ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

Cyperus Rotundus L. Phenols Extracts Enhance the Anticancer Effect of Oncolytic Newcastle Disease Virus on Digestive System Neoplasms

Russell A. Abo-Altemen[1], Ahmed Majeed Al-Shammari[2*], Muayd S. Shawkat[1]

1. *University of Baghdad, Collage of Science, Department of Biotechnology, Baghdad, Iraq.*
2. *Mustansiriyah University, Iraqi Center for Genetics and Cancer Research, Experimental Therapy Department, Baghdad, Iraq.*

Abstract

This study created novel cancer therapy consist of a combination of Secondary metabolites compound (Phenols) extracted from Cyperus rotundus L. and oncolytic Newcastle disease virus (NDV) which showed cytotoxic activity against different cancer cell lines in vitro. Moreover, it is safe and less toxic on normal cell lines. Three cancer cell lines were used, esophagus cancer cell line (SKG), mouse hepatocellular carcinoma cell line (HC), Human rectal cancer cell line (HRT), and normal cell line Rat Embryo Fibroblast (REF). MTT cell viability assay was used and analyzed for possible synergism using the Chou-Talalay analysis method. In vitro study showed that combination therapy of NDV with Phenols extract had enhanced synergistic anticancer activity on all cancer cell lines tested. Moreover, combination therapies showed antagonism and less cytotoxic effect on normal REF cell line.

Keywords: *Cancer cell line, Combination therapy, Newcastle virus, phenols, Cyperus rotundus.*

Introduction

Digestive system cancers is among the common primary cancers for both sexes [1]. Plants have been used for long time in the treatment of a different type of diseases, including cancer [2]. Family Cyperaceae is a family of monocotyledonous graminoid flowering plants known as sedges, which superficially resemble grasses; the family is large, with 5500 species described and about 109 genera worldwide [3].

Multivalent plant extensively used in traditional medicine around the world for treatment of various diseases, C. rotundus commonly known as Motha is abundantly available and it provides an excellent source of remedy to treat various ailments because it is growing wild, cheap, with broad range benefit and large-scale secondary metabolites [4].

C. rotundus, have been described to contain, phenols, glycosides, saponins, flavonoids, tannins [5]. Phenols which are one of the largest groups of phytochemicals in plants have amazing effects on human based on their toxicity against cells of foreign organisms. Therefore, the compounds detected may be responsible for the antibacterial activity, anticancer, antioxidant, anti-inflammatory and antimicrobial [3] Virotherapy is a promising tool for fighting cancer because of its safety and selectivity [6].

The Iraqi attenuated strain of Newcastle Disease Virus (NDV-ICCMGR-Najaf) has significant oncolytic activity and exceptional safety [7]. NDV is an RNA virus that causes deadly outbreak in avian [8]. Oncolytic NDV induces cancer cell death via apoptosis [9] there was induction of intrinsic pathway with association of extrinsic pathway of apoptosis [10]. Activation of endoplasmic reticulum stress pathway of death was also reported in NDV infected cancer cells [11].

NDV infection induce immune response stimulation by cancer cell antigenic surface modification by inserting its HN and F proteins in the infected cells membrane which increase its recognition by the immune system [12].

Furthermore, NDV infection in cancer cells downregulate angiogenesis factors secreted by tumor cells [13]. Cancers chemo resistant can be overcome by combination therapy to induce diverse death mechanisms [14]. Attenuated Iraqi oncolytic NDV strain showed enhanced antitumor activity when combined with various kinds of conventional cancer therapeutics such as chemotherapies; 5-fluorouracil [15], cyclophosphamide [16], Methotrexate [17], doxorubicin, and rituximab [18].

Moreover, it showed synergistic activity with non-conventional and novel therapeutics such as cobalt ferrite nanoparticles [19]. There is need for novel combination therapies of oncolytic viruses and Phytotherapy. So, this work explores the new anti-cancer combination therapy of *Cyperus rotundus L.* phenols extract with the oncolytic Iraqi attenuated strain of Newcastle diseases as anti-digestive system tumors.

Materials and Methods

Cell Culture

The Esophagus cancer cell line (SKG), Hepatocellular carcinoma cell line (HC), Human rectal cancer cell line (HRT), and normal Rat Embryo Fibroblast cell line (REF). Were cultured in an RPMI-1640 medium (US biological, USA) with 10% fetal bovine serum (Capricorn-Scientific, Germany), 100 unit/ML penicillin, and 100 µg/ml streptomycin and then incubated at 37 ^{o}C. All the cell lines were supplied by the Cell Bank Unit, Experimental Therapy Department, Iraqi Center for Cancer and Medical Genetic Research (ICCMGR). These cells are regularly assessed for standard growth characteristic, and they are regularly authenticated [20].

Plant Extract

Cyperus rotundus rhizomes were collected from local markets during April 2017, the plant documented in the Iraqi herbarium / Faculty of Science / University of Baghdad. Two hundred grams of *C. rotundus* rhizomes were used for extraction. The methanolic extract was obtained at 80% concentration in a 48-hour sterilization method [21]. The extract was filtered using the filter paper Whatman No.1 and sterilized using the 0.22 µm micro-filter [22]. Detection of secondary metabolites chemically revealed by Ferric chloride test, the result was positive for the appearance of a dark green color, in addition to an analytical analysis using GC-MS technique, confirming the emergence of twinty-five peaks of Phenols during retention time 25 min as shown in Fig.1. After drying at 40 °C and dissolve with phosphate buffer saline, multi half dilution of Alkaloids was prepared (50, 75, 100) mg/ml and dilute with Roswell Park Memorial Institute media (RPMI-1640) free of serum.

Virus Propagation

The attenuated Newcastle disease virus strain (Iraq/Najaf/ICCMGR/2013) [8, 23] was supplied by the Cell Bank Unit / Experimental Therapy Department / Iraqi Center for Cancer and Medical Genetic Research (ICCMGR), Mustansiriyah University. A stock of infectious virus was propagated in embryonated chicken eggs (Al-Kindi Company, Baghdad, Iraq), allantoic fluids were harvested and purified by centrifugation (3000 rpm, 30 min at 4 °C). The virus was quantified by a hemagglutination test then aliquoted and stored at -86 °C in deep freezer. Viral titers were determined by a 50% tissue culture infective dose ($TCID_{50}$) titration on RD cells according to the standard procedure [24].

Cytotoxicity Assays and Combination Therapy Analysis

The SKG, HRT, HC and REF cells were seeded at 10,000 cells/ well in 96 well microplates and incubated at 37°C overnight. Three different multiplicity of infection (MOI) of NDV first were added at (10, 5 and 3) for two hours and incubated at room temperature. The Plant extract then was added (100, 75 and 50µg/ml) and incubated for 72 h. The combination treatments were done by combining each virus concentrations with each plant extract concentrations.

Also, each agent alone was tested individually to compare the cytotoxicity. MTT cell viability assays were performed after 72 hrs of infection; the cell viability was measured by removing the medium, adding 28 µl of a 2-mg/ml solution of MTT (Bio-World, USA), and incubating for 1.5 hrs at 37 °C. The remaining crystals in the wells were solubilized by the adding of 130 µl of DMSO (dimethyl sulphoxide) (Santa Cruz Biotechnology, USA) and then incubated for 15 minutes with shaking.

Absorbency was determined on a microplate reader (Flourstar optima, BMG Labtech, Germany) at 492 nm (test wavelength). The assay was done in triplicate, and the experiments repeated three times. The mean viability of the treated cells for each dilution was calculated as a percentage relative to the control wells treated with media alone (100% survival) ± SEM [15]. The median effective doses (MED50) for the *Cyperus rotundus* L. extract, and NDV were calculated individually.

To determine synergism, NDV and *Cyperus rotundus* L. extract were studied as a non-constant ratio. To analyze the combination of NDV and the extract, Chou-Talalay combination indices (CI) were calculated using CompuSyn software (CompuSyn, Inc., Paramus, NJ, USA). The non-fixed ratios of NDV and Cyperus rotundus L. extract in addition to mutually exclusive equations were used to measure the CIs. A CI between 0.9 and 1.1 was considered additive, whereas CI<0.9 and CI>1.1 indicated synergism and antagonism, respectively [25].

Measurement of P_{53} Gene Expression

To quantify the P_{53} level in the infected cells, the cell lysates of the cells treated with the one synergistic and one antagonistic doses and untreated control cells were collected at two intervals (24 and 48 hours) to determine the level of P_{53} mRNA. All RNA in the cell lysate was isolated using an ExCellenCT Lysis Kit (Applied Biological materials ABM, Canada) according to the manufacturer's protocol.

The yield was quantified using spectrophotometer (Biodrop, Biochrom, UK). The isolated RNAs were reversed transcribed to produce double-stranded cDNA by the reverse transcriptase polymerase enzyme using KAPA SYBR FAST One-Step qRT-PCR universal kit (Kapa Biosystems, Cape Town, South Africa), and they were measured in real-time PCR (MX3005P Stratagene, Agilent Technologies, Germany). Specific primers were used to detect the P53 gene in mouse HC and human SKG and HRT cells. The primer sequence for mouse gene was gained from PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) [26]. And for human gene was obtained from [27].

5′ TAACAGTTCCTGCATGGGCGGC 3′ Sense	For	
3′ AGGACAGGCACAAACACGCACC 5′ Antisense		Human
5′GCGTAAACGCTTCGAGATGTT 3′ Sense	For	
5′ TTTTTATGGCGGGAAGTAGACTG 3′ Antisense		Mice

The amplification condition of the reactions that were used for human and mouse p53 amplification were as follows: 42 ºC for 5 min, denaturation at 95 ºC for 10 min, and amplification in 40 cycles, each of which at a denaturation temperature of 95 C° for 3 s, annealing temperature at 60 ºC for 20 s, and elongation temperature at 72 ºC for 20s. The SYBR green real-time PCR assay was included Controls in each run; for each primer pair, one sample with no cDNA (containing only RNase free water) was included.

The result of each sample was analyzed using a relative quantification to compare the difference between the sample and the control. The mean CT value of the genes were calculated in each sample (a duplicate replication for each sample) and used to normalize the expression level by applying the ΔΔCT method: The threshold cycle (Ct) was determined, and the relative gene expression was expressed as follows: fold change = 2^ (−ΔΔCt) [28].

Statistical Analysis

The MTT assay and the RT-PCR data (i.e., average mean after determining the ΔΔCT values) were statistically analyzed using the two-way analysis of variance test (ANOVA) (GraphPad prism version 6.5). The standard deviation of the mean was considered significant at P=0.05.

Results

Detection of (Phenols) in *Cyperus rotundus* Extract

Result obtained by qualitative chemical detection of Cyperus rotundus L. rhizomes extract indicate the presence of Phenols after it were purified in Ferric chloride reagent.

GC-MS Analysis of Alkaloid

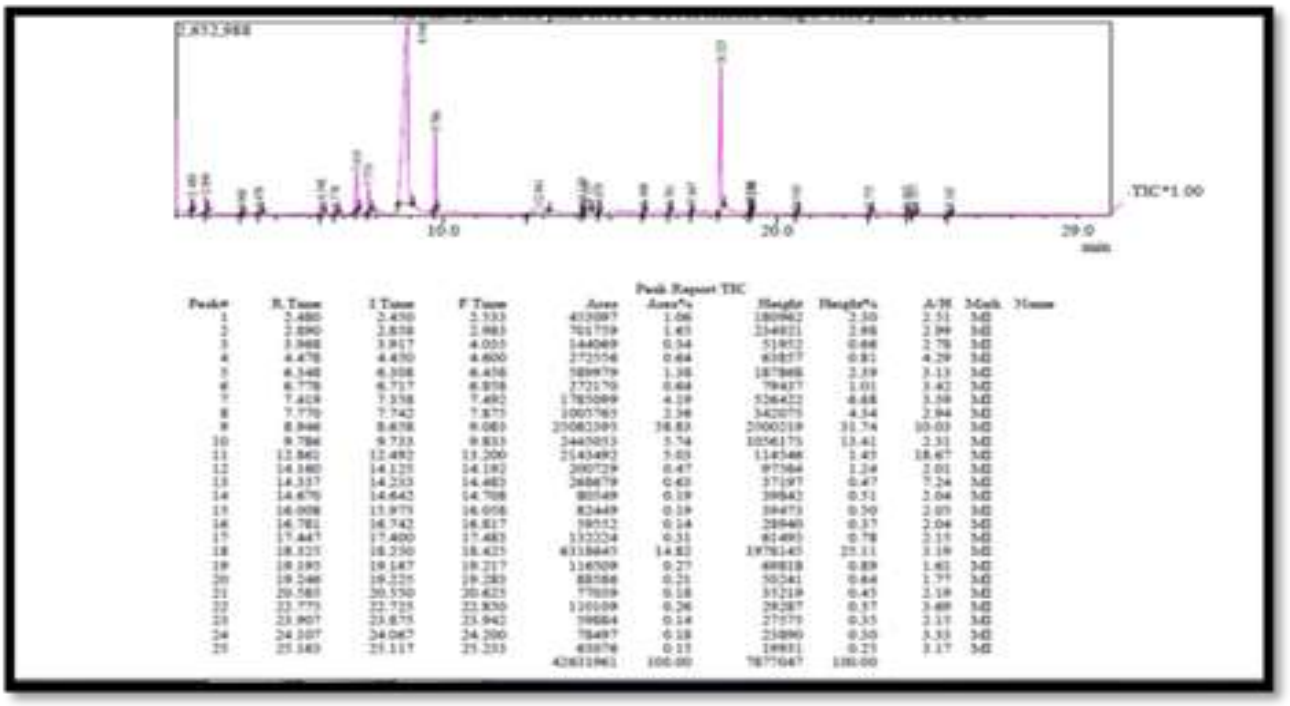


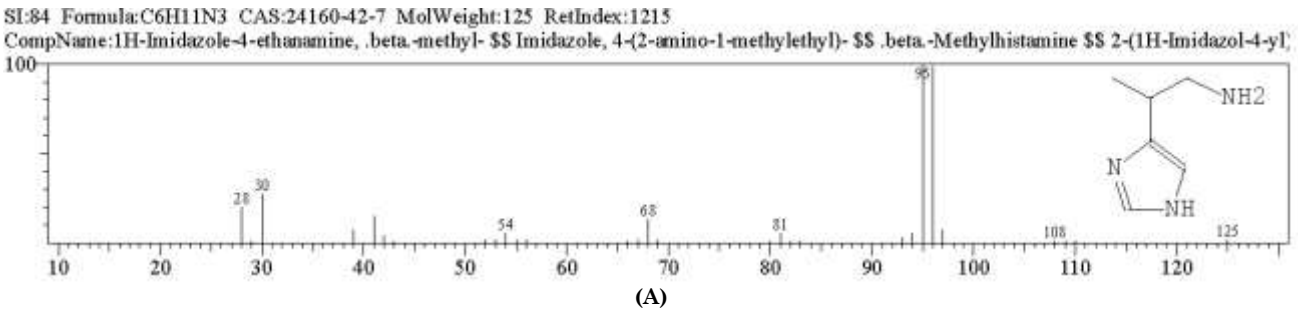
Figure 1: GC-MS analysis of the different constitutes of methanol extract of *Cyperus rotundus* extract

GC-MS Analysis of purified Alkaloid showed Twenty-five peaks during retention time 25 min .Figure 1, Table 1.

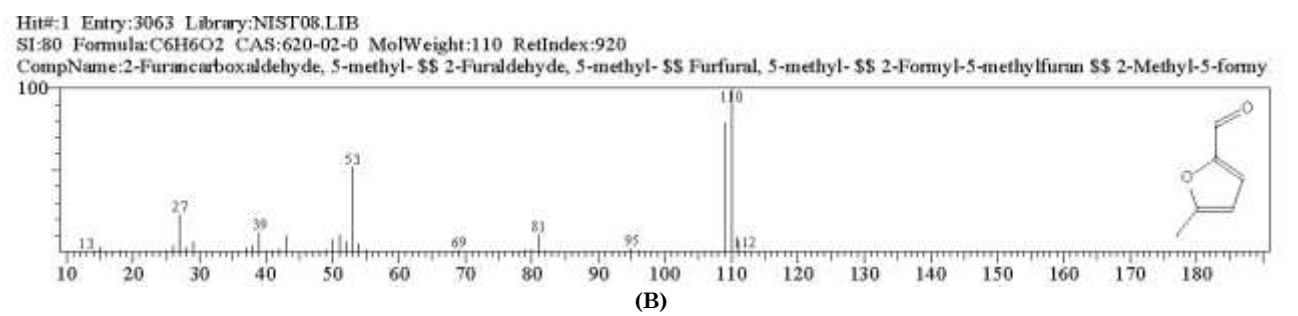
Table 1: GC-MS analysis of Phenols extract

Peak	R. Time	Area	Area%	Height	Height%	Compound Name
1	2.48	4539	1.06	1809	2.30	2,3-Butanediol, [R-(R*,R*)]- , 2,3-Butanediol # ,
2	2.89	7017	1.65	2349	2.89	2,3-Butanediol , Butane-2,3-diol $$ Dimethylethylene glycol , 2,3-Butylene glycol , 2,3-Dihydroxybutane , D-2,3-Butane diol ,
3	3.96	1440	0.34	5195	0.66	2,3-Butanediol, [S-(R*,R*)]- , (2S,3S)-(+)-2,3-Butanediol , 2,3-Butanediol .
4	4.487	2725	0.64	6385	0.81	2-Hydroxypropanoic acid , Lactic acid , L-(+)-Lactic acid , Propanoic acid, 2-hydroxy-, (S)- , Lactic acid, L- , Espiritin ,
5	6.34	5899	1.38	18786	2.39	Propylene Glycol , 1,2-Propanediol , .alpha.-Propylene glycol , Methyl glycol , Methylethyl glycol , Methylethylene glycol ,
6	6.78	2721	0.64	79437	1.01	Isopropyl Alcohol ,2-Furfural , 2-Furancarboxaldehyde , 2-Furaldehyde , .alpha.-Furole , Artificial ant oil , Fural , Furaldehyde , Furale , Furanc
7	7.41	1785	4.19	52642	6.68	Methyltartronic acid, 2-Hydroxy-2-methylmalonic acid.
8	7.77	10057	2.36	342075	4.34	3-Furaldehyde, 3-Furancarboxaldehyde.
9	8.94	2508	5.83	25002	31.74	4-Penten-2-ol $$ 1-Penten-4-ol $$ 4-Hydroxypent-1-ene. CH2=CHCH2CH(OH)CH3 ,
10	9.78	24450	5.74	10561	13.41	1H-Imidazole, 4,5-dimethyl- , Imidazole, 4,5-dimethyl- , 4,5-Dimethyl-1H-imidazole .
11	12.86	21434	5.03	11456	1.45	1H-Imidazole-4-ethanamine, .beta.-methyl- , Imidazole, 4-(2-amino-1-methylethyl)- , .beta.-Methylhistamine , 2-(1H-Imidazol-

						4-yl)
12	14.67	20072	0.47	9756	1.24	1H-Pyrazole, 1,3-dimethyl- , Pyrazole, 1,3-dimethyl- , 1,3-Dimethylpyrazole , 2,5-Dimethylpyrazole , 1,3-Dimethyl-1H-pyrazole
13	14.3	2686	0.63	37197	0.47	1-Methoxy-2-propyl acetate ,Propylene glycol methyl ether acetate ,2-Propanol, 1-methoxy-, acetate ,Acetic acid, 2-methoxy-1-
14	14.67	80549	0.19	3984	0.51	Propane, 1-(1-methylethoxy)- , Ether, isopropyl propyl $$ Isopropyl propyl ether , Propyl isopropyl ether , 1-Isopropoxypropane .
15	16	82449	0.19	3947	0.50	Oxirane, 2,3-dimethyl-, trans- , Butane, 2,3-epoxy-, trans- , trans-2-Butene Oxide , trans-2-Butylene Oxide , trans-2,3-Dimethylox
16	16.78	59552	0.14	2894	0.37	Propanoic acid, 2-oxo- , Pyruvic acid, .alpha.-Ketopropionic acid, Acetylformic acid, BTS. Pyroracemic Acid , 2-Oxopropan
17	17.47	13222	0.31	6149	0.78	Ethyl Acetate, Acetic acid, ethyl ester, Acetic ether , Acetidin , Acetoxyethane , Ethyl acetic ester , Ethyl ethanoate , Vinega.
18	18.32	63186	14.82	1978145	25.11	2-Furancarboxaldehyde, 5-methyl- , 2-Furaldehyde, 5-methyl- , Furfural, 5-methyl- , 2-Formyl-5-methylfuran , 2-Methyl-5-formyl
19	19.19	11650	0.27	6981	0.89	3,4-Furandimethanol
20	19.24	8858	0.21	5024	0.64	p-Mesyloxyphenol
21	20.58	7703	0.18	3521	0.45	Pyrazole-4-carboxaldehyde, 1-methyl- , 1-Methyl-1H-pyrazole-4-carbaldehyde ,
22	22.77	1101	0.26	2928	0.37	Pyrazine, methoxy- , Methoxypyrazine , 2-Methoxypyrazine ,
23	23.9	59884	0.14	2757	0.35	Methyl 2-furoate , 2-Furancarboxylic acid, methyl ester , 2-Furoic acid, methyl ester , Methyl pyromucate $$ Methyl 2-furancarboxy
24	24.10	7849	0.18	2389	0.30	3-Furancarboxylic acid, methyl ester , 3-Furoic acid, methyl ester , Methyl 3-furancarboxylate , Methyl 3-furoate .
25	25.16	6307	0.26	10031	0.25	Furyl hydroxymethyl ketone, Ethanone, 1-(2-furanyl)-2-hydroxy- , Ketone, 2-furyl hydroxymethyl, 2-(Hydroxyacetyl) furan.

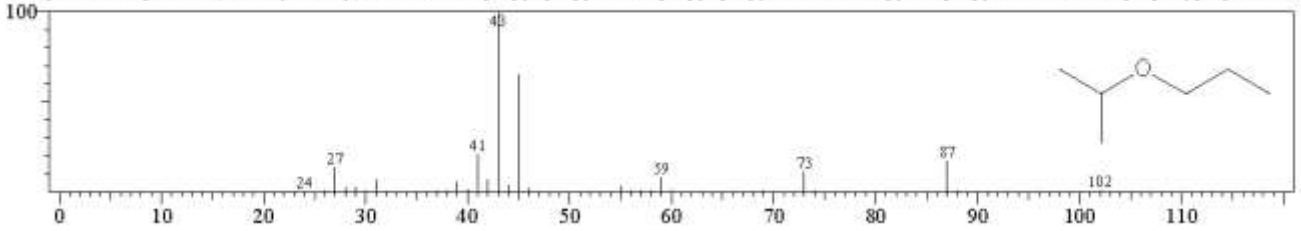


(A)



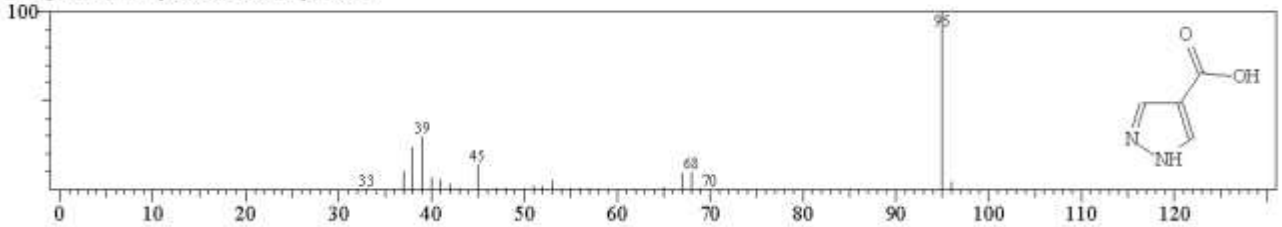
(B)

SI:84 Formula:C6H14O CAS:627-08-7 MolWeight:102 RetIndex:629
CompName:Propane, 1-(1-methylethoxy)- $$ Ether, isopropyl propyl $$ Isopropyl propyl ether $$ Propyl isopropyl ether $$ 1-Isopropoxypropane # $



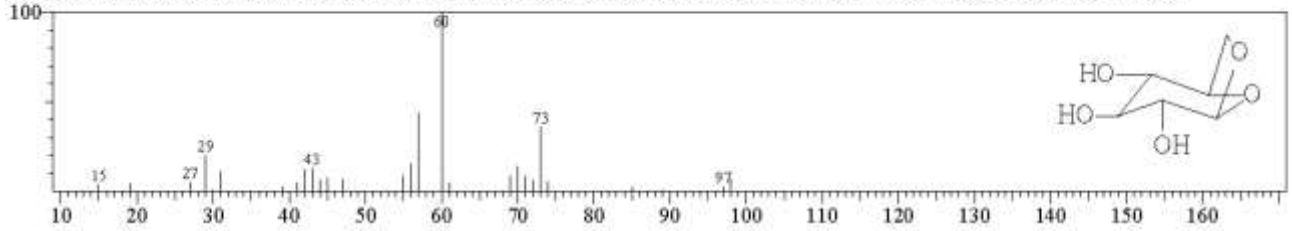
(C)

SI:86 Formula:C4H4N2O2 CAS:0-00-0 MolWeight:112 RetIndex:1277
CompName:1H-Pyrazole-4-carboxylic acid



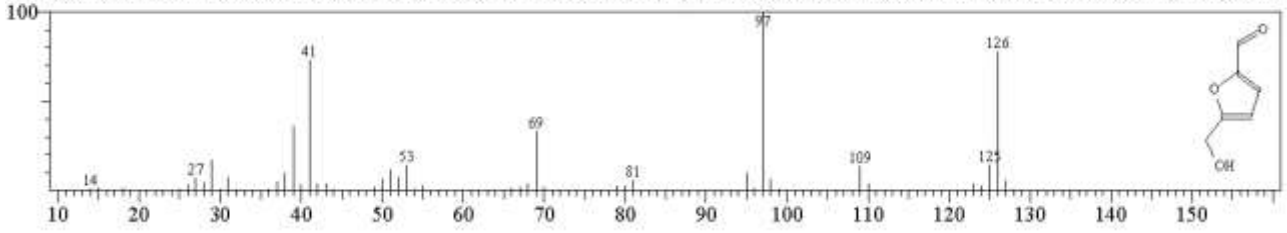
(D)

SI:90 Formula:C6H10O5 CAS:498-07-7 MolWeight:162 RetIndex:1404
CompName:.beta.-D-Glucopyranose, 1,6-anhydro- $$ Anhydro-d-mannosan $$ Levoglucosan $$ 1,6-Anhydro-.beta.-D-glucopyranose $$



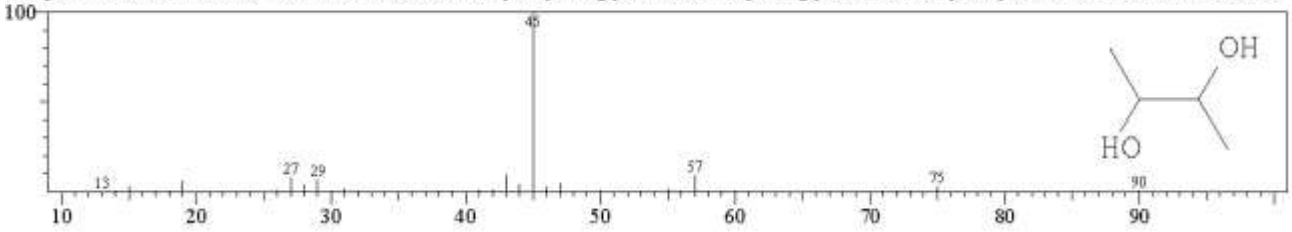
(E)

SI:84 Formula:C6H6O3 CAS:67-47-0 MolWeight:126 RetIndex:1163
CompName:2-Furancarboxaldehyde, 5-(hydroxymethyl)- $$ 2-Furaldehyde, 5-(hydroxymethyl)- $$ 5-Hydrxoymethylfurfural $$ Hydroxymethylfurfu



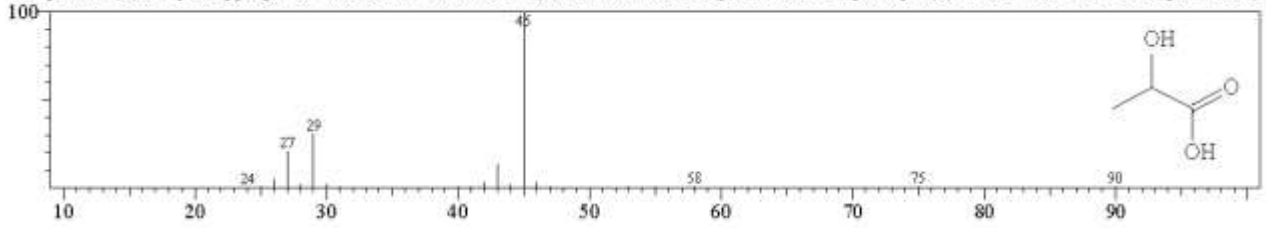
(F)

SI:93 Formula:C4H10O2 CAS:513-85-9 MolWeight:90 RetIndex:743
CompName:2,3-Butanediol $$ Butane-2,3-diol $$ Dimethylethylene glycol $$ 2,3-Butylene glycol $$ 2,3-Dihydroxybutane $$ D-2,3-Butane diol $$



(G)

SI:91 Formula:C3H6O3 CAS:79-33-4 MolWeight:90 RetIndex:858
CompName:(S)-2-Hydroxypropanoic acid $$ Lactic acid $$ L-(+)-Lactic acid $$ Propanoic acid, 2-hydroxy-, (S)- $$ Lactic acid, L- $$ Espiritin $$ (



(H)

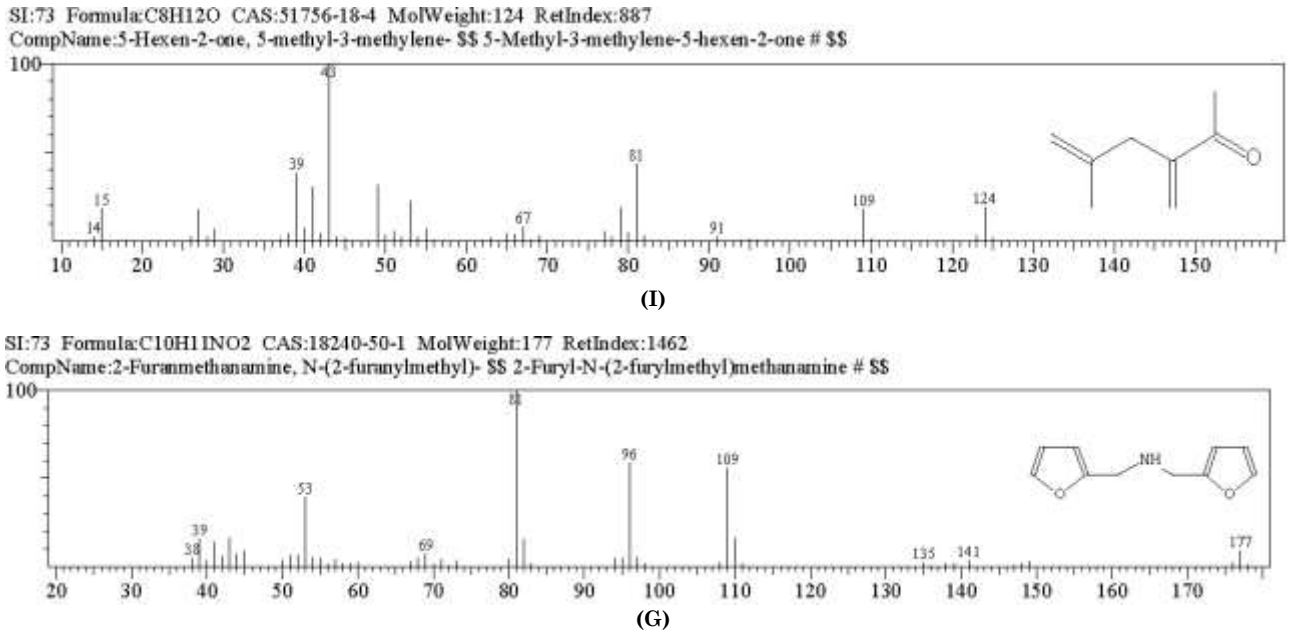
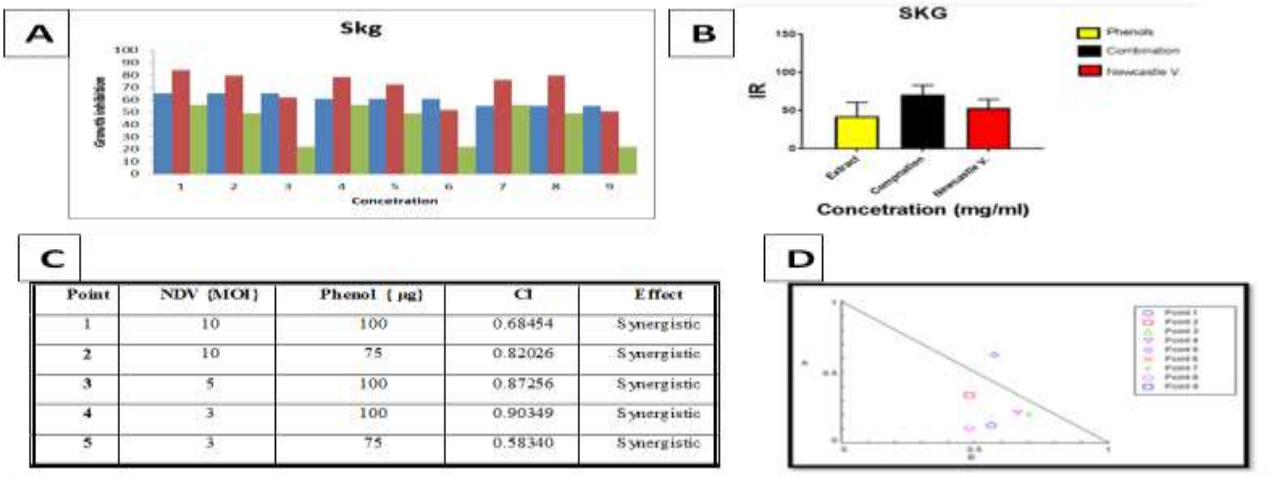


Fig.2: Mass spectra from full scan analysis of phenol extract: (A) 4-Penten-2-ol , 1-Penten-4-ol , 4-Hydroxypent-1-ene , CH2=CHCH2CH(OH)CH3 .(B) 3-Furaldehyde , 3-Furancarboxaldehyde .(C) 1H-Imidazole, 4,5-dimethyl- , Imidazole, 4,5-dimethyl-, 4,5-Dimethyl-1H-imidazole .(D) 1H-Pyrazole, 1,3-dimethyl-, Pyrazole, 1,3-dimethyl-, 1,3-Dimethylpyrazole, 2,5-Dimethylpyrazole, 1,3-Dimethyl-1H-pyrazole(E) Methyltartronic acid, 2-Hydroxy-2-methylmalonic acid .(F) Furyl hydroxymethyl ketone ,Ethanone, 1-(2-furanyl)-2-hydroxy- , Ketone, 2-furyl hydroxymethyl , 2-(Hydroxyacetyl)furan .(G) 2,3-Butanediol, [R-(R*,R*)]-, 2,3-Butanediol .(H) 2-Hydroxypropanoic acid , Lactic acid, L-(+)-Lactic acid . Propanoic acid, 2-hydroxy-, (S) - .Lactic acid, L- .Espiritin, (I) 3-Furancarboxylic acid, methyl ester, 3-Furoic acid, methyl ester, Methyl 3-furancarboxylate, Methyl 3-furoate.(J) Propanediol , .alpha.-Propylene glycol , Methyl glycol , Methylethyl glycol $$ Methylethylene glycol.

In Vitro Anti-tumor Cytotoxicity and Synergism Determination in Combination Therapy

Cell viability (MTT) assay were used assess the therapeutic effect of *C. rotundus* Phenols extract-NDV and its cytotoxicity against digestive system cancers and in normal cells. In SKG, an esophageal carcinoma cell line, combination treatment of 10 moi and 100ug/ml resulted in 88.07% growth inhibition (G.I.), and was statistically significant (p: 0.0001), whereas NDV alone induce 65.1% G.I.

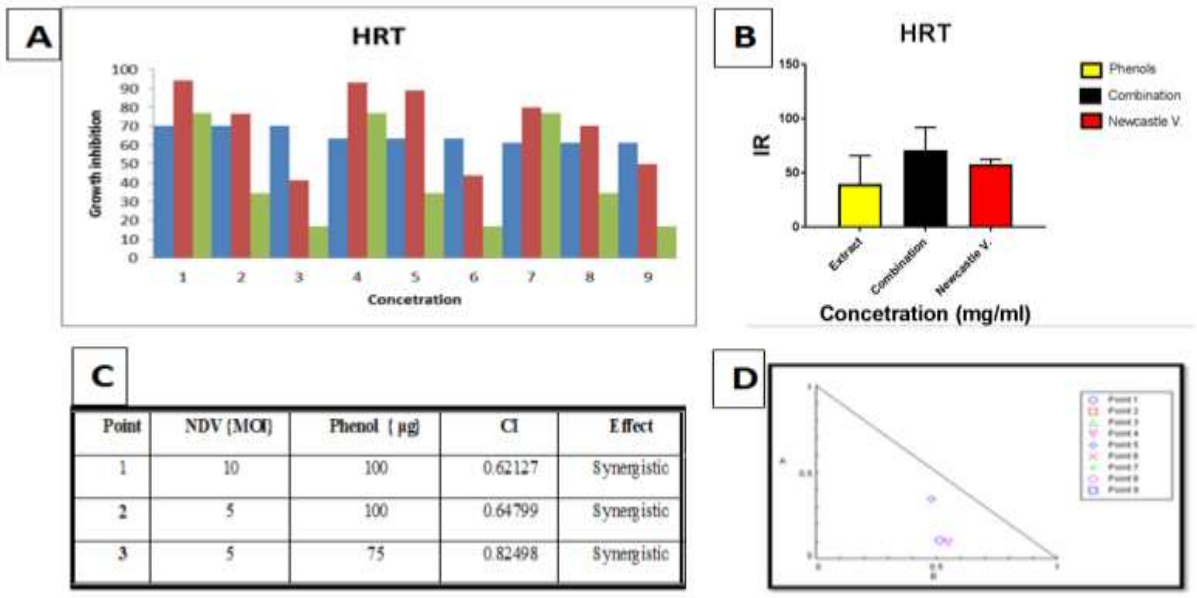
Phenols extract at 100 ug/mL produced only 55.91% growth inhibition (Figure-3a). The combination therapy was statistically significantly more cytotoxic than each monotherapy alone (figure-3b). Possible interactions between NDV and C. rotundus Phenols extract were assessed using Chou–Talalay equations. Combination index table (Figure-3c) and dose-oriented isobologram technique, showed that combination therapy tested on SKG cell line had synergism at 50% growth inhibition doses, as shown in (Figure 3d). three point were at synergy.



Point	NDV (MOI)	Phenol (µg)	CI	Effect
1	10	100	0.68454	Synergistic
2	10	75	0.82026	Synergistic
3	5	100	0.87256	Synergistic
4	3	100	0.90349	Synergistic
5	3	75	0.58340	Synergistic

Figure 3 Cytotoxicity of NDV and C. rotundus Phenols extract combination on SKG, esophageal carcinoma cell line. (a) Cytotoxic effect of Phenols extract, NDV, or combination, on SKG cells in vitro; (b) One-way ANOVA test showing combination therapy is more significant than monotherapies, and (c) showing the combination index data for each synergistic dose. (d) isobologram analysis displays strong synergism between NDV and Phenols extract in three points of the combination as they located at the lower left of the hypotenuse, demonstrating the effect is synergistic at a 50% cytotoxicity dose

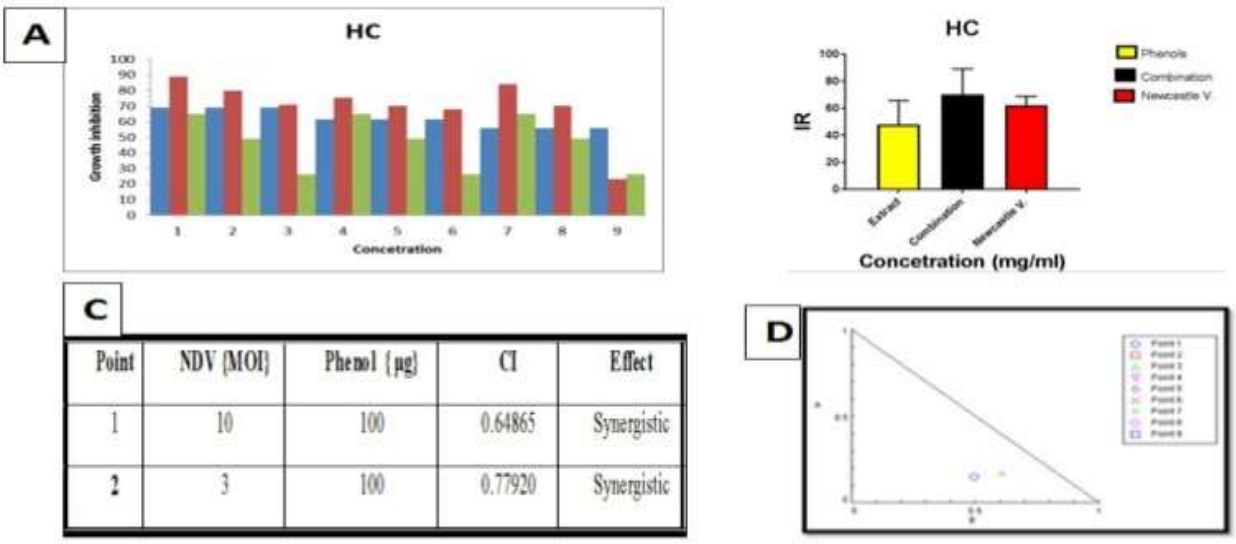
In HRT, a Human rectal cancer cell line, NDV and C. rotundus Phenols extract combination therapy induces 94.37% cytotoxicity at 10moi; 100ug/ml. Whereas NDV treatment alone at 10moi titer was also induced 70% cytotoxicity, while, Phenols extract alone at 100ug/mL induced less growth inhibition 67.95% (Figure 4a, b). The in vitro data were investigated further through Chou–Talalay equations and the dose-oriented isobologram technique. There was synergism between NDV C. rotundus Phenols extract at most doses tested as represented in Figure 3b, and c.



Point	NDV (MOI)	Phenol (µg)	CI	Effect
1	10	100	0.62127	Synergistic
2	5	100	0.64799	Synergistic
3	5	75	0.82498	Synergistic

Figure 4: Combination therapy cytotoxicity on Human rectal cancer cell line. (a) Cytotoxic effect of C. rotundus Phenols extract, NDV, or combination. The results for the treatment groups were stated as cell growth inhibition compared with untreated control cells; (b) One-way ANOVA test showing combination therapy is of no significant effect than monotherapies. (c) Combination index for one dose revealing strong synergism. (d) isobologram analysis exhibitions NDV and Phenols extract in multiple points of the combination as they located at the lower left of the hypotenuse, demonstrating the effect is synergistic at a 50% cytotoxicity dose

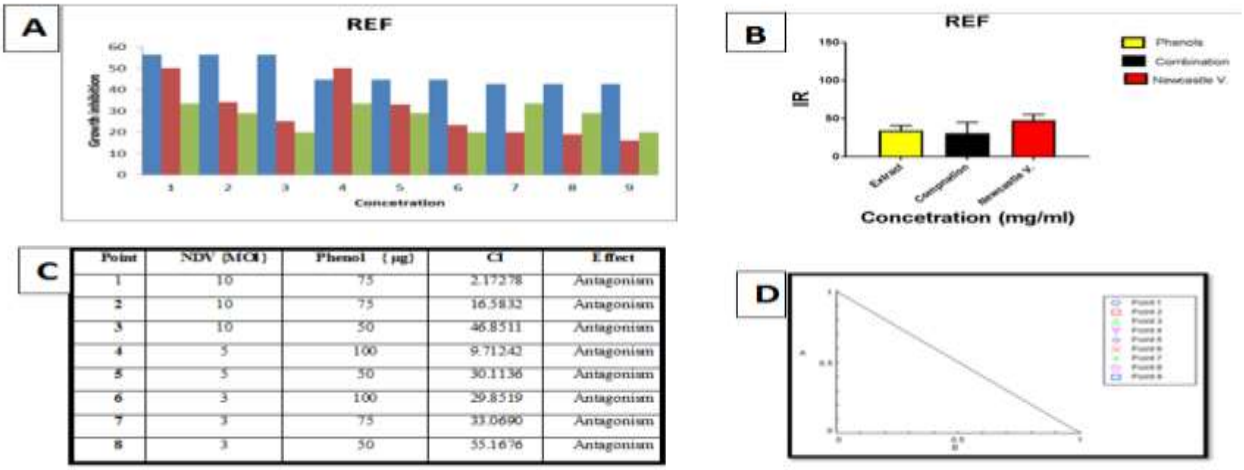
In HC, murine Hepatcellular carcinoma cell line, NDV and C. rotundus Phenols extract. Combination therapy at higher dose 10moi; 100ug/ml was very sufficient to induce (Figure-3c) 89% significant cytotoxicity (Figure-4b). Whereas C. rotundus Phenols extract produce 64.88% cytotoxicity against HC cells.



Point	NDV (MOI)	Phenol (µg)	CI	Effect
1	10	100	0.64865	Synergistic
2	3	100	0.77920	Synergistic

Figure 5: Combination therapy cytotoxicity on murine Hepatcellular carcinoma cell line. (a) Cytotoxic effect of C. rotundus Phenols extract, NDV, or combination. The results for the treatment groups were stated as cell growth inhibition compared with untreated control cells; (b) One-way ANOVA test showing combination therapy is of no significant effect than monotherapies. (c) Combination index for one dose revealing strong synergism. (d) isobologram analysis exhibitions NDV and Phenols extract in multiple points of the combination as they located at the lower left of the hypotenuse, demonstrating the effect is synergistic at a 50% cytotoxicity dose

In REF, a Rat embryo fibroblast , NDV and C. rotundus Phenols extract combination therapy induces 50.1% cytotoxicity at 10moi; 100ug/ml. Whereas NDV treatment alone at 10moi titer was also induced 45.84% cytotoxicity, while, Phenols extract alone at 100ug/mL induced less growth inhibition 33.4% (Figure 64a, b). The in vitro data were investigated further through Chou–Talalay equations and the dose-oriented isobologram technique. There was synergism between NDV C. rotundus Phenols extract at most doses tested as represented in Figure 6b, and c.

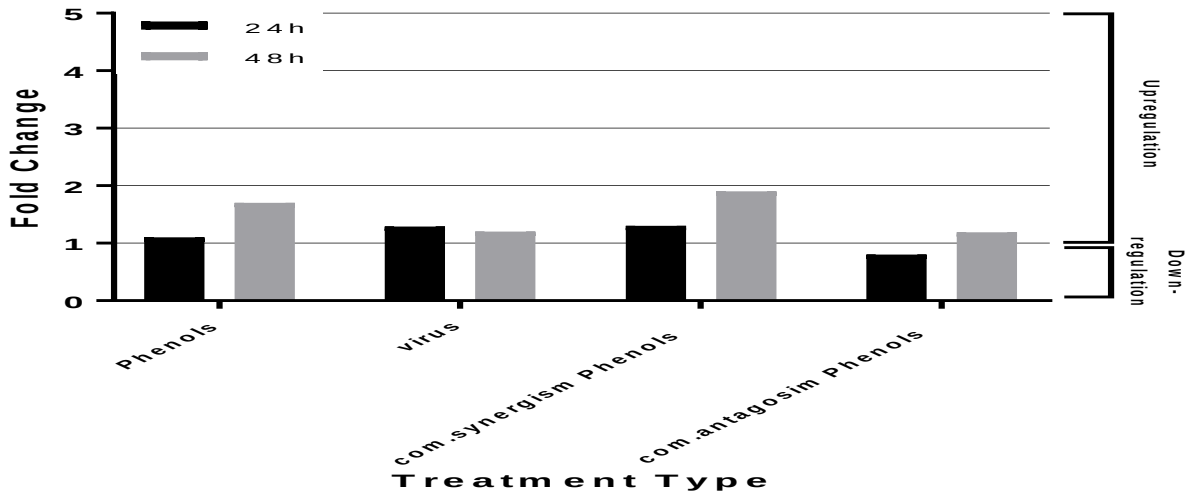


Point	NDV (MOI)	Phenol (µg)	CI	Effect
1	10	75	2.17278	Antagonism
2	10	75	16.5832	Antagonism
3	10	50	46.8511	Antagonism
4	5	100	9.71242	Antagonism
5	5	50	30.1136	Antagonism
6	3	100	29.8519	Antagonism
7	3	75	33.0690	Antagonism
8	3	50	55.1676	Antagonism

Figure 6: Combination therapy cytotoxicity Rat embryo fibroblast.(a) Cytotoxic effect of C. rotundus Phenols extract, NDV, or combination. The results for the treatment groups were stated as cell growth inhibition compared with untreated control cells; (b) One-way ANOVA test showing combination therapy is of no significant effect than monotherapies. (c) Combination index for one dose revealing strong synergism. (d) isobologram analysis exhibitions NDV and Phenols extract in multiple points of the combination as they located at the lower left of the hypotenuse, demonstrating the effect is synergistic at a 50% cytotoxicity dose

Analysis of P_{53} Gene Expression in Cancer and Normal Cells Lines

In cell exposed to Alkaloid- NDV combination, inhibition was significantly higher than single treatment in the three-cell line compared to the untreated control cells., RT-PCR was chosen to assess the expression at two-time point following the Phenols - NDV, NDV alone, and Phenols alone. In SKG cell line Phenols alone and NDV alone was upregulated at both 24hrs and 48hrs incubation duration as well as in combination it was upregulated. For HRT cell line p53 gene expression was upregulated for NDV alone, Phenols alone and Phenols-NDV combination at 24hrs and for 48hrs incubation duration. In HC cell line upregulated at both 24hrs and 48hrs incubation duration was observed in Phenols alone and NDV alone and in combination therapy at 24hrs and 48hrs incubation, duration. Figure 9.



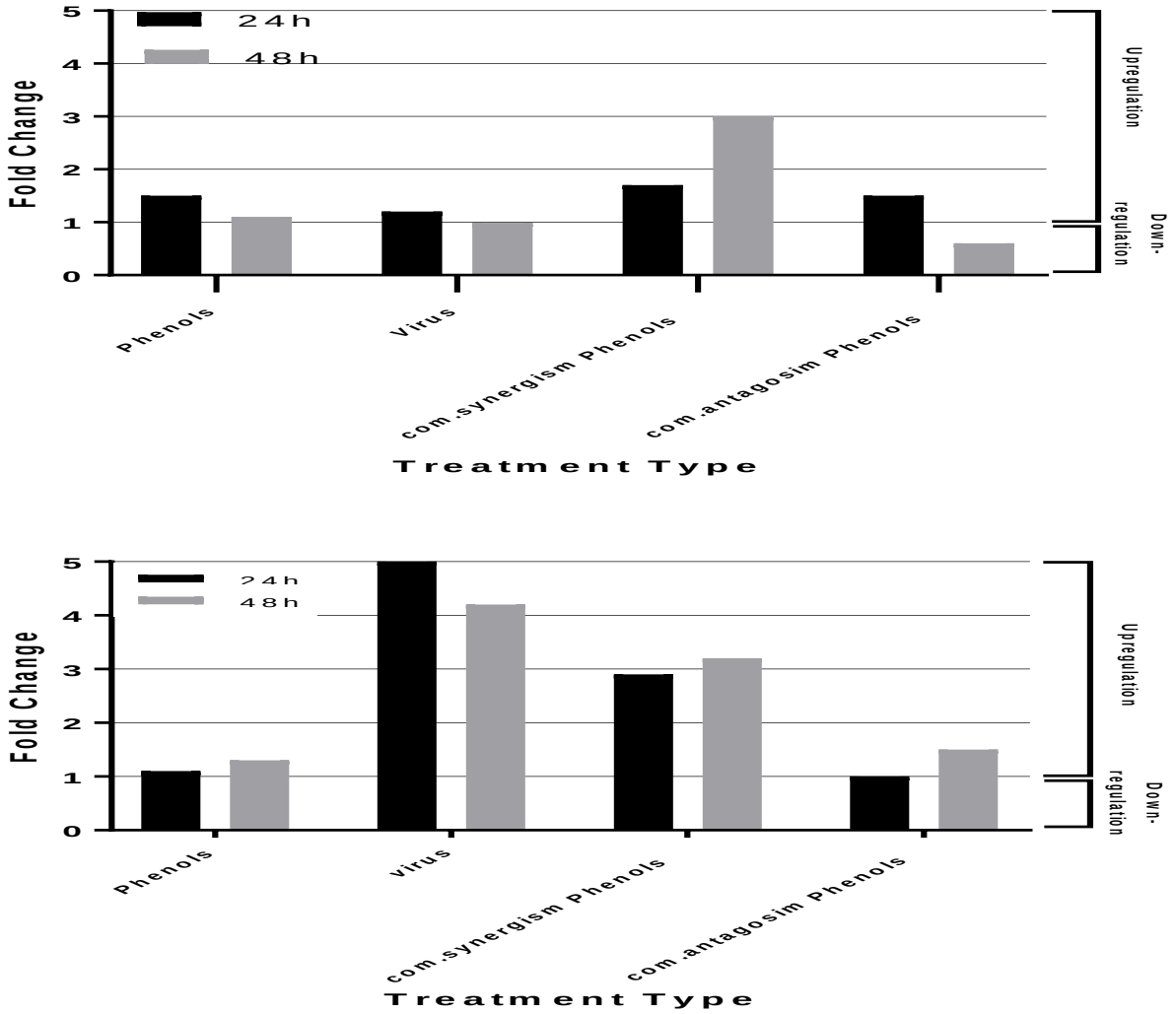


Figure 7(A) Gene expression in SKG, (B) Gene expression in HRT (C) Gene expression in HC

Discussion

The present study compared the effect of our novel combination therapy of NDV and Phenols on in vitro mouse and human digestive cancer cell line. Digestive cancer is responsible for more cancers and more deaths from cancer than any other system in the body [29]. Drug combination is most extensively used in treating the most dangerous diseases, such as cancer. The main aims are to achieve a synergistic therapeutic effect, dose and toxicity reduction [30].

The result of the current work provides evidence that combining Secondary metabolites (Phenols) with oncolytic virotherapy (NDV) could synergize in vitro to induce tumor cell death and inhibit tumor growth, this combination of agent was more effective than either agent administrated alone in inducing the imbibition of cancer cell growth. The aim of the study was to evaluated lower concentration both of Phenols and NDV alone and the synergistic combination of different concentration that enhanced the cytotoxic effect. An MTT assay was conducted using several different concentrations of Phenols and NDV. The result indicates that the NDV, Phenols, and combination of Phenols -NDV effectively reduced the viability of cancer cells and showed insignificant cytotoxicity in the normal cells. The combination of NDV and Phenols were evaluated using Compusyn. Synergism and antagonism determined by the combination index values, the result demonstrated synergistic cytotoxicity in almost all the concentration tested on the cancer cell.

Moreover, the combination of Phenols and NDV has little effect on normal fibroblast cells at most doses tested which prove safety. Several previous studies reported that Cyperus rotundus enhance anti-cancer effect of other chemotherapeutics [31, 32]. However, our current study aimed to use secondary metabolites product to demonstrate the presence of synergism between Phenols and oncolytic NDV.

To explain this synergistic enhancement of anti-tumor action, we notice that growth inhibition became high by increasing concentrations of both Phenols and NDV, and higher when Phenols and NDV combine. Basically secondary metabolites phenols used medicinally as anticancer or antitumor due to its definite antioxidant properties [33].

Hence, based on the result of this study, we conclude that oncolytic NDV with secondary metabolites extracted from Cyperus rotundus improve effect on cancer cells, while has an Intangible or little effect on normal cells compared with cancer cells. Our result showed that P_{53} gene protein upregulated in a combination of Phenols-NDV treated HC, SKG and HRT cancer cells compared with the untreated control cells. NDV alone and Phenols alone were also upregulated P_{53} but to lesser degrees. Interestingly, the expression levels of P_{53} were upregulated in NDV-treated cells equally or more than in the Phenols -treated cells especially in hepato cancer cell lines; this indicated that NDV might play an important role in inhibition cancer cell through three mechanisms depended on the degree of virus replication and virulence.

The first mechanism is by cytolysis secondary to viral replication [34]. The second mechanism involves inducing apoptosis[30, 35]. Since replication of NDV is independent of host cell DNA replication (which is the target of many cytostatic drugs and radiotherapy) and because of other findings, oncolytic NDV is a candidate agent to break therapy resistance of tumor cells [36]. Furthermore, NDV is reported to induce antigenic modifications to the tumor cells surface, making them more recognizable by the immune system. Using real-time PCR assays, we confirmed that the P53 gene was transcriptionally upregulated generally by the combination Phenols -NDV.

The significant upregulation of p53 by the combination therapy could explain the notable enhancement of cancer cell cytotoxicity. It is also suggested that NDV and the combination induced inhibition of p53 expression. Since the oncolytic effect of the Iraqi NDV strain was previously proved to have several anti-tumor mechanisms. One of these mechanisms is apoptosis induction in the infected tumor cells both in vitro and in vivo. Therefore, the inhibition of p53 was shown to be primary anti-tumor mechanism and novel target [37]. Suggested that induction of cancer cells inhibition may relate to the observed increased expression of p53 that, in turn, contributes to the upregulated in P53 and observed suppression of cell growth in both melanoma cell lines. Mention that Phenols that extracted from brown Rice inhibit the growth of human breast and colon cancer cells. Moreover phenolic compounds extracted from blueberry induced anti-proliferation and apoptosis induction effects using two colon cancer cell lines, HT-29 and Caco-2 and increases in DNA fragmentation [38].

In contrary when Phenols combined with NDV have significant effect and be even remarkable by dose augmentation for three cancer cells [39]. GC-MS of phenol extract reviled twenty-five intra-components through retention time 25 min, the most prominent components and higher concentration 58.83 in 9 min was Imidazole derivatives are valuable in treatment of many systemic fungal infections [40],furthermore other study conducted that when Imidazole mixed with DMSO-imidazole-tetrachloro ruthenatedemon strated to be anti-metastatic activity as a anticancer Agent [41].

Mesyloxyphenol with 14.82 concentration in 9 min is a classical phenolic antioxidant, its considered one of phenolic antioxidants that scavenger oxygen radicals [42]. Propionic acid with 5.03 concentrations in 10 min its occurring as carboxylic acid which inhibits the growth of mold and some bacteria As a result; most propionic acid produced is consumed as a preservative for both animal feed and food for human consumption, Propionic acid is also useful as an intermediate in the production of other chemicals, especially polymers like cellulose and esters [43].

4-heptene with concentration 2.36 in 7 min is a liquid that is a mixture of isomers It is used as an additive in lubricants, as a catalyst, and as a surfactant industrially and showed antibacterial activity against Helicobactor pylori pharmacology [44]. Major of studies concentrate in GC/MS analysis of Cyperus rotundus either on essential oil [45], [46] or crude extract [47]. The GC-MS elucidated bioactive compounds present in

the Phenol. The differences in retention time may due to the difference in mobile phase, and the extraction method, on the other hand, the different dielectric constant of solvents and polarity may affect the quantity of the extracted active compound [48]. The variant in component between alkaloids extract include ten components, and phenols extract that include twenty-five and the difference in retention time maybe it has chemotaxonomically significant for the plants [49]. In Conclusion, the result of our study supports a novel therapeutic strategy that provides a rationale for combining viro therapy using the oncolytic NDV Iraqi strain with secondary metabolites phenols in a treatment pattern that targets cancer cells. This study is the first that used secondary metabolites product extracted from C. rotundus which has a significant anti-tumor response in vitro. However, further investigation of its activity, in vivo, is necessary to elaborate on and exploit this promise.

Acknowledgments

The authors acknowledge the efforts of the staff of the Experimental Therapy Department at ICCMGR

References

1. Ashktorab H, Kupfer SS, Brim H, Carethers JM (2017) Racial Disparity in Gastrointestinal Cancer Risk. Gastroenterology, 153(4):910-23.
2. Memariani T, Hosseini T, Kamali H, Mohammadi A, Ghorbani M, Shakeri A, et al (2016) Evaluation of the cytotoxic effects of Cyperus longus extract, fractions and its essential oil on the PC3 and MCF7 cancer cell lines. Oncology letters, 11(2):1353-60.
3. Babu H, Savithramma N (2014) Screening of secondary metabolites of underutilized species of Cyperaceae. Int. J. Pharm. Sci. Rev Res., 24:182-7.
4. Singh N, Pandey B, Verma P, Bhalla M, Gilca M (2012) Phyto-pharmacotherapeutics of Cyperus rotundus Linn.(Motha): An overview. Indian Journal of Natural Products and Resources, 3(4):467-76.
5. Daswani PG, Brijesh S, Tetali P, Birdi TJ (2011) Studies on the activity of Cyperus rotundus Linn. tubers against infectious diarrhea. Indian J. Pharmacol., 43(3):340-4.
6. Al-Shammari AM, Ismaeel FE, Salih SM, Yaseen NY (2014) Live attenuated measles virus vaccine therapy for locally established malignant glioblastoma tumor cells. Oncolytic Virother, 3:57-68.
7. Al-Shammari A, Yaseen N, Alwan M (2010) 536 Newcastle disease virus Iraqi local isolate as a therapy for murine mammary adenocarcinoma: In vitro and in vivo study. European Journal of Cancer Supplements, 8(7):171.
8. Al-Shammari AM, Al-Nassrawei HA, Kadhim AMA (2014) Isolation and sero-diagnosis of Newcastle Disease Virus Infection in Human and Chicken Poultry Flocks In Three cities of Middle Euphrates. Kufa Journal For Veterinary Medical Sciences, 5(5):16-21.
9. Al-Shammary AM, Hassani HH, Ibrahim UA (2014) Newcastle disease virus (NDV) Iraqi strain AD2141 induces DNA damage and FasL in cancer cell lines. Journal of Biology and Life Sciences, 5(1):1.
10. Al-Shammari AM, Humadi TJ, Al-Taee EH, Al-Atabi SM, Yaseen NY 439 (2015) Oncolytic Newcastle Disease Virus Iraqi Virulent Strain Induce Apoptosis In Vitro through intrinsic Pathway and Association of Both Intrinsic and Extrinsic Pathways In Vivo. Molecular Therapy, 23:S173-S4.
11. Al-Shammari AM, Yaseen NY, Alwan MJ (2012) Newcastle Disease Virus Iraqi Oncolytic Strain Induce Apoptosis in Tumor Cells through Endoplasmic Re-ticulum Pathway. Iraqi J. Cancer Med. Genet., 5:34-41.
12. Al-Shamery A, Yaseen N, Alwan M (2009) Study the Antigenic modification of tumor cell surface by NDV infection. Iraqi Journal of Cancer and Medical Genetics, 2(1):95.
13. Al-Shammari AM, Al-Hili ZA, Yaseen NY 647 (2013) Iraqi Newcastle Disease Virus Virulent Strain as Cancer Antiangiogenic Agent. Molecular Therapy, 21:1.
14. Al-Shammari AM, Al-Juboory AA, Al-Mukhtar AA, Ali AM, Al-Hili ZA, Yaseen

NY (2014) Abstract 1221: Establishment and characterization of a chemoresistant glioblastoma cell line from an Iraqi patient. Cancer Research, 74(19):1221.

15. Al-Shammari AM, Salman MI, Saihood YD, Yaseen NY, Raed K, Shaker HK, et al (2016) In vitro synergistic enhancement of Newcastle Disease Virus to 5-fluorouracil cytotoxicity against tumor cells. Biomedicines, 4(1):3.
16. Al-Shammari A, Yaseen N, Alwan M, editors (2011) Cyclophosphamide synergistically enhance Newcastle disease virus Iraqi strain oncolytic activity. Proceedings of the 6th International Conference on Oncolytic Viruses as Cancer Therapeutics, Las Vegas, NV, USA.
17. Al-Shammari A, Yaseen N (2012) In Vitro Synergistic enhancement of Newcastle Disease Virus to Methotrexate cytotoxicity against tumor cells. Al-Anbar J. Vet. Sci., 5:102-9.
18. Al-Shammari AM, Rameez H, Al-Taee MF (2016) Newcastle disease virus, rituximab, and doxorubicin combination as anti-hematological malignancy therapy. Oncolytic virotherapy, 5:27.
19. Al-Shammari AM, Zainal IG, Kachi W, editors (2018) Oncolytic Virotherapy and Cobalt Ferrite Nanoparticles as Breast Cancer Therapy. Molecular Therapy, Cell Press 50 Hampshire ST, Floor 5, Cambridge, MA 02139 USA.
20. Al-Shammari AM, Alshami MA, Umran MA, Almukhtar AA, Yaseen NY, Raad K, et al (2015) Establishment and characterization of a receptor-negative, hormone-nonresponsive breast cancer cell line from an Iraqi patient. Breast Cancer (Dove Med Press). 7:223-30.
21. Nkumah OC (2015) Phytochemical analysis and medicinal uses of Hibiscus sabdariffa.
22. Hanafy M, Saadawy F, Milad S, Ali R (2012) Effect of some natural extracts on growth and chemical constituents of Schefflera arboricola plants. Journal of Horticultural Science & Ornamental Plants, 4(1):26-33.
23. Al-Shammari AM, Al-Nassrawei HA, Murtadha A (2014) Molecular diagnosis of Newcastle disease Iraqi Virulent strain virus HN gene by specific primers design. Kufa Journal For Veterinary Medical Sciences, 5(2).
24. Phuong LK, Allen C, Peng KW, Giannini C, Greiner S, TenEyck CJ, et al (2003) Use of a vaccine strain of measles virus genetically engineered to produce carcinoembryonic antigen as a novel therapeutic agent against glioblastoma multiforme. Cancer Res, 63(10):2462-9.
25. Chou T-C (2010) Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method. Cancer Research, 70(2):440-6.
26. Wang X, Seed B (2003) A PCR primer bank for quantitative gene expression analysis. Nucleic Acids Res, 31(24):e154.
27. Chew YC, Adhikary G, Wilson GM, Xu W, Eckert RL (2012) Sulforaphane induction of p21(Cip1) cyclin-dependent kinase inhibitor expression requires p53 and Sp1 transcription factors and is p53-dependent. J. Biol. Chem., 287(20):16168-78.
28. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. methods, 25(4):402-8.
29. Mbuk E (2017) Retrospective study on gastrointestinal tract tumors in humans in Zaria, Kaduna State, Nigeria. Journal of Cancer Research and Experimental Oncology, 9(1):1-6.
30. Chou T-C (2010) Drug combination studies and their synergy quantification using the Chou-Talalay method. Cancer research, 0008-5472. CAN-09-1947.
31. Al-Shammari A, Yaseen N (2012) In Vitro Synergistic enhancement of Newcastle Disease Virus to Methotrexate cytotoxicity against tumor cells. Al-Anbar Journal of Veterinary Sciences, 5(2):102-9.
32. AL-Hilli ZA, AL-Jumaily EF, Yaseen NY (2010) Role of volatile oils fraction of cyperus rotundus in induction of apoptosis on cancer cell lines in vitro. Iraqi J. Biotech., 9(2):286-98.
33. Matkowski A (2008) Plant in vitro culture for the production of antioxidants-a review. Biotechnology advances. 2008;26(6):548-60.
34. Phuong LK, Allen C, Peng K-W, Giannini C, Greiner S, TenEyck CJ, et al (2003) Use of a vaccine strain of measles virus

genetically engineered to produce carcinoembryonic antigen as a novel therapeutic agent against glioblastoma multiforme. Cancer research, 63(10):2462-9.

35. Wang X, Seed B (2003) A PCR primer bank for quantitative gene expression analysis. Nucleic acids research, 31(24):e154-e.
36. Schirrmacher V, Griesbach A, Ahlert T (2001) Antitumor effects of Newcastle Disease Virus in vivo: local versus systemic effects. International journal of oncology, 18(5):945-52.
37. Hsieh T-c, Wang Z, Hamby CV, Wu JM (2005) Inhibition of melanoma cell proliferation by resveratrol is correlated with upregulation of quinone reductase 2 and p53. Biochemical and biophysical research communications, 334(1):223-30.
38. Yi W, Fischer J, Krewer G, Akoh CC (2005) Phenolic compounds from blueberries can inhibit colon cancer cell proliferation and induce apoptosis. Journal of agricultural and food chemistry, 53(18):7320-9.
39. Tian S, Nakamura K, Kayahara H (2004) Analysis of phenolic compounds in white rice, brown rice, and germinated brown rice. Journal of agricultural and food chemistry, 52(15):4808-13.
40. Sundberg RJ, Martin RB (1974) Interactions of histidine and other imidazole derivatives with transition metal ions in chemical and biological systems. Chemical reviews, 74(4):471-517.
41. Rademaker-Lakhai JM, van den Bongard D, Pluim D, Beijnen JH, Schellens JH (2004) A phase I and pharmacological study with imidazolium-trans-DMSO-imidazole-tetrachlororuthenate, a novel ruthenium anticancer agent. Clinical Cancer Research, 10(11):3717-27.
42. Kjällstrand J, Petersson G (2001) Phenolic antioxidants in wood smoke. Science of the Total Environment, 277(1-3):69-75.
43. Thenappan T, Devaraj AP (2006) Dielectric studies on binary polar mixtures of propanoic acid with esters. Journal of molecular liquids, 123(2-3):72-9.
44. Zhang B-B, Dai Y, Liao Z-X, Ding L-S (2010) Three new antibacterial active diarylheptanoids from Alpinia officinarum. Fitoterapia, 81(7):948-52.
45. Aghassi A, Naeemy A, Feizbakhsh A (2013) Chemical composition of the essential oil of Cyperus rotundus L. from Iran. Journal of Essential Oil Bearing Plants, 16(3):382-6.
46. Kumar V. Medicinal Uses, Phytochemistry and Pharmacological Activities of Motha (Cyperus rotundus Linn.): A Potential Herb.
47. Al-Hilli Z (2004) Effect of cyperus rotundus crude extracts on cancer cell lines M. Sc: thesis. College of Science Univ Baghdad Iraq.
48. Al-Snafi AE (2016) A review on Cyperus rotundus A potential medicinal plant. IOSR Journal of Pharmacy, 6(7):32-48.
49. Ekundayo O, Oderinde R, Ogundeyin M, Stahl-Biskup E (1991) Essential oil constituents of Cyperus tuberosus Rottb. rhizomes. Flavour and fragrance journal, 6(4):261-4.

