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Anticancer Activity Assay of Some Purification Compounds Extracted from Pleurotus Ostreatus

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Abstract--- *The present study is designed to evaluate some compounds extracted from Pleurotus ostreatus and purification using analytical and preparative HPLC technique, then tested the anticancer activity by invitro crystal violate (C.V) cytotoxicity assay on cancer and normal cell line, chemical description of these compounds were done by GC-MS technique. Analytical and preparative HPLC carried out with 25%methanol with 1% formic acid mobile phase and description about 12 fractions with retention time (9,10,11.5,12,12.5,13.5,14.7,15.5,17,18,19.5-21,21.8)at 254nm respectively. In vitro C.V cytotoxicity assay on SW-480 colon cancer cell line appeared the excellent inhibition with (1, 5, 6 ,7 ,12) fractions with (1000, 500,250,125,62.5 µg/ml), while the same fractions have no effect on vero-101normal cell line. GC-MS analysis data gave us the most probability anticancer compounds in these fractions such as Quinoline, 1H indol, Silane compounds and N-hexadecanoic acid.*

Keywords--- *Pleurotus Ostreatus, Analytical and Preparative HPLC Technique, Anticancer, (C.V) Cytotoxicity Assay, IC50, GC-MS.*

I. INTRODUCTION

Cancer is a non-specific term for a few sorts of illnesses that can be chronic and are in charge of numerous die around the world. When all is said in done, malignancy is an irregular development of cells that have a tendency to multiply in an uncontrolled path and, now and again, to metastasize or spread to other part of the body (1,2).

Uncontrolled cell prolifiration can be prompted by many components (biotic and abiotic), counting chemicals, physical, or biological factors (3,4).

This for the most part brings about an irregularity or tumor. Neoplasms can be additionally arranged into amiable, conceivably dangerous (pre-cancer), or cancer (malignancy) (5). In spite of the fact that there has been extensive advance in current disease treatment look into, troubles in understanding the molecularmanner of different sorts of cancer and the various symptoms experienced by patients from medicines implies that this entire branch of knowledge is as yet risky .As a steady help to the medications specified above, organic treatment is presently increasing more consideration, since it significantly lessens the reactions and defeats tumor development (6).

Mushrooms draw in more interest as a crude material for the creation of such medications. Remedial properties of restorative mushrooms were misused by people pharmaceutical all through the world since antiquated circumstances (7). Organically dynamic substances of therapeutic mushrooms with anticancer activity contain polysaccharides, polysaccharide-protein edifices, dietary fiber, certain kinds of proteins, terpenoids, steroids, phenols, and so forth. The principal show of mushroom extricate antitumor movement has been led in 1957 by (8).

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what's more, the individuals who utilizing separates from the fruiting assortments of Boletus edulis and different holobasidiomycetes against Sarcoma-180. The methanol concentrates of P. ostreatusand P. salmoneostramineus demonstrated suppressive impact against development of HT-29 cell line with survival rates of 39.9 and 40.7% at the focus 500 µg/ml, while survival rate was over half when P. cornucopiae methanol extricate was utilized (9).(10) reported that P. ostreatus mushrooms cultivated on date waste possess a potent antitumor activity against Ehrlich ascites Carcinoma.Along with renewed interest in using mushrooms in anticancer therapy and in establishing their medicinal properties therefore the presents studyhas been designed toExtraction and purification of some bioactive compounds from P. ostreatusand assaying its anticancer activity.

II. MATERIAL AND METHODS

Fruiting Bodies of P. Ostreatus

About one kilogram of dried fruiting bodies were gained from Ministry of Science and Technology- Department of Prevention-National Center For Organic Agriculture-Baghdad.

Prepared of Fungal Extracts

The fruiting bodies were drudge using a blender. The crashed biomass 100 g was suspended in 400 ml of absolute methanol and incubated at 200 rpm for 48 hrs. and 37°C. , the suspension was filtered with Whatman No. 2 to remove the biomass, this process was repeated twice. The supernatant was concerted in a rotary evaporator at 50°C under reduced pressure. The subsequent dried biomass was melted in distilled water to make stocks 50 mg/ml and stored at 4°C.this method according to (11) with modification.

Prepare Liquid –Liquid Extract

Equal volume of deionized distilled water and chloroform were added to above dried extracts and shaking at 250 rpm for 1 hrs then lifted until two layers formed upper water layer was gained for addition HPLC analytical (12).

Separation and Purification by HPLC Technique

Shimadzu LC-6AD gradient pump, SPD-M20A prominence diode array detector, and HPLC 2D Chemstation software (Hewlett-Packard, Les Ulis, France). The chromatographic separation was performed with a reversed-phase column (Bio wide pare C18 ,25cm*4.6mm,5Mm supelco analytical column and Shim-pack prep-ODS 250*20 mml.Dpreprative column.).CBM-20A controller, DGU-20Adegaseos. FCR-10A, shimadzu fraction collector.

Development of Separation Protocol by Analytical DAD-HPLC

The column temperature adjusted at 30 C° at a flow rate of 1.0 ml/min to achieve the optimum resolution of the separation of many compounds . The injection volume was maintained at 20 µl of watery extract, different mobile phase had been employed to achieve the best ***separation condition fractionation protocol by preparative HPLC:*** The column temperature adjusted at 30 C° at a flow rate of 10 ml/min to achieve the optimum resolution of the separation of compounds . The injection volume was maintained at 500 and 1000µl of watery extract.

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Preparation of Fractions for Cytotoxicity Assays

10 mg of 12 lyophilized compound fractions were dissolved in 2ml deionized distilled water then filtrated through 0.2 µM Nalgin filter and prepared concentrations(1000,500,250,125,62.5)µg/ml then kept at $4C^{\circ}$.

Anticancer Activity Assay

Sw-480 human colon cancer cells and monkey normal vero-101 fibroblasts cells were prepared and cultured in RPM1-1640 medium , According to (13), the cytotoxicity assays were applied for determination of the effect of 12 compounds fraction on SW-480 and Vero-101 cell line culture. Different concentrations of compounds were tested for a defined time durations. When the growth in the flask became as monolayer before it reached the exponential phase, the cell monolayer were harvested and re-suspended with a growth medium in a concentration of $5*10^5$ cell / ml and seeded in a 96 well microtiter plate. Since the cell growth reaches 80%, the wells were exposed to serial dilutions of the test fractions.Then all plate was covered with self plastic lid and incubated for 48hrs. After the end of the exposure the wells washed with 200 µl of a sterile PBS. The effect on cell line growth was assessed by C.V assay (13)

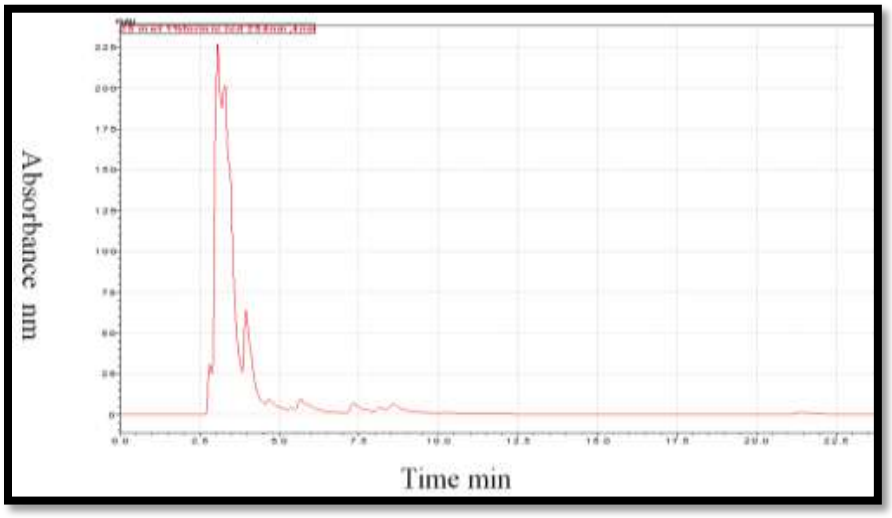
Detection of Chemical Compositions of P. Ostreatus Methanolic Extracts by (GC/MS)

The chemical compositions of the methanolic extracts of *P. ostreatus* fruiting bodies samples were detected by GC/MS analysis according to (14).

III. RESULTS AND DISCUSSION

Development of Separation Protocol by Analytical DAD-HPLC

254nm and 25%methanol 1%formic acid was exhibited the best separation method ,that may be related to polarity of liquid extract that interact with non-polar column C18 and medium polarity of mobile phase according to hydrophobicity. On the other hand the addition of acid related to suppress the ionization of the silanol groups in silica that causing peak tailing and generally increasing retention because of the less polar nature of the non-ionized analytes (15)(Figure,1)



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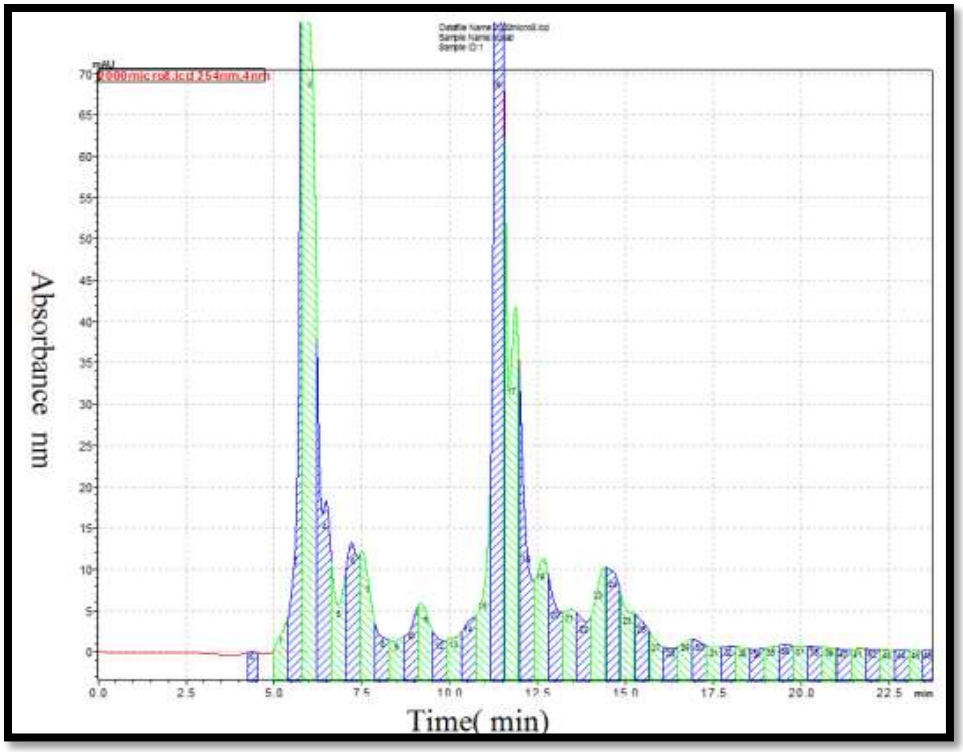

Figure 1: Developed Separation Method by Using Analytical and Preparation HPLC (25%Methanol +1%Formic Acid)

Fractionation by Preparative HPLC

Results of preparative HPLC revealed the 12 fractions were collected with desired amounts of each compound when using fraction collector in depend on the mobile phase system in analytical HPLC (25% methanol +1% formic acid) at 254 nm and cycling up was employed to increase the separation efficiency, each fraction were collected at specific retention time (min) (Table ,1; Figure ,1).

Table 1: Fraction Number and Retention TIME (min) Used Preparation HPLC with Separation System (25%Methanol+1%Formic Acid)

Fr.no	1	2	3	4	5	6	7	8	9	10	11	12
RT(min)	**9**	**10**	**11.5**	**12**	**12.5**	**13.5**	**14.7**	**15.5**	**17**	**18**	**19.5-21**	**21.8**

Anticancer Activity Assays

1. Cytotoxicity Assay

All 12 fractions were exposure on SW-108 cells line for anticancer activity assay then the best 5 fractions in activity were selected for other tests ,so we founded the excellent results as concerned with concentration in fraction (1and12),so the viable cells number effect still low at fifth serial dilution (62µg/ml) ,while other fractions stopped activity at fourth serial dilution (125µg/ml) (Figure2).

Also the same 5 fractions were tested on vero-101kidney fibroblast cell line as normal cell control ,the results showed in figure (3) that observed first fourth fractions have no significant inhibition effect on these cells

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comparison with control, according to dose depended method, these results agreed with (16), this is may be due to their oyster mushroom components that have beneficial effects on health through immunomodulatory, anti-neoplastic, and lipid-reducing properties.

Some study focused on conditions used to produce polysaccharides from the edible mushroom. *P. ostreatus* is a legitimate candidate to look for chemopreventive elements since it has been shown to exhibit a wide variety of medicinal properties, including anti-tumor activity (17,18).

In this study, the fractions separation from *P. ostreatus* had selective cytotoxicity. The Separation fractions can effectively inhibit the proliferation of SW-480 colon cancer cells, at the same time they exhibited little cytotoxicity against human fibroblast kidney Vero-101cells.

The results show that no adverse side-effects and a moderatelylesser cytotoxicity on human normal cells. That isconfirmed that no antiproliferative effect of the fractions from Oyster mushroom on normal cells, implying that they had non direct cytotoxicity to non-cancerous cells.

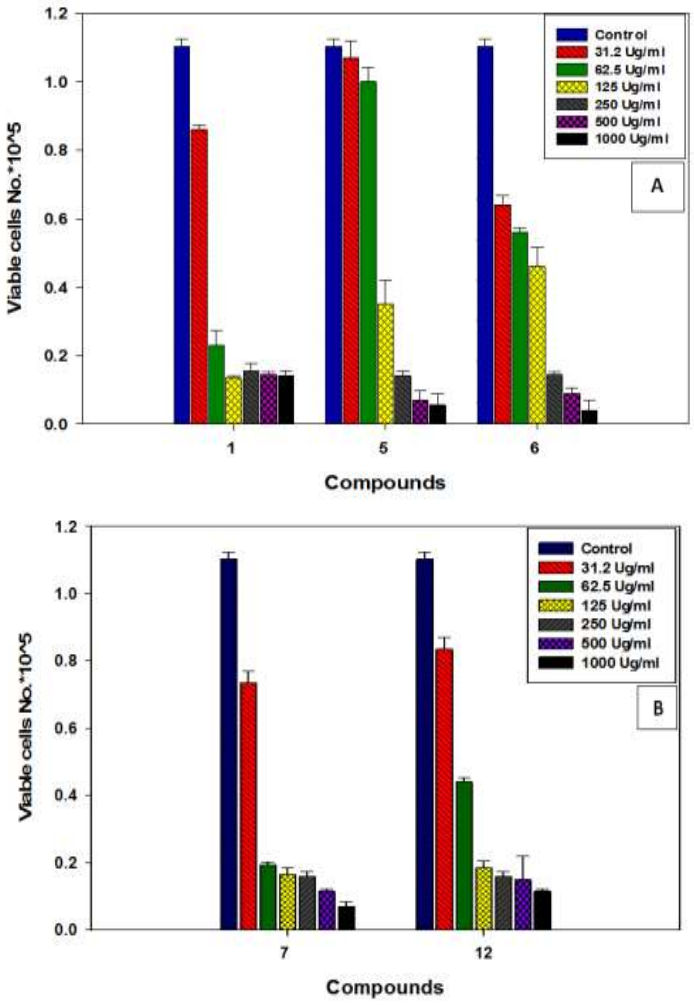

Figure 2A and 2B: Cytotoxicity Assay of P. Ostreatus 5 Fractions Presented by Viable Cells ±Sd by C.V Assay for 48 h on SW-148 Cell Line

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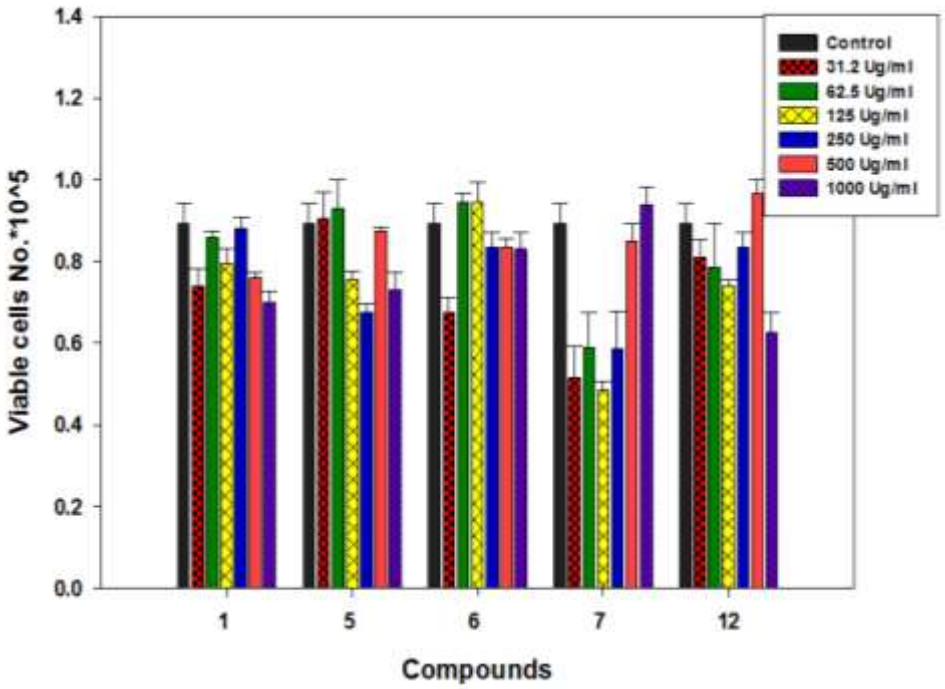

Figure 3: Cytotoxicity Assay of P. Ostreatus 5 Fractions Presented by Viable Cells ±Sd by C.V Assay for 48 h Onvero-101 Cell Line

2. Dose Dependent Response Curve and Determination IC50 Value

Inhibition rate of cytotoxicity *invitro* assay for more than five fraction active against SW480 colon cancer cell line were calculated for seven serial dilution started with 1000µg/ml and ending with 15.62 µg /ml , so the inhibition ratio were according with dose dependent response ,then the application of concentration with inhibition value and solved the equation to IC50 value were gained, which were (59.84, 72.21, 41.35, 33.24 and 41.91) of (1,5,6,7 and 12)fractions respectively.**(Table,2; Figure ,4-5-6)**

Table 2: Inhibition Rate of SW480 Colon Cancer Cell by (1,5,6,7,12) Fraction 48 h

Conc.µg/ml	**Fraction number**						
	1	**5**	**6**	**7**	**12**	**Con.**	
	Inhibition rate %						
1000	87.11	90.96	93.84	91.63	88.46	**0**	**L.S.D.$_{0.05}$=1.7**
500	86.15	91.53	91.82	87.98	89.51	**0**	
250	85.67	87.4	86.44	83.17	84.9	**0**	
125	82.34	61.25	66.23	75.86	79.42	**0**	
62.5	80.76	40.75	52.71	70.3	62.98	**0**	
31.25	17.88	32.8	41.44	46.53	45.25	**0**	
15.62	9.49	19.92	38	26.75	21.8	**0**	
L.S.D$_{0.05}$=2.2							
IC50	**59.84**	**72.21**	**41.35**	**35.33**	**41.91**		

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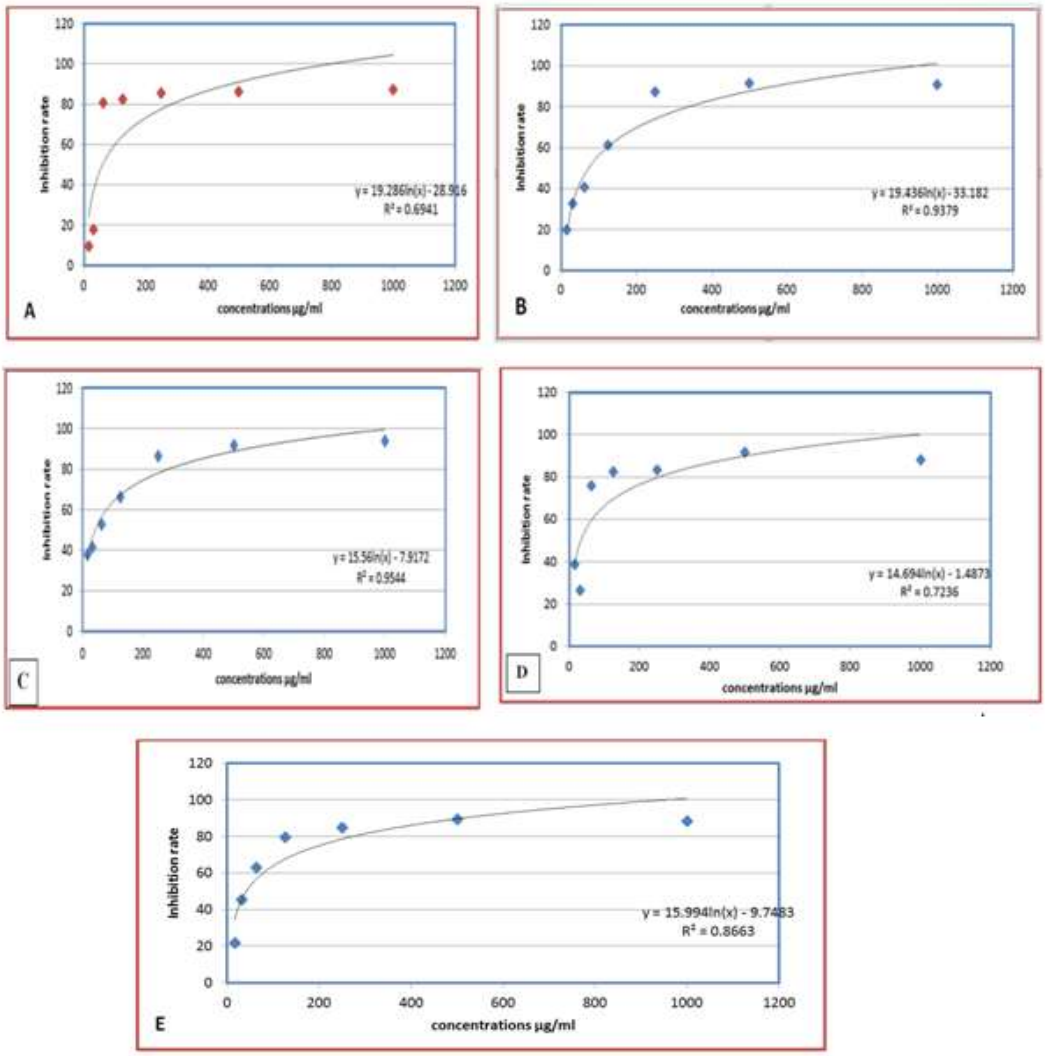

Figure 4: Dose Response Curve of Growth Inhibition of P. Ostreatus 5 Fractions on SW-184 Cell Lines Presented by Plotting of Concentration Versus GI% Values.(A):Fractino1compounds.(B):Fraction2 Compounds.(C):Fraction3 Compounds.(D):Fraction 4 Compounds.(E):Fraction5 Compounds

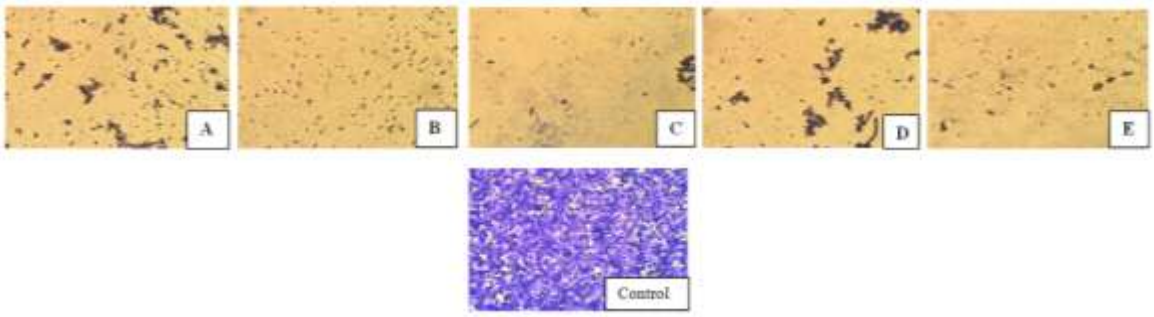

Figure 5: Images of Inverted Phase Contrast Microscope After 48 h Exposure of SW-480 Cells to: (A):Fractino1 Compounds. (B):Fraction5 Compounds. (C):Fraction6 Compounds. (D): Fraction 7 Compounds. (E):Fraction12 Compounds. Comparising with (Control) without Treatment

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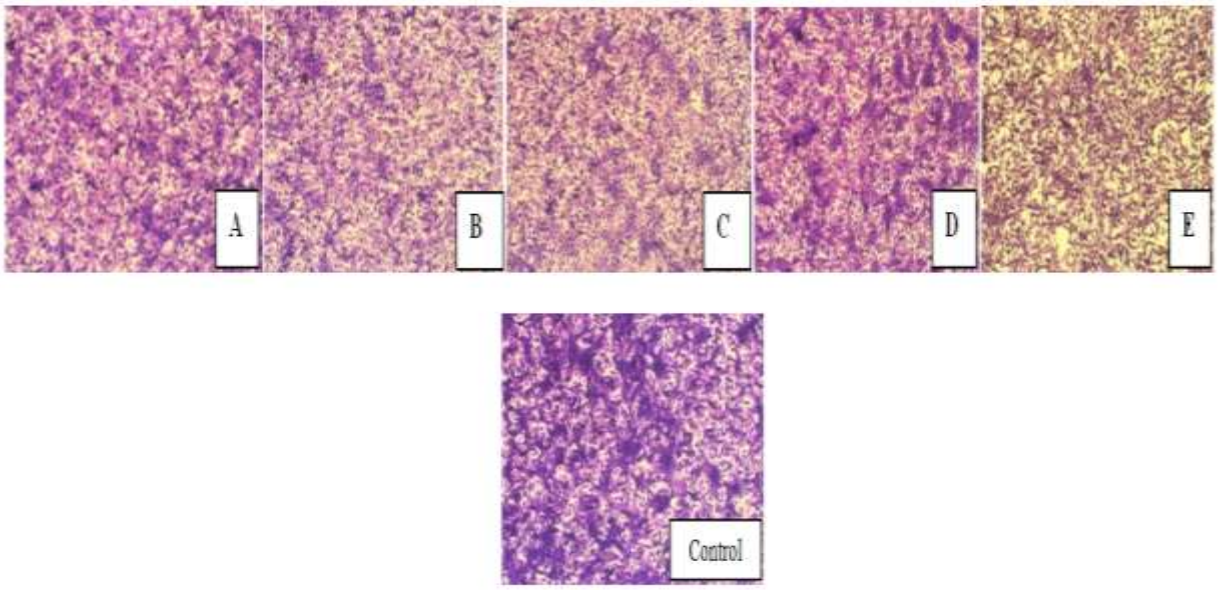

Figure 6: Images of Inverted Phase Contrast Microscope After 48 h Exposure of Vero-101 Cells to: (A):Fractino1 Compounds.(B):Fraction5 Compounds.(C):Fraction6 Compounds.(D):Fraction 7 Compounds.(E):Fraction12 Compounds. Comparising with (Control) without Treatment

3. Most Dominant Compound in More Anticancer Activity Fractions According to (GC-MS) Analysis

According to GC-MS data analysis in tables (3,4,5,6 and 7) we found many of anticancer compounds derivatives and modification other such as Quinoline that shows an important character in anticancer drug enlargement as their derivatives have shown excellent results through altered mechanism of action like growth inhibitors by cell cycle arrest, apoptosis, inhibition of angiogenesis, disturbance of cell migration, and inflection of nuclear receptor responsiveness. The anticancer potential of several of these derivatives have been demonstrated on various cancer cell lines (19).

Also 1H indol derivatives are known to have cytotoxicity alongside human carcinoma cell lines. This compound therefore, has a potential to be used as a chemotherapeutic agent against cancer (20).Silane modification compounds which addition of chemotherapy drug of cancer after coated upconversion nanoparticles to offerdecent biocompatibility.(21).The cytotoxic activityof N-hexadecanoic acid is due to its interaction with DNA topoisomerase-I and itcould be explored further for its anticancer cytotoxic potential with other cancer drug target proteins.(22).

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Table 3: Different Compounds Analyzed by GC-MS of P. Ostreatus HPLC Fraction(1) (25%Methanol +1%Formic Acid)

RT	Area	Compound
10.359	10.64	Quinoline-2 ,5 (1H.6H) –dione, 8-tetrahydor-7,7-dimthy1-4 (3-nitrophenyl)-
11.007	2.77	Morphinan, 5,6,8,14-tetradehydro-3 ,6-dimethoxy-17-methyl-
13.965	7.37	Fluoren-9-ol ,3, 6-dimethoxy-9-henylethynyl-
14.381	1.21	Silane, dithyl (2-ethoxyethyloxy) octadecyloxy-
15.164	2.49	3`-methyl-4`-tramethylsilyloxyphe nyl) propane
17.639	0.33	2-Deoxy-D-galactose
18.751	3.58	1(3H) –Isobenzofuranone, 6-(dimethyl lamino)-3,3-bis[4-(dimrthylamion)phenyl]-
18.964	1.52	2-[(p-Trimethylsilyoxy) phenyl]-2-[(p-trimethylsilyloxyethylenoxy) phenyl] propane
19.128	13.27	1,7-Di(3-ethylphenyl) -2,2,4,4,6,6-hexamethyl-1,3,5,7-tetraoxa-2,4,6-trisilaheptane
19.293	0.25	Purin-2,6-dion, 1,3-dimthyl-8-[2-nitrophenethy1]-
19.476	0.80	6,8-Difluoro-2,2,4,4,6,7,7,8,9,9-d ecamethyl-[1,3,5,2,4,6,7,8,9] trioxahexasilonane
20.095	1.94	7-Chloro-2,3-dihydro-3- (4-N, N-dimo thylaminobenzylidene) -5-phenyl-1H-l, 4-benzodiazepin-2-one
21.304	0.54	Acetic acid, hydrazide
21.613	0.52	Pterin-6-carboxylic acid
23.431	5.63	7-chloro-2,3-dihydro-3-(4-N, N-dime thylaminobenzylidene) -5-phenyl-1H-1,4-benzodiazepin-2-one
38.439	2.04	Purin-2,6-dione, 1,3-dimethyl-8-[-nitrophenethenyl]
39.793	0.29	Adenosine, 1,2-dihydro-2-oxo-
42.123	1.97	4-pyridinecarboxamide, 6-chloro-4, 5-dicyano-2-(cyclohexylidenamino)

Table 4: Different Compounds Analyzed by GC-MS of P. Ostreatus HPLC Fraction (1) (25%Methanol +1%Formic Acid)

RT	Area	Compound
9.702	1.49	Hydrazinecarboxamide
13.037	1.22	1H,1H,7H-Dodecafluoro-1-heptanol
13.095	0.91	Cyacetacide
13.588	1.06	2-propanone,1-hydroxy-,oxime
15.802	0.99	carbohydrazide
19.032	1.33	2,3,4,6-Tetrafluorophenyl isothiocyanate
21.729	1.04	Silicic acid ,diethyl bis(trimethylsilyl) ester
22.031	23.56	Hexadcanoic acid ,ethyl ester
22.184	1.23	1,5-pentanediol
24.408	1.58	1-Nitro-9,10-dioxo-9,10-dihydro-anthracene-2-carboxylic aciddiethylamide
26.356	1.22	Arsenous acid ,tris(trimethylsilyl)ester
27.902	12.20	Octadecanoic acid, ethyl ester
28.430	2.79	Hexahydropyridine ,1-methyl-4-(4,5-dihydroxyphenyl)-
28.836	0.84	1,4-Bis(trimethylsilyl)benzene
29.890	0.76	2-Ethylacridine
31.379	1.19	Methyl (5-hydroxy-1H-benzimidazol-2-yl)carbamate
35.314	7.66	1H-Indole-2-Indole-2-carboxylic acid ,6-(4-ethoxyphenyl)-3-methyl-4-oxo-4,5,6,7-tetrahydro-isopropyl ester
37.160	1.08	Indol-2-one,2,3-dihydro-N-hydroxy-4-methoxy-3,3-dimethyl-

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Table 5: Different Compounds Analyzed by GC-MS of P. Ostreatus HPLC Fraction (1) (25%Methanol +1%Formic Acid)

RT	**Area**	**compound**
5.172	18.14	Silane,trithoxymethyl
5.9	5.08	Disiloxane,1,3-diethoxy-1,1,3,3tetramethyl
8.491	1.91	Benzonitrile,2-(2-hydroxy-5-nitrobenzylidenamino)
9.805	0.78	Silicic acid,diethyl bis(trimethylsilyl)ester
10.864	5.02	Fluoren-9-ol,3,6-dimethoxy-9-henylethynyl)
12.878	1.24	2-chloroethyl chlorobromoethyl sulfoxide
13.937	0.55	Dimethyl sulfoxide
14.079	0.94	Pyrimidine-4,6(3H,5H)-dione, 2-butylthio
14.277	1.77	Cycloheptasiloxane,tetradecamethyl
15.781	0.93	Carbonic acid,heptadecyl propyl ester
17.133	0.59	Benzofuran-2-one, 2,3-dihydro-3,3-dimethyl-4-nitro
18.712	1.07	Hexahydropyridine,1-methyl-4-(4-5-dihydrophenyl)
27.108	1.34	Acetic acid,chloro-,hexadecyl ester
31.079	0.60	4-bromo-3-chloroaniline
35.646	0.54	Benzenamine,4-bromo-2-chloro-

Table 6: Different Compounds Analyzed by GC-MS of P. Ostreatus HPLC Fraction (1) (25%Methanol +1%Formic Acid)

RT	Area	Compound
6.004	8.62	Trisiloxane, 1,1,3,3,5,5-hexamathy -l
8.112	3.43	Butanamide, 2,2,3,3,4,4-heptaflu oro-N-[2-[(trimethylsilyl) oxy]-2-[4-[(trimethylsiyl) oxy] phenyl] ethyl]-
8.699	4.91	Cyclopentasiloxane, decamethyl-
11.564	10.63	Cyclohexasiloxane, dodecamethy1-
14.325	8.83	Cycloheptasiloxane, tetradecamethy l-
16.811	8.87	Cycloheptasiloxane, hexadecamethyl-
18.986	8.21	Cycloheptasiloxane, octadecamethyl-
21.511	7.47	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11 ,13,13,15,15-hexadecamethyl-
22.182	10.15	Hexadecanoic acid, ethyl ester

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Table 7: Different Compounds Analyzed by GC-MS of P. ostreatus HPLC Fraction (1) (25%Methanol +1%Formic Acid)

RT	Area	compound
5.909	6.44	Disiloxane ,1,3-diethoxy-1,1,3,3-tetramethyl
5.995	13.28	Trisiloxane1,1,3,3,5,5-hexamethyl
6.345	5.68	Cyclotetrasiloxane,octamethyl
8.699	4.72	Cyclopentasiloxane ,decamethyl
10.864	3.14	Benzenamine,N-(4-chlorobenzylidene)-4-iodo
11.546	5.69	Cyclohexasiloxane,dodecamethyl-
22.409	34.56	Hexadecanoic acid,ethyl ester
28.215	39.98	Octadecanoic acid,ethyl ester
44.563	34.73	Cis ,6-octadecenoic acid, trimethylsilyl ester
45.064	2.83	Octadecanoic acid

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