp show drastic variation of colour and pattern, thus distinguish different species of this genus[2021]. The mature spore mass produces a characteristic carrion like smells which attracts different insects to disperse the spore, and make it one of the most easily recognizable species of fungi. According to Index Fangorum 2016, genus *Phallus* contains 31 species. Reported *Phallus* species show wide range of distribution pattern in different climate type from tropical, subtropical and temperate forest but predominantly occupying the tropical and subtropical habitat[22-26].There are a large number of *Phallus* sp. reported from different habitat and location of India[2026-28].

So, in this present study we have tried to assess the impact of both ethyl acetate and methanolic extracts of wild stinkhorn (*Phallus* sp.) on different cancer line (MCF-7, MOLT-4, REH) *in vitro* and try to compare the phytochemical constituents profiling of two extracts through GC-MS analysis. The antioxidant profile of the methanolic extract of this mushroom was also evaluated.

MATERIALS AND METHOD

Chemicals

All chemicals used were of analytical grade and freshly prepared. RPMI 1640 (Sigma Aldrich), DMEM (Sigma Aldrich), FBS (Sigma Aldrich), Penicillin and Streptomycin (Sigma Aldrich), MTT [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Aldrich), DMSO, Phosphate Buffer Saline (Himedia) , 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) (SRL) , 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma-Aldrich), sodium persulfate (SRL) , Gallic acid (Merck), Sodium carbonate (Merck), Folin Ciocalteau (Merck), Methanol, Ethyl acetate, Hexane,. Quercetin (SRL), Sodium nitrite (Himedia), Aluminum chloride (Merck).

Sample collection and identification

The fresh mature fruit body of *Phallus* was collected from Lolegaon (27°00'50.6"N 88°33'40.4"E), a small Lepcha village of Kalimpong district of West Bengal during the month of May 2019 .Their morphological characteristics such as color, size, shape were recorded on spot immediately after collection.

Molecular identification of the mushroom

DNA extraction-Total genomic DNA was extracted from the air-dried specimen (38 mg) by using the Fungal gDNA Mini Kit (Xcelris Genomics, Ahmedabad, India) following the manufacturer instructions.

Polymerase Chain Reaction (PCR)- Internal transcribed spacer (ITS) region 1, 2 and the 5.8s rDNA were amplified by using a pair of primer sequence ITS1 (forward primer 5' TCC GTA GGT GAA CCT GCG G 3') and ITS4 (reverse primer 5' TCC TCC GCT TAT TGA TAT GC 3')[29]. The PCR reaction mixture were contained following components :10X PCR buffer (Thermo#EP0702) , 8X dNTP mix 2 mM each (Thermo #R0241) , 100 mM of each primer, 30 ng of extracted genomic DNA, 5 U/µL *Taq* DNA polymerase (Thermo#EP0702), 20 mM MgCl (Thermo#EP0702) and nuclease free water to make final volume 50 µL. Amplification reaction was performed by using 96 well thermal cycler (Veriti, Applied Biosystems) programmed as initial denaturation for 4 min at 94°C followed by 35 cycles consisting of 1 minutes at 94°C (denaturation) then 1 min in 56°C for annealing, 1 min at 72°C (extension) and finally one cycle for 10 min at 72°C for final elongation.

The PCR amplicons were checked by electrophoresis on 2% agarose gel containing ethidium bromide.

Sequencing- PCR amplicons were then further purified and were subjected to automated DNA sequencing on an ABI3730XL-15104-028 DNA Analyzer (Applied Biosystems, USA) using same sets of primers identical with amplicons for the ITS rDNA region. The newly generated sequences were then deposited in GenBank (*www.ncbi.nlm.nih.gov*).

Extraction procedure

Fresh material was air-dried and grinded by mixer grinder. Powdered basiodiocarp (5g) was first defatted with 50 mL of hexane for 48 hours. After that residue was air-dried and extracted with 50 mL of ethyl acetate for 48 hours. The mixture was filtered through whatman-4 and then lyophilized to ethyl acetate fraction (EAP). The residue was further re-extracted with 50 mL methanol 48 hours and same procedure was followed for obtaining methanol fraction (MEP).

Cell line and culture

MCF-7 (human breast cancer cell line), MOLT-4 (human acute T lymphoblastic leukaemic cell line), REH (human B cell precursor leukemia cell line) cell lines were used for the evaluation of anti-proliferative activity of both EAP and MEP extracts. Above cell lines were cultured in either DMEM or RPMI 1640 supplemented with 2 mM L-glutamine, 10% (v/v) FBS (heat inactivated), 10 U/mL penicillin and streptomycin and incubated at 37°C in a humidified atmosphere of 5% CO_2 incubator (HF90).

Cell viability assay

In order to determine the impact of both EAP and MEP extracts on cell proliferation, MTT assay[30] was performed. Briefly, $5X10^4$ cells was seeded in 96-well cell culture plate in presence of MEP and EAP (10-100µg/mL) for 48 h. After that, cell culture media was replaced with 5% FBS-containing phenol red-free DMEM and MTT (200 µL; 0.5 mg/mL) and incubated for 4h in a humidified incubator containing 5% CO_2. Finally, the media was discarded and formazan crystals was dissolved in DMSO and was measured at 560 nm (iMark, Biorad). 5% SDS lyses buffer was used for making the 100% full lysed cell and it was also spectrophotometrically measured at 560 nm (iMark, Biorad) .The percentage of cell viability was calculated according to the following equation: % cell viability = (O.D. sample – O.D. 100%lysis) / (O.D. 0%lysis – O.D.100%lysis) × 100.

Comparative phytochemical profiling of EAP and MEP by Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Phytochemical investigation of both EAP and MEP extracts were performed by gas chromatographic analysis 7890A (Agilent Technologies) coupled with a mass spectrometer (MSD 7000). For gas chromatographic analysis a capillary non-polar column HP-5MS (5% Phenyl Methyl Silox: Agilent 19091S-433) with the dimension of 30 m X 250 µm, along with film thickness: 0.25µm was used. Helium was used as a mobile phase with a flow rate of 2.25 mL min^{-1}. The injection volume of the sample was 1 µL with a split ratio of 2:1 at 320 °C. The oven was programmed at 60 °C, then raised to 320 °C for 12 min at 6 °C min^{-1} rate. For mass spectrometric analysis the system was programmed in full scan, with electron impact 70 eV in a range of 50-550 (m/z). The compound was identified by comparing its fragmentation profile with spectra present in the library NIST version 2.2.

Evaluation of antioxidant potential of MEP

The DPPH and ABTS•+ free radical scavenging assay are widely used methods for evaluation of the antioxidant capacities of natural products[31]. This aforementioned assays are performed by spectrophotometric techniques based on the quenching of stable colour radicals (DPPH and ABTS•+) and thus indicate the radical scavenging potentiality of antioxidant compounds even when present in complex biological mixtures[31].

To determinate the impact of MEP on artificially generated free radical, DPPH free radical scavenging assay and ABTS•+ free radical scavenging assay were carried out.

DPPH free radical scavenging Assay

The free radical scavenging activity of the MEP on DPPH radicals was measured according to the method of Hajra *et al.*[32] and Brand-Williams *et al.*[33] with slight modification. Briefly 0.9 mL of DPPH solution (0.1 mM) was added to a test tube of 100 µL mushroom extract, concentration range 100-500 µg/mL diluted with methanol. Control was prepared with methanol instead of the mushroom sample. The reaction mixture was incubated for 2 h at room temperature and the absorbance was measured at 515 nm with a spectrophotometer (Shimadzu). The percent of reduction of DPPH was calculated according to the following equation: % DPPH reduction = [{(Ac–As)/Ac}×100] (Where; As is the absorbance of sample, Ac is the absorbance of control).

ABTS•+ free radical scavenging assay

The free radical scavenging activity of the MEP on ABTS•+ radicals was measured according to the method of Re *et al.*[34] with slight modification. The long-lived ABTS•+ radical cation chromophore 2, 2´-azinobis-(3—ethylbenzothiazoline-6-sulphonate) was generated by reacting 7 mM of ABTS stock solution (prepared in deionised water) with 2.45mM potassium persulfate (prepared in deionised water) at a ratio 1:1 (v/v) and the mixture was incubated in the dark at room temperature for twelve hours before use. Working solution of ABTS•+ was prepared by diluting ABTS stock solution with 5mM phosphate buffer solution (PBS), pH 7.4, and equilibrated an absorbance of 0.7 (± 0.2) at 734 nm at 30°C. The assay was performed in 96 wall microplate by following protocol, 10 µL of methanolic extract (concentration range, 100-500 µg/mL diluted methanol) of mushroom were mixed with 290µL of ABTS•+ working solution and incubated for ten minutes at 37°C.The reduction of ABTS•+ radical by mushroom extract, containing antioxidant compounds was measured by the change of absorbance of ABTS•+ radical at 734nm (Shimadzu). The percent of reduction of ABTS•+ was calculated according to the following equation: % ABTS•+ reduction = [{(Ac–As)/Ac} ×100] (Where; As is the absorbance of sample, Ac is the absorbance of control.)

Assessment of antioxidant compound present in MEP

The antioxidant potentiality of the mushroom extract depends on the antioxidant compound present in the extracts. The phenol and flavonoid are the major antioxidant compounds present in biological mixtures. So the total phenol and flavonoids content of MEP were also quantitatively evaluated.

Total Phenolic content determination

Total phenolic content of MEP was quantitatively measured by the method of Singleton *et al.*[35] and Mridha *et al.*[36] using Folin-Ciocalteu reagent with vary minor modification. 200 µL of mushroom extract was mixed with 1 mL of Folin-Ciocalteu reagent. After 5 minutes, 0.8 mL of 10% sodium carbonate solution was added and volume was adjusted with help of 50% methanol. Then the mixture was incubated in dark for 20 minutes and finally the absorbance was read at 765 nm by spectrophotometer. Different concentration of Gallic acid (0-10 µg/mL) was used as a standard. Finally, the result was expressed as mg of Gallic acid equivalent (GAEs) present per g of mushroom dry weight.

Total flavonoid content determination

Total flavonoid content of MEP was quantitatively assessed by following the method of Shing *et al.*[37] using aluminium chloride colorimetric method . Briefly 200 µL of mushroom extract was diluted with 1 mL of double distilled water and then 75 µL 5% sodium nitrate solution was added. After 6 minutes of incubation 75 µL of 10% aluminium chloride solution was added to the reaction mixture and kept for 5 minutes in room temperature. Finally, 500 µL of 1M sodium hydroxide solution was added to the mixture, and it was vortexed and absorbance was taken at 510 nm. Different concentration of Quercetin (10-100 µg/mL) was used as a standard. Finally, the result was expressed as mg of Quercetin equivalent (QEs) present per g of mushroom dry weight.

RESULT

Identification of the mushroom by DNA Barcoding

DNA barcoding is one of the most emerging molecular technique for identification and taxonomic revision of the biological specimen[38-40]. DNA sequence based specimen identification method was applied for this present study to verify the morphologically identified species. Genomic DNA extracted from this specimen was amplified with combination of both ITS1 and ITS4 primers and 365 bp long stretch of DNA sequence was generated. The generated sequence was submitted to gene bank with accession number MT007520. The closest hit of MT007520 was *Phallus coronatus* (GenBank : MG678522), sequence identity=313/324 (97%); gaps= 4/324 (1%).

Cell viability assay

Both EAP and MEP extracts of *Phallus* showed very effective anti-proliferative activity *in vitro* when subjected to MTT assay at the time point of 48 hours (Figure 1). IC_{50} is the particular concentration of the drug, where 50% of cancer cells are killed. The IC_{50} of both MEP and EAP against different cancer cell line were represented on Table 1 (Figure 1C). The IC_{50} value of MEP against the MCF-7 and REH were 8.544±2.812 µg/mL and 25.987±2.696 µg/mL respectively. IC_{50} value of EAP against the MCF-7 and REH were 35.279±2.864 µg/mL and 51.484±1.481 µg/mL respectively. MOLT-4 cell line showed minimal activity than aforementioned two cell lines. IC_{50} value of MOLT-4 cell line were 131.266±9.806 µg/mL against MEP extract and 126±2.111 µg/mL against EAP extract. Both extracts showed very negligible cytotoxic activity against Peripheral Blood Mononuclear Cells or PBMC isolated from healthy donor (Figure 1A,1B). So, it can be assumed that both extract of *Phallus* showed the specific cytotoxic effect on cancer cells, no such derivation on the ground of cell viability were recorded from normal PBMC cells.

Comparative study of photochemical profiling of EAP and MEP by Gas Chromatography-Mass Spectrometry (GC-MS) analysis

GC-MS chromatogram of both MEP and EAP were represented in Figure 2 and Figure 3 respectively. Total 43 and 114 compounds were identified by comparing its fragmentation profile with spectra present in the library NIST version 2.2. Among them the major compounds which occupied minimum 1% of total area were listed in Table 2 for EAP and Table 3 for MEP respectively. Total 16 major compounds were found in MEP and 29 major compounds were found in EAP and 9 compounds were shared by both of extracts.

EAP extract were contained two major compounds namely ergosta-5,22-dien-3-ol,(3β,22E,24S) and 9,12-octadecadienoic acid (Z,Z)

Table 1: Comparative analysis of IC_{50} (µg/mL) values of EAP and MEP of *Phallus* sp. tested in the following cell line through MTT assay. Results are the mean value of three independent experiments with standard deviation.

Cancer cell line	IC_{50} (µg/mL)	
	EAP	MEP
MCF-7	35.279 ± 2.863	8.544 ± 2.812
REH	51.484 ± 1.480	25.987 ± 2.696
MOLT-4	126.702 ± 2.111	131.266 ± 9.805

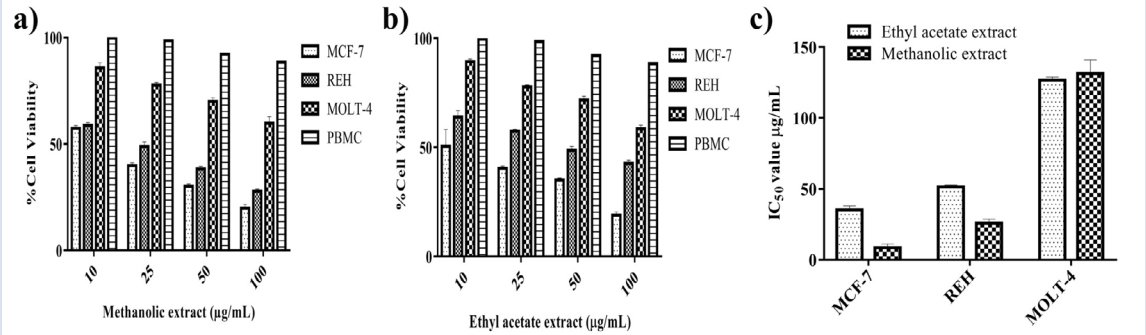

Figure 1: Selective anti-proliferative activity of EAP and MEP extracts of *Phallus* sp. (10-100 µg/mL) on different cancer cell lines including MCF-7, REH, MOLT-4 and normal cell PBMC. A) Methanolic extract (MEP), B) Ethyl acetate extract (EAP), C) Comparative account of IC_{50} values.

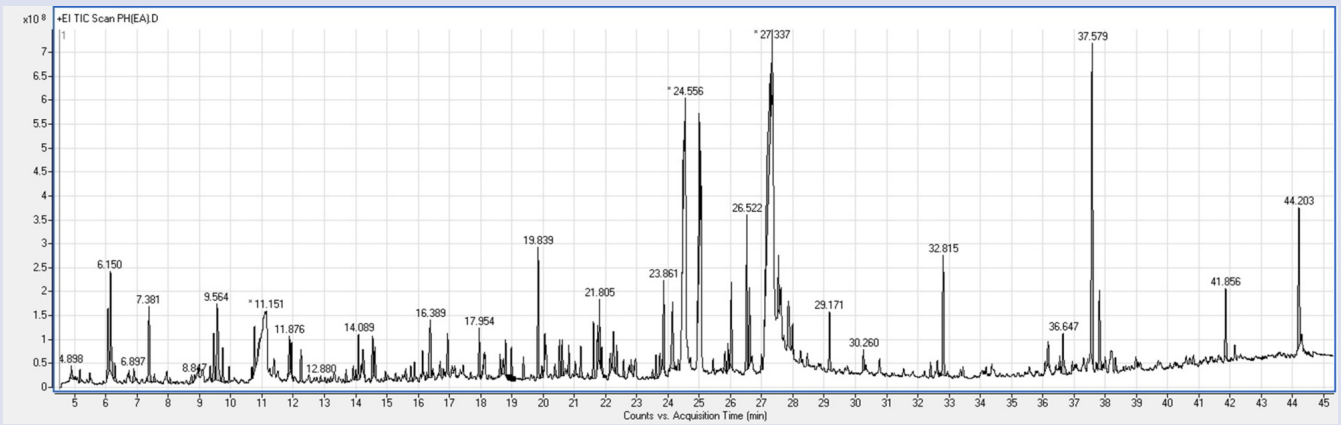

Figure 2: GC-MS chromatogram of Ethyl acetate extract of (EAP) of *Phallus.*

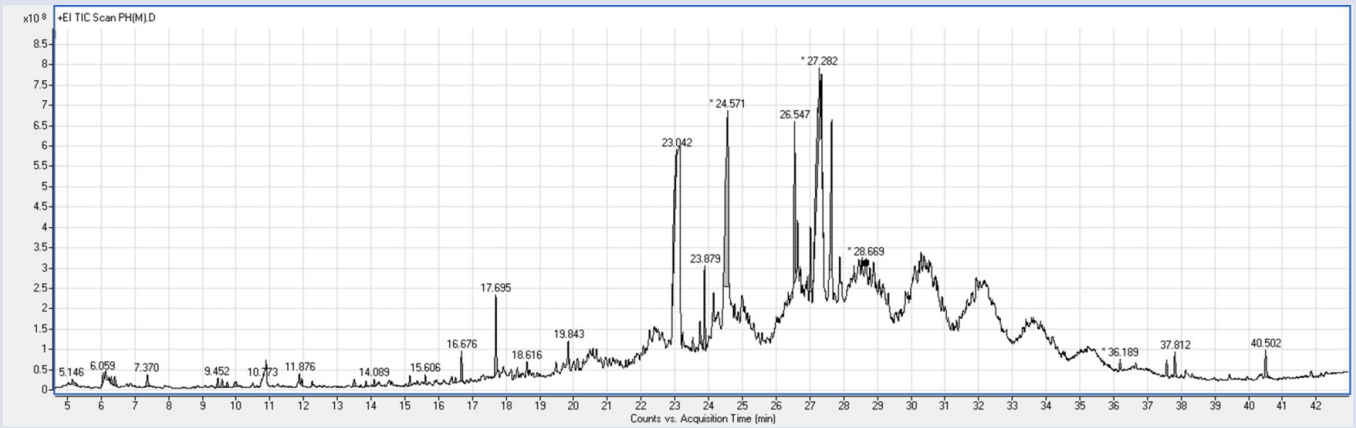

Figure 3: GC-MS chromatogram of Methanolextract of (MEP) of *Phallus.*

Table 2: Major phytoconstituents identified in EAP extract of *Phallus* sp. and its biological uses.

Sl No.	RT	Area	Name of the compound	Molecular weight	Molecular formula	CAS #	Biological Activity	Reference
1	37.58	8.61	Ergosta-5,22-dien-3-ol,(3β,22E,24S)	398	$C_{28}H_{46}O$	17472-78-5	anticancer	41
2	27.27	6.76	9,12-octadecadienoic acid (Z,Z)	280	$C_{18}H_{32}O_2$	60-33-3	antiinflammatory and antiarthritic	42
3	44.20	4.03	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)4-hydroxy-,octadecyl ester	530	$C_{35}H_{62}O_3$	2082-79-3		
4	26.52	3.33	9,12-octadecadienoic acid (Z,Z)-methyl ester	294	$C_{19}H_{34}O_2$	112-63-0	anticancer	43
5	32.82	3.30	Di-n-octyl-phthalate	390	$C_{24}H_{38}O_4$	117-84-0		
6	25.00	3.27	Chlorpyrifos	349	$C_9H_{11}Cl_3NO_3PS$	2921-88-2	insecticides	44
7	19.84	2.80	Dodecyl acrylate	240	$C_{15}H_{28}O_2$	2156-97-0	antifungal	45
8	9.56	2.70	Isopulegol	154	$C_{10}H_{18}O$	89-79-2	antifungal	46
9	6.15	2.43	D-limonene	136	$C_{10}H_{16}$	5989-27-5	anticancer	47
10	7.38	2.18	Bezene, (1-methylenepropyl)	132	$C_{10}H_{12}$	2039-93-2		
11	37.82	2.16	Ergosta-5,7,9(11),22-tetraen-3-ol, (3β,22E)	394	$C_{28}H_{42}O$	516-85-8		
12	24.15	2.14	Palmitoleic acid	254	$C_{16}H_{30}O_2$	373-49-9	antiinflammatory	48
13	23.86	2.00	Hexadecanoic acid, methyl ester	270	$C_{17}H_{34}O_2$	112-39-0	antibacterial and antifungal	4950
14	16.39	1.95	2,4-Di-tert-butylphenol	206	$C_{14}H_{22}O$	96-76-4	antimicrobial , antifungal , antioxidant , antitumor	515253
15	26.03	1.90	2(1H)-Naphthalenone,octahydro-4a-phenyl-,trans	228	$C_{16}H_{20}O$	22844-36-6	antimicrobial	54
16	27.85	1.75	Acetamide,N-[2-(1H-indol-3-yl) ethyl]-	202	$C_{12}H_{14}N_2O$	1016-47-3		
17	41.86	1.71	Ergosta-7,22-dien-3-ol, (3β,5α)	440	$C_{30}H_{48}O_2$	1449-60-1		
18	6.07	1.67	1,3,8-p-Menthatriene	134	$C_{10}H_{14}$	18368-95-1	aromatic volatile compound	55
19	26.61	1.54	9-Octadecenoic acid(Z)-,methyl ester	296	$C_{19}H_{36}O_2$	112-62-9	anticancer	4350
20	24.49	1.31	n-Hexadecanoic acid	256	$C_{16}H_{32}O_2$	57-10-3	antiinflammatoy, antioxidant, hemolytic, 5-Alpha reductase inhibitor.	5657
21	29.17	1.24	(4-Isopropylidenebicyclo[3.2.0] hept-2-en-6-ylidene)acetic acid,methyl ester	204	$C_{13}H_{16}O_2$			
22	36.65	1.22	3α-5-cyclo 5α-ergosta-6,8(14).22t-triene	378	$C_{28}H_{42}$	24352-51-0		
23	21.62	1.19	1-Nonadecene	266	$C_{19}H_{38}$	18435-45-5	antimicrobial, antioxidant and anticancer	58
24	9.45	1.13	1H-Indene,1-methylene	128	$C_{10}H_8$	2471-84-3		
25	14.54	1.11	Naphthalene,1,7-dimethyl-	156	$C_{12}H_{12}$	575-37-1		
26	22.25	1.10	Benzene,(1-pentyloctyl)-	260	$C_{19}H_{22}$	4534-49-0		
27	21.81	1.08	2,8-Decadienedioic acid,diethyl ester	254	$C_{14}H_{22}O_4$	4921-68-0	antibacterial	59
28	10.76	1.07	1-Phenoxypropan-2-ol	154	$C_9H_{12}O_2$	770-35-4		
29	27.34	1.04	Oleic acid	282	$C_{18}H_{34}O_2$	112-80-1	antibacterial	60

which appeared at 37.58 and 27.27 minutes interval with 8.61% and 6.78% peak area respectively. There were also many minor compounds such as bnzenepropanoic acid, 3,5-bis (1,1-dimethylethyl)4-hydroxy-,octadecyl ester (4.03%), Di-n-octyl-phthalate (3.30%), Chlorpyrifos (3.27%), Isopulegol (2.70%), D-limonene (2.43%) etc. traced in EAP extract. n-Hexadecanoic acid (23.34%) and 9,12-Octadecadienoic acid (Z,Z) (12.23%) were found as a major compound in MEP extract and were appeared at 24.571 and 27.282 minutes time interval respectively. Several minor compound such as Octadecadienoic acid (9.72%); 9,12-Octadecadienoic acid (Z,Z) methyl ester (9.52%); 1,4-Benzenedicarboxylic acid, bis (2-methylpropyl) ester (7.14%); hexadecanoic acid, methyl ester (4.64%); aromadendrene (4.54%) were also detected from MEP extract. Nine compounds namely n-Hexadecanoic acid; 9,12-Octadecadienoic acid(Z,Z); 9,12-Octadecadienoic acid (Z,Z) methyl ester; hexadecanoic acid, methyl ester; oleic acid, 9-Octadecadienoic acid (Z) methyl ester; ergosta-5,7,9(11)22-tetraen-3-ol(3β,22E)-; 9,12-Octadecadienoic acid (Z,Z) were shared by both of the MEP and EAP. It was clearly observed that most of the fatty acid and its methylated derivatives are common for both of the extracts. Reported biological activities of the detected compounds were also listed in the Table 2 and Table 3 respectively.

Table 3: Major phytoconstituents identified in MEP extracts of *Phallus* sp. and its biological uses.

Sl No	RT	Area	Name of the compound	Molecular weight	Molecular formula	CAS #	Biological Activity	Reference
1	24.571	23.34	n-Hexadecanoic acid	256	$C_{16}H_{32}O_2$	57-10-3	antiinflammatoy, antioxidant, hemolytic, 5-Alpha reductase inhibitor.	5657
2	27.282	12.23	9,12-Octadecadienoic acid(Z,Z)	280	$C_{18}H_{32}O_2$	60-33-3	antiinflammatory and antiarthritic	42
3	27.646	9.72	Octadecadienoic acid	284	$C_{18}H_{36}O_2$	57-11-4		
4	26.547	9.52	9,12-Octadecadienoic acid (Z,Z) methyl ester	294	$C_{19}H_{34}O_2$	112-63-0	anticancer	43
5	23.042	7.14	1,4-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	278	$C_{16}H_{22}O_4$	18699-48-4		
6	23.879	4.64	Hexadecanoic acid, methyl ester	270	$C_{17}H_{34}O_2$	112-39-0	antibacterial and antifungal	4950
7	17.695	4.54	Aromadendrene, dihydro	202	$C_{15}H_{22}$		anticancer	61
8	27.344	3.16	Oleic Acid	282	$C_{18}H_{34}O_2$	112-80-1	antibacterial	60
9	27.017	3.06	Methyl stearate	298	$C_{19}H_{38}O_2$	112-61-8	anticancer	62
10	40.502	1.97	Ergosta-7,22-dien-3-ol, (3β,5α,22E)-	398	$C_{28}H_{46}O$	2465-11-4		
11	26.638	1.78	9-Octadecadienoic acid (,Z) methyl ester	296	$C_{19}H_{36}O_2$	112-62-9	anticancer	4350
12	16.676	1.70	Aromadendrene,dehydro-	202	$C_{15}H_{22}$		anticancer	61
13	37.812	1.54	Ergosta-5,7,9(11)22-tetraen-3-ol, (3β,22E)-	394	$C_{28}H_{42}O$	516-85-8		
14	24.134	1.27	Hexadecanoicacid,Z-11-	254	$C_{16}H_{30}O_2$	2416-20-8		
15	23.745	1.12	Dibutyl phthalate	278	$C_{16}H_{22}O_4$	84-74-2	antibacterial	63
16	27.883	1.07	9,12-Octadecadienoic acid (Z,Z)	280	$C_{18}H_{32}O_2$	60-33-3	anticancer	62

Evaluation of antioxidant potential of MEP

DPPH free radical scavenging Assay

DPPH free radical scavenging assay was carried out with different concentration range (100-500 µg/mL) of the MEP extract (Figure 4A). Result indicated that with increase of the mushroom concentration the percentage of scavenging of DPPH radical proportionally increase in a linear manner. The percentage of scavenging of DPPH radical increase 10.073±0.8250 % to 61.276±8.286% at concentration range 100 µg/mL to 500 µg/mL (Table 4). The IC_{50} value is the particular concentration at which 50% of free radical is scavenged by antioxidant molecule of the extract[5]. The IC_{50} value of MEP against the $DPPH^{+}$ free radical was 426.723±17.574 µg/mL (Table 5).

ABTS•+ free radical scavenging assay

free radical scavenging assay was also performed same concentration range (100-500 µg/mL) of the MEP extract (Figure 4A). The percentage of scavenging of **ABTS•+** free radical proportionally increased with the concentration of MEP extract of mushroom in a linear manner (Figure 4). The percentage of scavenging of **ABTS•+ free** radical increase 27.295±1.974% to 91.790±2.277% at concentration range 100 µg/mL to 500 µg/mL (Table 4) . The IC_{50} value of MEP against the **ABTS•+** free radical was 209.671±6.314 µg/mL (Table 5).

This result indicated that antioxidant molecule present in MEP extract of *Phallus* was more effective on ABTS•+ free radical than DPPH free radical (Table 5).

Assessment of antioxidant compound present in MEP

Total phenol content

Total phenol content of MEP of *Phallus* sp. was expressed as Gallic acid equivalent of phenol par gram of mushroom dry weight. Different concentration of Gallic acid (0-10 µg/mL) was used as equivalent standard and finally the equivalent concentration of total phenol content of MEP was determined from the equation $y=0.138x+0.062$, $R^2=0.996$ (x = gallic acid concentration, y = absorbance of MEP). The MEP contented 6.771±0.031 mg Gallic acid equivalent of phenol content present par gram of mushroom dry weight Table 6 (Figure 4B).

Total flavonoid content determination

The total flavonoid content of MEP of this mushroom were expressed as Quercetin equivalent of flavonoid per gram of mushroom dry weight. Quercetin (concentration range 0-100 µg/mL) was used as equivalent standard and equivalent concentration of total flavonoid content of MEP was determined from the equation $y=0.008x+0.012$, $R^2=0.976$ (x = Quercetin concentration, y = absorbance of MEP). The MEP contented 32.628±0.082 mg Quercetin equivalent of flavonoid per gram of mushroom dry weight Table 6 (Figure 4B).

The major antioxidant compound present in the MEP extract was flavonoid molecules. The total flavonoid content of the MEP was higher than phenol content.

Table 4: Comparative analysis of the free radical scavenging potentiality of different concentration (100-500 µg/mL) of MEP extract on DPPH and ABTS•+ free radical. Results are the mean value of two independent experiments with standard deviation.

MEP (µg/mL)	(%) DPPH free radical	(%) ABTS•+ free radical
100	10.073 ± 0.825	27.294 ± 1.973
200	20.674 ± 0.904	50.893 ± 3.993
300	33.395 ± 0.999	69.085 ± 6.993
400	44.979 ± 3.960	84.145 ± 0.813
500	61.276 ± 8.285	91.790 ± 2.276

Table 5: Comparative analysis of IC_{50} (µg/mL) values of MEP of *Phallus* sp. on DPPH and ABTS•+free radical. Results are the mean value of two independent experiments with standard deviation.

	DPPH free radical (µg/mL)	ABTS•+ free radical (µg/mL)
IC_{50} of MEP	426.723 ± 17.574	209.671 ± 6.314

Table 6: Comparative analysis of antioxidant compound profile of MEP of *Phallus* sp. Results are the mean value of two independent experiments with standard deviation.

Total Phenol Content (mg Gallic acid equivalent / gram of dry weight)	Total Flavonoid Content (mg Quercetin equivalent / gram of dry weight)
6.771 ± 0.031	32.628 ± 0.082

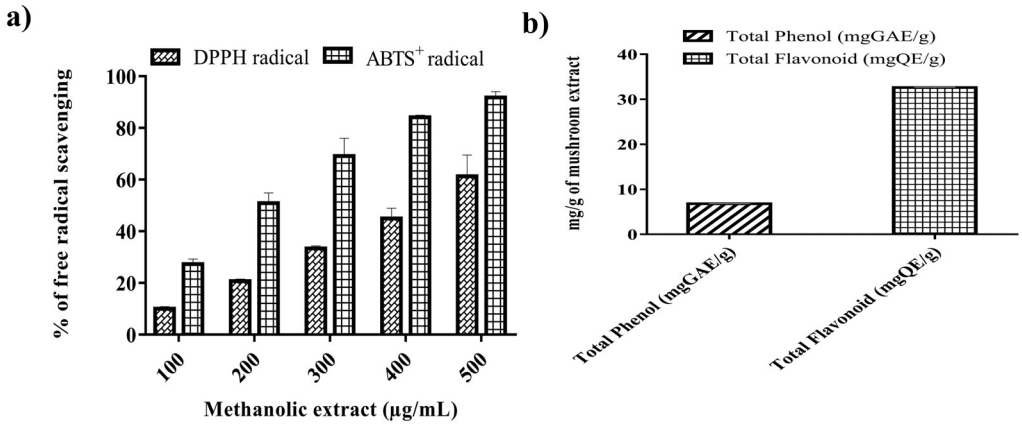

Figure 4: Evaluation of antioxidant potential of MEP and assessment of antioxidant compound A) Comparative study of DPPH and ABTS•+ free radical scavenging potential of MEP B) Comparative study of total phenol and flavonoid content of MEP.

DISCUSSION

Both MEP and EAP extracts showed very appreciable anti-proliferative activity with IC_{50} value against MCF-7 8.54±2.81 µg/mL 35.27±2.86 µg/mL respectively and negligible effect on normal PBMCs. Moderate activity of MEP and EAP on REH cell line and almost no anti-proliferative effect were observed. Data taken together indicates the effectiveness of *Phallus* extract specifically on breast cancer. Different derivatives of ergosterol such as Ergosta-5,22-dien-3-ol,(3β,22E,24S)-;Ergosta-5,7,9(11),22-tetraen-3-ol, (3β,22E);Ergosta-7,22-dien-3-ol, (3β,5α);3α-5-cyclo 5α-ergosta-6,8(14).22t-triene;Ergosta-7,22-dien-3-ol, (3β,5α,22E)-;Ergosta-5,7,9(11)22-tetraen-3-ol, (3β,22E)-were found on both of EAP and MEP extracts of this mushroom. Previous authors reported that ergosterol extracted from *Amauroderma rude* was very effective against human breast cancer cells MDA-MB-231, it up-regulated expression of Foxo-3 which further down regulate several down-stream signaling molecules like Fas, FasL, BimL, and BimS thus further enhance apoptotic stimuli[64]. This observation also supports our findings. As GC data clearly indicate that MEP extract of the *Phallus* contained Ergosta-7,22-dien-3-ol, (3β,5α,22E)-(1.97%) and Ergosta-5,7,9(11)22-tetraen-3-ol (1.54%) which probably responsible molecule for its high anti-proliferation activity. Several ergosterol derivatives of were also traced from EAP extract of *Phallus.* Probably ergosterol derivativs present in MEP extract was more potent than EAP extract with respect to its anticancer property.

Different sets of bioactive compounds were also found in both EAP and MEP extracts with various bioactive properties such as anti-bacterial, anti-fungal, anti-inflammatory, anti-diabetic etc. Among several conventional cancer treatments, chemotherapy is most frequently carried out to treat malignant cancer. Most of the chemotherapeutic drugs show a plethora of side effects. The most of the commonly used anticancer drugs, especially designed for targeting DNA molecule are associated with various adverse reactions such as excessive production of reactive oxygen species (ROS) and subsequent build up of oxidative stress. To allay these undesired side effects, antioxidants are used as an adjuvant in chemotherapy[65]. As the MEP extract of the *Phallus* sp. had shown very potent anticancer activity, the antioxidant potentiality of MEP was also evaluated.

Flavonoid and phenolic compounds are large groups of naturally occurring polyphenol compounds which depict the antioxidant profile

of any biological mixtures. The MEP showed higher flavonoid content than phenol content. MEP also showed moderate antioxidant activity and very prominent impact on ABTS•+ free radical**.** So, MEP extract of *Phallus* may be selected as future safe alternative anticancer drug and potent antioxidant adjuvant for chemotherapy. So, MEP extract of *Phallus* sp. can be tapped to isolate and purify novel anticancer compound in future for development of anticancer drug and antioxidant adjuvant for chemotherapy.

CONCLUSION

This wild mushroom *Phallus* sp. showed very promising anti-proliferative activity on human breast cancer cell line MCF-7 followed by REH. Both extracts showed its effect specifically towards the cancer cells, irrespective of normal PBMC cells. ROS is generated due to oxidative stress, is very harmful for living system. Our study showed that methanolic extracts was very potent for quenching of the free radicals. Our study also showed that methanolic extract of this wild stinkhorn is very potent candidate for not only as a safe alternative anticancer drug but also an effective chemotherapeutic antioxidant adjuvant. Presence of various anticancer and antioxidant molecule were detected from GC-MS analysis, which also supports above conclusion. Different derivatives of ergosterol may be probable anticancer molecules. So, it can be concluded that the methanolic extract of this wild stinkhorn can be tapped to isolate and purify potent anticancer compound in future.

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CONFLICTS OF INTEREST

The authors report no conflict of interest.

AUTHOR CONTRIBUTION

Entire design of the experimental work was done by Prof. Santanu Paul. Amrita Pal and Ribhu Ray collected this mushroom. Molecular identification, extract preparation, phytochemical profiling study and antioxidant profiling was carried out by Ribhu Ray. Amrita Pal performed the cell viability assay. Prof. Santanu Paul and Ribhu Ray analysed data and wrote the manuscript. All authors approved the final manuscript.

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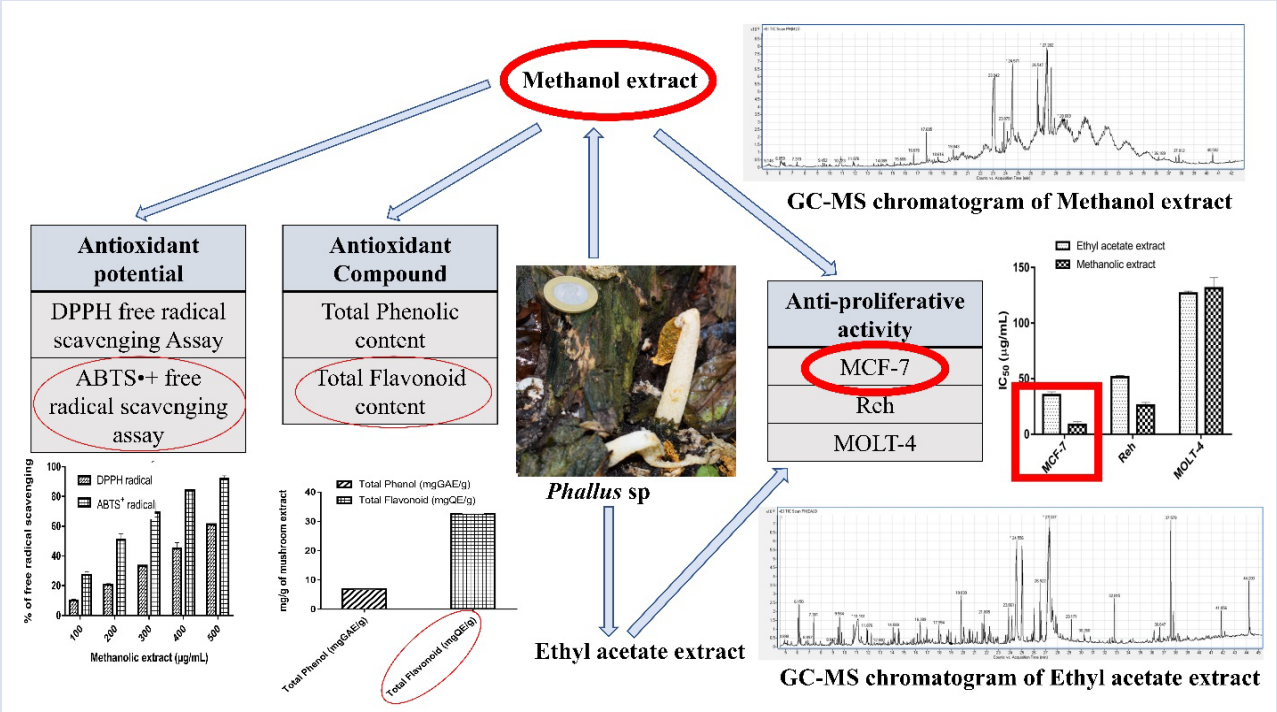
GRAPHICAL ABSTRACT



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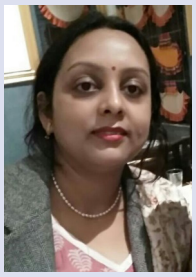

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