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Pilot Study: New Aqueous Extracts of the Mistletoe (Viscum Album L.) Plant, Diminish In Vitro Cell Proliferation of The NSCLC Cell Line with EGFR Mutations HCC 827

New extracts of V. album diminish cell proliferation

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Abstract

Non-small cell lung adenocarcinoma with EGFR mutations continues to be a vexing problem due to the ability of the cancer to rapidly develop drug resistance. In vitro studies with the non-small cell lung adenocarcinoma cell line HCC827 (with an acquired mutation in the EGFR tyrosine kinase domain, E746 - A750 deletion) are relevant to the finding of increasingly effective treatments. Pharmaceutically processed, extracts of mistletoe, Viscum album Loranthaceae, have been shown since the 1920's to have potential in cancer therapy. They are currently the most frequently used natural preparations in complementary cancer treatments. In this pilot study we demonstrate that newly prepared standardized extractions of mistletoe (Viscum album, L.), from different host trees, are shown to significantly diminish the cell proliferation of the HCC 827 cell line.

Keywords: Mistletoe; Viscum album; Iscador; HCC827; non-small cell lung cancer; Adenocarcinoma; EGFR; Extracts; Lili Kolisko; Rudolf Steiner

Abbreviations: V: Viscum, NSCLC: Non-Small Cell Lung Cancer; ADC: Adenocarcinoma

Introduction

Globally and specifically in the United States, lung cancer is the leading cause of cancer-related mortality [1,2]. Tobacco smoking remains the predominant risk factor for lung cancer development. Non-tobacco risk factors include environmental and occupational exposures, chronic lung disease, lung infections, and lifestyle factors [3]. Despite significant treatment advances, advanced stage lung cancer shows a poor prognosis (80% mortality rate by 5 years) due to the acquisition of resistance to treatments and the late detection of the disease. Unfortunately, the present "gold standard" modalities are often not tolerated well by patients due to significant side effects, such as fatigue, debilitating nausea, and severe diarrhea, or since they lead to secondary clinical complications, stopping therapeutic efficacy or more. Thus, not surprisingly, one finds that lung cancer patients are seeking non- conventional therapies to supplement or replace the more customary modalities.

Among lung cancers 80-85% are non- small cell lung cancer (NSCLC). Moreover, research from 2020 shows that EGFR Mutations occur particularly in NSCLC tumors of adenocarcinoma (ADC) histology (NSCLC/ADC) and estimates are that 32.4 % of NSCLC cases have the EGFR mutation [4,5]. The treatment of NSCLC tumors is complicated by the fact that different mechanisms in tumor cells emerge to resist the targeting agent. Most commonly in EGFR-mutant NSCLC, secondary resistance mutations occur on the target kinase domain [6-8]. Moreover NSCLC/ADC treated with an EGFR targeting drug may transform into squamous cell carcinoma, a deadlier form of the disease [9]. For this reason, NSCLC/ADC cell lines with an acquired E746-A750 deletion in the EGFR tyrosine kinase exon 19 domain have been extensively studied, and the HCC827 cell line is an example. A Pub Med search (https://pubmed.ncbi.nlm.nih.gov) with the key words HCC827 resulted in 719 returns. Studies such as these have contributed

to the discovery of important chemotherapies and other immune therapies (Rybrevant, Gilotrif, Tagrisso, and others). Mistletoe is used in Europe for treatment of various conditions including diabetes, heart disease, and bacterial infections, [9,10] and was introduced by Rudolf Steiner in 1920 for the treatment of cancer [11]. In vitro study has elucidated the multiple antitumor mechanisms of action of mistletoe, including effects on membrane receptors, enzymes, ion channels, transporter proteins and transcriptional targets [12]. In the present study, we have validated a growth inhibition assay for the HCC827 cell line using the Promega Cell Titer Glo assay, and used this system to demonstrate that Viscum Kolisko, a newly developed mistletoe extraction from a multitude of host trees developed by the Kolisko laboratory, has an inhibiting effect on the HCC 827 cell line, and, therefore, may be a candidate treatment for NSCLC/ADC.

Materials and Methods

A 20mg vial of Iscador U c. Hg was purchased commercially. Iscador U c. Hg has as its host tree elm, (Ulmus Ulmaceae). The undiluted contents of this vial is considered as the mother tincture. The mistletoe raw material used for our own extractions was obtained from Europe. Mistletoe used in this experiment came from different host trees: poplar (Populus tremulus, Salicaceae), the willow (Salix alba, Salicaceae), acacia (Robinia pseudo-acacia, Fabaceae) and pear (Pyrus malus, Rosaceae).

Mistletoe Extraction and Potentization

The mother tincture (Ø) of each mistletoe was obtained through a proprietary method of extraction. The mistletoe was harvested from each host tree in two different opposing seasons (e.g., summer and winter). After the separate extraction of each seasonal harvest, the summer harvest extraction was allowed to drip by gravity downwards into the centrifugally rotating extract from the winter season. The resultant final mother tincture was designated "Viscum Kolisko… (host tree name)". This final mother tincture contained the extracted contents of 200mg dry mistletoe per mL. The "potency", a dilution prepared in "homeopathic" style, was designated as D1, and was made by 1:10 dilution using 1 ml of the mother tincture and 9 ml sterile ddH2O, succussed for two minutes, followed by one minute of rest. The D2 "potency" was made by 1:10 dilution of D1 as before.

Cell Culture

HCC827, a human lung cancer cell line (ATCC, CRL-2868), was typically seeded at 20% confluency in 75cm2 flasks, and grown to confluency at 37˚C, 5% CO2, which typically took three days. The medium was RPMI-1640 (ATCC, 30-2001) supplemented with 10% Fetal Bovine Serum (FBS; ATCC, 30-2020) and 1% penicillin streptomycin (ATCC, 30-2300). Once confluent, the medium was removed, and the cells rinsed with Dulbecco's Phosphate Buffered Saline (D-PBS). The cell monolayer was then harvested by incubation at 37˚C with 3cmL 0.25% Trypsin, 0.53 mM EDTA for 5 minutes, followed by 7 mL fresh medium. The suspension was transferred to a 15-ml centrifuge tube, and sedimented at 130 x g for 7 minutes. The supernatant was removed, and the pellet resuspended in fresh media. Countess 3 (Invitrogen, AMQAX2000) was used to count cell inoculum and to check viability.

Calculation of IC 50 of Viscum Extracts

In results the values shown are the Log10 of the dilution of the mother tincture, of each extracted viscum album L., prepared by extracting 200mg of the corresponding mistletoe tissue per mL. The Log10 IC50 values are derived from adjacent concentrations, whose % growth is above and below 50, respectively, using the following formula:

$$\text{Log10 IC50} = A - ((A-B)(C - 0.5(100C - 0C))/(C-D)$$

Where:

A = log10(upper sample conc.)

B = log10(lower sample conc.)

100C = 100% control RLU (maximum cell growth)

0C = 0% control RLU (initial cell inoculum)

C = lower sample conc. RLU

D = upper sample conc. RLU

For each extract except Pyrus, there were 32 replicates of every dilution, and 16 replicates of the 0% and 100% control. For Pyrus, there were 8 replicates of every dilution and of the controls. The Log10 IC50 was determined for every combination of control and sample values, 262,144 in total, 4096 for Pyrus. The values shown are the mean ± standard deviation of those 262144, or 4096, values.

HPTLC plant identification

Two methods for plant identification were used: first organoleptically then the mistletoe leaves received from Romania were confirmed to be Viscum album L. by the HPTLC method by the (third party independent) Alkemist Lab, www.Alkemist.com.

Cell Viability Assay

Cells were plated in white, 96-well plates (Costar, CLS3917) at 1 x 104 cells per well, 0.1 ml medium per well. 10% of the medium was either ddH2O (0% and 100% controls), or Ø, D1, or D2 (samples), with four replicates per treatment or control. Plates were left for 2 hours at room temperature, before subsequent incubation at 37˚C and 5% CO2, to produce uniform cell distribution on the growth surface and reduce edge effect (35). Cell growth was assessed using the CellTiter-Glo luminescent cell growth assay (Promega, G9243), using 100 µl of reagent per well according to the manufacturer's instructions. The luminescence was measured in a BioTek, Synergy LX plate reader. Cell growth was assessed for 0% control wells after an initial 18 hours of incubation, while sample and 100% control wells were assessed

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after an additional 48 hours of growth.

Calculation and Statistics

Cell count analysis was performed on four measurements, 16 wells in total (two sets of quadruplicates on two different dates). Data are expressed as mean ± standard deviation. Significance ($p<0.05$) was determined by T test in Microsoft Xcel. The percent growth was measured according to the following formula:

%Growth = 100 x (Sample – 0% Control)/ (100% Control – 0% Control)

Results

For the HCC827 cell line we first determined the most appropriate cell inoculum, 1 x 104 cells, and incubation time which was 48 hours. The cells had a viability of 95% or greater. In the experiment the addition of Viscum Kolisko mother tincture or its potencies D1 or D2 showed an inhibition of the cell proliferation - as indicated in the table and chart. In many cases, the suppression of growth is more than 100%. This likely indicates that the extract not only suppresses growth, but is also toxic, and is reducing the cell number to levels below that seen in the 0% control. The mother tinctures of several of our preparations are apparently less inhibitory than the D1 or D2 samples. This appears to be caused by an artifactual production of luminescence in the CellTiter Glo assay caused by the tincture alone. This effect does not prevent assessment of the potency of our preparations, because the IC50 occurs at much higher dilutions where no luminescence would be observed from the mistletoe alone. Since the standardized and commercially available mistletoe extract Iscador U c. Hg is known to have been used successfully in the treatment of lung cancer case it was used as a rough comparison to gage the strength of our mistletoe extraction. The lesser potency of Iscador as compared to our extracts likely demonstrates an extract concentration below that of our undiluted extract. Mistletoe as a semi-parasitical plant is assumed to take on nutrient elements from the host tree that it grows on. This is evident in the fact that our mistletoe extractions show a different inhibitory effect depending on which tree they grew on (Figures 1 & 2) (Table 1).

IC50 Results

(Table 2).

HPTLC Results

(Figure 3).

Table 1: Calculations of the influence of mistletoe (Viscum album L. Kolisko) from different host trees on the HCC827 cells growth and % growth compared to control. Measurement of mean growth (arithmetic mean) and % growth in RLU's (Relative Light Units). All Viscum mistletoes represent Viscum Kolisko extractions. The (*) represents the standard deviation of the percent growth calculated as a fractional standard deviation. The V. acacia had the most overall inhibition of the HCC827 cells followed by the mistletoes from the other host trees.

	Control		∅	D1	D2	Total Inhibition
Viscum acacia	Mean	275801.4	6975.875	29725.125	48391.44	-532 .353
	% Growth	0	-192.7898	-176.475	-163.0884	
	Std. Dev. *	14.89667	-28.06744	-15.04086	-8.141682	
Viscum poplar	Mean	208461.1	43242.63	12986.5	45496.5	-448.14
	% Growth	0	-141.932	-167.285	-139.463	
	Std. Dev. *	39.80564	-20.1889	-33.2732	-16.9819	
Iscador U c. Hg	Mean	30482.5	1427.25	6397.667	24205.96	-399.072
	% Growth	0	-195.1496	-161.7658	-42.15639	
	Std. Dev. *	26.81831	-49.02974	-15.92129	16.78754	
Viscum salix	Mean	166187.6	105955.3	33811.81	43969.44	-344.872
	% Growth	0	-65.9807	-145.009	-133.882	
	Std. Dev. *	17.22867	5.404422	-6.39676	-5.18291	
Viscum pyrus	Mean	225157.6	190973.3	23113.81	29445.5	-324.441
	% Growth	0	-25.6768	-151.76	-147.004	
	Std. Dev. *	27.93477	13.1595	-8.729716	-10.35971	

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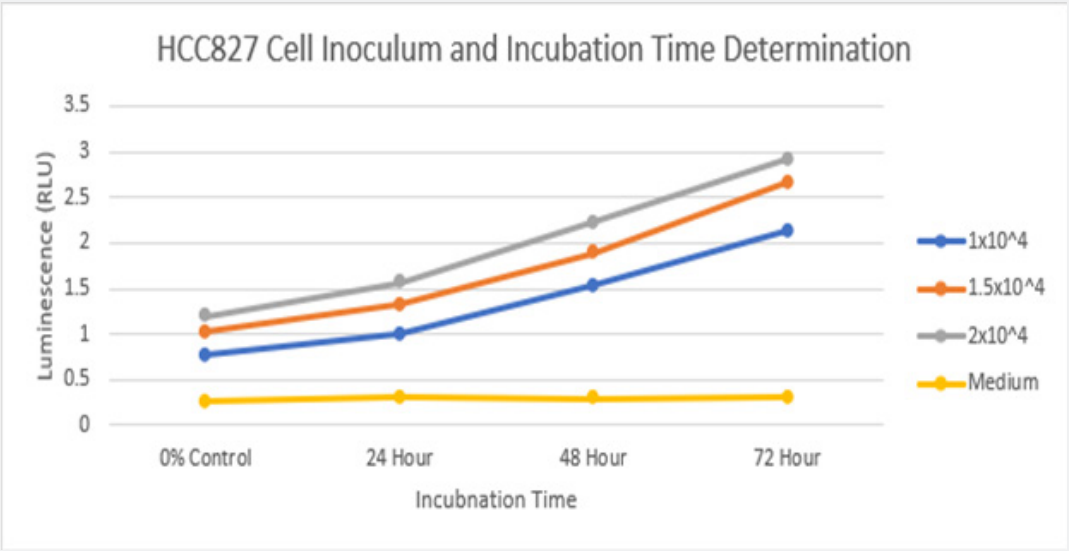

Figure 1: Incubation time and cell inoculum determination of HCC827 cells to use for future studies.

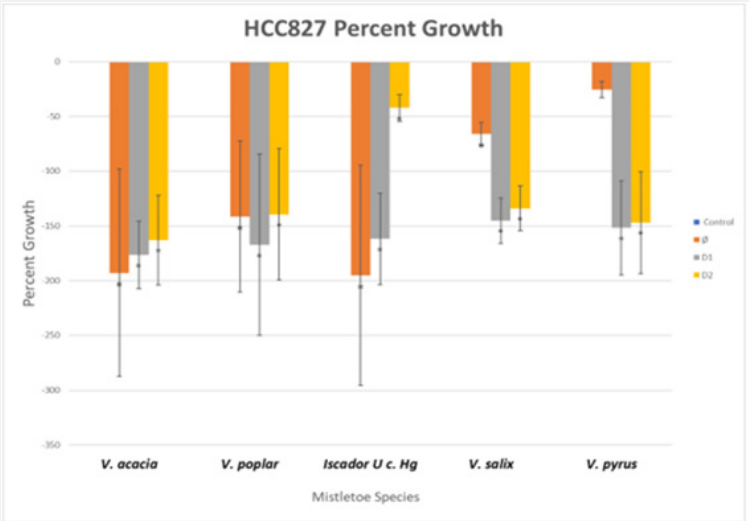

Figure 2: Inhibiting influence of mother tincture and potencies D1 and D2 mistletoe (Viscum album L, Kolisko) from different host trees on HCC827 cells. The control is set to "0" and each mistletoe shows inhibition of percent growth of HCC827 (* represents p = <0.05) when compared to the control. The bars are the standard deviation of the percent growth. The Viscum (V.) represents Viscum Kolisko extractions. RLU=Relative Light Units.

Table 2: The table shows the log10 IC50 values for mistletoe extracts from four different species of tree. Of the four extracts tested, the Acacia extract seems the most potent, being 0.84 log (6.9x) more potent than the Poplar extract, 0.67 log (4.68x) more potent than the Salix extract, and 1.86 log (72.44x) more potent than the Pyrus extract. The values shown are the mean ± standard deviation of those 262144, or 4096, values.

Extract	LogIO Dilution
Poplar	-2.66±0.23
Acacia	-3.50±0.41
Salix	- 2.83±0.29
Pyrus	-1.64±0.11

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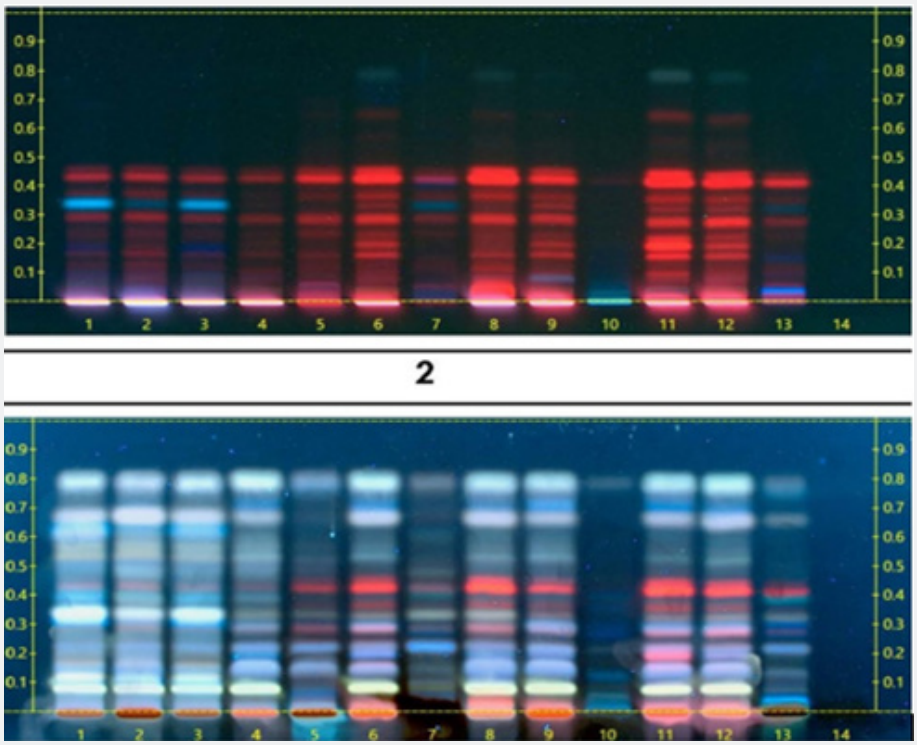

Figure 3: The two images represent the same plate with different detection conditions. This way different types of compounds can be revealed since some compounds might be naturally fluorescent under UV light, while others may require specific chemical reagents or UV light for visualization. Multiple detection techniques enhance the chances of visualizing a wide range of compounds in the samples. The image on the top shows the plate under UV light before and then after derivatization with sulfuric acid.

Results

Lanes 4, 6, 8, 9, 11, 12 are the test samples of Viscum album, grown on different host trees. Lanes 1, 2, 3 are the in-house reference samples of Viscum album used for comparison. Lanes 5, 7, 10, 13 are the in-house reference samples of various host trees used for comparison. The test samples detect the presence of Viscum album. The viscum samples do not detect the presence of their host trees.

Discussion

We demonstrated in this study that our Viscum album L. extracted at the Kolisko Institute from different host trees was able to inhibit cell proliferation in the HCC 827 cell line. The degree of inhibition by each type of mistletoe was different depending on the provenance from its host tree. Thus, the acacia mistletoe from the Robinia pseudo-acacia tree was more potent than the mistletoe coming from the willow (Salix) tree, which in turn was more inhibiting than the mistletoe poplar tree and finally the mistletoe grown on the pear tree (Pyrus) was weakest. This experimental finding supports the biological expectations that there are differences between the mistletoes originating on different host trees since the mistletoe as a semi-parasitic plant takes on nutrients from the host tree and thus would be expected to manifest different characteristics, even if small overall. Iscador U c. Hg was chosen since it is the only standardized mistletoe that we have found to have been specifically used in a few lung cancer cases [13,14]. It was chosen as a comparison to our mistletoes as its "total" inhibitory strength fell between the poplar and willow mistletoe. In view of the paucity of mistletoe studies both in vitro and in clinical situations of the effects of mistletoe standardized extracts on NSCLC this study is important since it warrants further studies to elucidate the mechanisms of action of mistletoe on NSCLC. It also encourages further study on the differentiation of anti-proliferative activity of mistletoe extracts by the host tree provenance. The role of the mistletoe extracts in cancer therapy is increasingly being accepted because it does not only not interfere with other "gold standard" therapies but rather it acts beneficially in a "complementary" fashion; it may be synergistic with the other therapies, ameliorates some side effects of conventional treatments and increases survival time and quality of life. Thus, any further study of its role in cancer therapy is relevant.

Authors participation

R. Rentea MD- designed the study; also participated in the extraction of the mistletoe material.

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M. Mueller M. Sc – executed the experimental data and wrote the Materials & Methods and Results sections.

M. Kamsler MD – participated in the extraction of mistletoe material.

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Conflict of Interest

R. Rentea MD and M. Kamsler MD are on the Board of the Kolisko Institute but had no influence on the experimental results obtained by Malory Mueller M.Sc.

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