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Comparative Evaluation of Anti-Inflammatory & Anti-Tumorigenic efficacy of "The Walking Mango Tree" Bark extracts

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General Note
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ABSTRACT

Stem bark of mango tree (Mangifera indica) has been used in herbal medicine as anti-phlogistic, liver protective, kidney protective, hypotensive, diuretic, anti-cough and analgesic agent. However, the anti-cancer activity and the potential anti-cancer mechanisms of walking mango tree bark have not been elucidated. We performed in vitro study to investigate whether WMTBE shows anti-inflammatory and anti-cancer activity. This was compared with the NMTBE. In anti-inflammatory activity proteins regulating NF-κB signaling were analyzed by Western blot. In anti-cancer activity, cell growth was measured by MTT assay. HPLC, MALDI- TOF & GC

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MS were carried out to reveal the molecule beyond this phenomenon. These findings suggest that WMTBE exerts anti-cancer activity, but more trials have to be carried on anti-inflammatory activity.

Keywords: Phlogistic, Hypotensive, Analgesic, Inflammatory, Western blot.

Abbreviations: WMTBE- Walking mango tree bark extract, NMTBE- Normal mango tree bark extract, MTT- [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide], HPLC- High performance liquid chromatography, MALDI- Matrix assisted laser desorption/ionization time of flight, GC MS- Gas chromatography mass spectroscopy.

1. INTRODUCTION

Nature has provided a complete storehouse of remedies to cure ailments of mankind (Gangadhar *et al*., 2012; Rahman *et al*., 2011). There is a widespread belief that green medicines are healthier, though the recovery of disease by plant medicine is slow, the therapeutic use of medicinal plant is becoming popular because of its inability to cause side effects and effective for antibiotic resistant microorganisms (Perumalsamy *et al*., 2008). Interest in natural compounds has grown in recent years because of concerns about drug costs and safety. This illustrates the need for new sources for drug discovery, and natural sources provide valuable information for research in this area. During the past decade, tremendous progress has been made toward understanding the cellular and molecular mechanisms underlying the process of carcinogenesis, leading to the development of potential cancer prevention options termed chemoprevention (Sporn M.B, 1979).

Cancer is a complicated disease that may develop in humans over a number of years. Development of a tumor starts with a normal cell that is transformed through the activation of proto-oncogenes and the suppression of tumor suppressor genes such as p53. The transformed cell no longer behaves like a normal cell but begins to exhibit the properties of a cancer cell. Such transformation in the cells makes them self-sufficient in growth signals and resistant to antigrowth signals, resulting in uncontainable proliferation. In addition, these cells are able to avoid apoptosis, resulting in tumor growth. This whole process of transformation may take 10–20 years. The growth of the tumor is aided by angiogenesis, which not only provides nutrition to the tumor but also enables its invasion to surrounding tissues, and its metastasis to distant tissues; the latter is usually lethal. Inflammation, which occurs as a response to cancer, has two stages, acute and chronic. Acute inflammation, the initial stage of inflammation, represents innate immunity; it is mediated through the activation of the immune system, lasts for a short period and generally is regarded as therapeutic inflammation. If the inflammation persists for a long period of time, however, the second stage, chronic inflammation, sets in (Lin W.W., 2007). Chronic inflammation has been linked with most chronic illnesses, including cancer, cardiovascular disease, diabetes, obesity, pulmonary disease, and neurologic disease (Aggarwal B.B., 2006).

Mangifera indica L. (Anacardiaceae) is one of the most important tropical plants, part of this plant is used in all system of medicine throughout the globe (Sharma *et al*., 1997). Parts of the mango, such as stem bark, leaves, and pulp are known for various biomedical applications, including anti-oxidative and free radical scavenging (Ajila, Bhat, & Prasada Rao, 2007; Rocha Ribeiro *et al*., 2007), anti-inflammatory (Hernandez *et al*., 2007), and anticancer (Percival *et al*., 2006) activities. Additionally, the ability of the mango bark extract to inhibit proliferation of various cancer cells was investigated. Because the elimination of cancer in the early stages is an integral part of chemoprevention, measuring antiproliferative properties against cancer cells using common assays, such as the MTT assay, provide useful insight on the chemoprotective potential of natural extracts. Hence our results showed the ability of WMTBE for inhibiting the growth of the tested cancer cell lines, to varying degrees.

Thus, the objective of this study was to examine the efficacy of walking mango tree bark as an inhibitory effect on human cancer cell proliferation in comparison with the normal mango tree bark.

2. MATERIALS & METHODOLOGIES

2.1. Plant material collection:

The WMT & NMT bark were collected from Gujarat & Bengaluru respectively. Sample were air dried at room temperature and crushed into small particles using mortar& pestle; stored in a Ziploc bags for further use.

2.2 Soxhlets extraction:

The extraction was carried out using Acetone (least polar), Aqueous (high polar), Chloroform (lipophilic), Methanol (high polar) & Petroleum Ether (non-polar) (separately). 100 gm of dry bark were extracted with the above solvents using Sox-eight hour till the color of the solvent returned colorless. Solvent was evaporated under reduced pressure using Rotary evaporator apparatus. Extract was finally allowed to dry at air at room temperature till complete dryness. The determined samples are stored at 4°C for further use.

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2.3 Cell Lines:

The human cancer cell lines, A375 (Malignant melanoma), HepG2 (Hepatocellular carcinoma), MCF7 (Adenocarcinoma), HCT 116 (p53+/+) (Colorectal carcinoma), HCT 116 (p53-/-)(Colorectal carcinoma),HeLa (Adenocarcinoma), U937 (Histiocytic lymphoma) were maintained in 75mm culture dishes (Nunc) at 37ºC in a humidified incubator containing 5% CO2 in growth medium (DMEM supplemented with 10% fetal calf serum).

2.4 Media & Reagents used:

DMEM (1X): Dulbecco's Modified Eagle Medium
RPMI (1X): Roswell Park Memorial Institute Medium
PBS pH 7.4 (1X): Phosphate Buffered Saline
1X TE: Tris EDTA
MTT dye: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
MTT lysis buffer-DMSO

2.5 Instruments used:

Soxlets apparatus
Rota-evaporator: Heidolph
Heating mantle: IKA®C-MAG HS 7
Recirculating cooler: Julabo F25
Flat shaker: IKA® KS 260 basic
BIO RAD: Model 680; microfigure reader
HPLC- UV/DAD
MALDI- TOF
GC-MS

2.6 Cell Proliferation Assay:

The cells were figure d at 100,000 cells in 60-mm tissue culture dishes. After 18 h of culture, cells were treated with different concentrations of DMSO-dissolved MSP (0, 12.5, 25 and 50 µg/mL At 24 h, cells were collected by trypsinization, stained with trypan blue, and the cell number in suspension was counted in duplicate using a hemocytometer. Data were the average of three independent experiments.

2.7 Statistical Analysis:

All data are expressed as means + standard deviation (SD) unless stated otherwise. Differences between groups were calculated using the Student's unpaired t-test. Dose-dependent effect was calculated using simple linear regression. $P < 0.05$ was considered statistically significant.

3. RESULTS

The performed experiments were evaluated and represented in the tabular & graphical form for a better understanding.

Table 21: MCF7- WMT Extract (27-7-2015; 8:00am)

	Aqueous [x] (nm)			Average [y]	Xa	Xb	Xc	STDEV [Xa: Xb: Xc]	Cell Survival (100%), [z]	Cell Death [100-z]	t- Test
Control	0.92	0.93	0.90	0.92	100.07	101.82	98.11	1.86	100.00	0.00	NA
Positive Control	0.04	0.06	0.05	0.05	4.59	6.12	5.79	0.81	5.50	94.50	0.0001
Solvent	0.42	0.40	0.41	0.41	46.32	43.92	44.68	1.23	44.97	55.03	NA
10µg	0.51	0.53	0.54	0.53	55.17	58.01	58.99	1.99	57.39	42.61	*** 0.0017
100µg	0.27	0.25	0.28	0.27	29.83	26.88	30.37	1.88	29.02	70.98	*** 0.0009
1mg	0.36	0.45	0.49	0.44	39.44	49.60	53.86	7.41	47.63	52.37	*** 0.0081
10mg	0.67	0.70	0.65	0.67	72.87	76.91	70.68	3.16	73.49	26.51	*** 0.0009

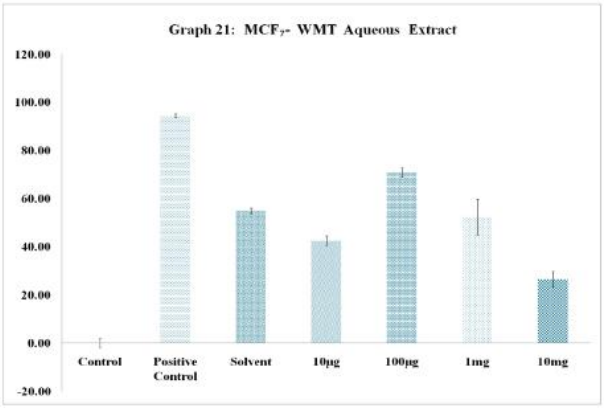

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Table 26: MCF$_7$- NMT Extract (27-7-2015; 8:00pm)

	Aqueous [x] (nm)			Average [y]	Xa	Xb	Xc	STDEV [Xa: Xb, Xc]	Cell Survival (100%), [z]	Cell Death [100-z]	t- Test
Control	0.92	0.93	0.90	0.92	100.07	101.82	98.11	1.86	100.00	0.00	NA
Positive Control	0.04	0.06	0.05	0.05	4.59	6.12	5.79	0.81	5.50	94.50	0.0001
Solvent	0.64	0.69	0.64	0.66	70.25	75.49	70.25	3.02	72.00	28.00	NA
10µg	0.72	0.68	0.70	0.70	78.11	74.18	75.58	1.98	76.29	23.71	*** 0.0068
100µg	0.71	0.80	0.75	0.76	80.52	87.51	81.17	3.86	83.07	16.93	*** 0.0059
1mg	0.61	0.63	0.69	0.65	67.08	69.16	75.60	4.44	70.61	29.39	** 0.0134
10mg	0.57	0.93	0.89	0.80	62.27	101.06	97.76	21.45	86.96	13.04	0.4028

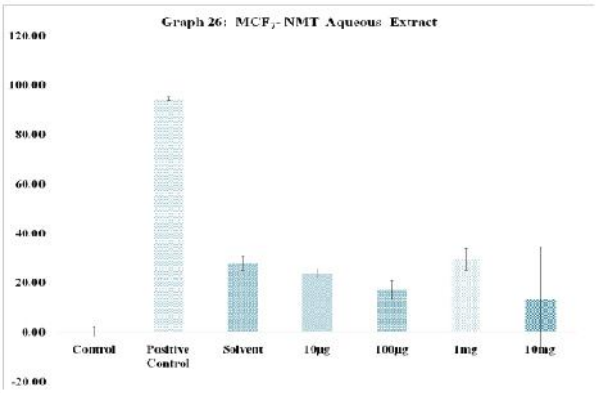

Table 31: A375- WMT Extract (28-7-2015; 8:00pm)

	Aqueous [x] (nm)		Average [y]	Xa	Xb	STDEV [Xa: Xb]	Cell Survival (100%), [z]	Cell Death [100-z]	t- Test
Control	2.49	2.49	2.49	100.04	99.96	0.06	100.00	0.00	NA
Positive Control	0.08	0.09	0.08	3.33	3.41	0.06	3.37	96.63	0.0005
Solvent	2.42	2.40	2.41	97.15	96.31	0.60	96.73	3.27	NA
10µg	1.94	1.69	1.82	77.88	67.96	7.01	72.92	27.08	0.1144
100µg	2.08	1.97	2.02	83.58	78.92	3.29	81.25	18.75	0.0773
1mg	1.30	1.32	1.31	52.31	53.11	0.57	52.71	47.29	*** 0.0059
10mg	0.20	0.23	0.22	8.19	9.19	0.71	8.69	91.31	*** 0.0038

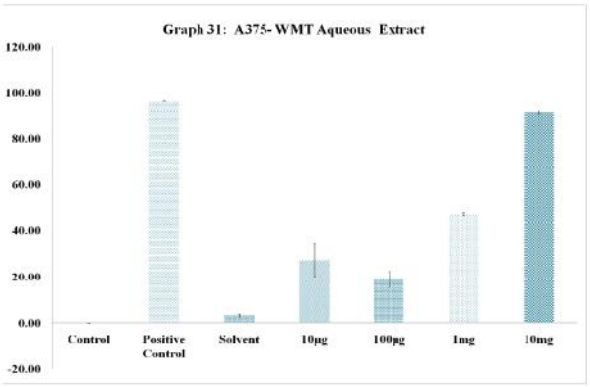

Table 36: A375- NMT Extract (28-7-2015; 8:00pm)

	Aqueous [x] (nm)		Average [y]	Xa	Xb	STDEV [Xa: Xb]	Cell Survival (100%), [z]	Cell Death [100-z]	t- Test
Control	2.49	2.49	2.49	100.04	99.96	0.06	100.00	0.00	NA
Positive Control	0.08	0.09	0.08	3.33	3.41	0.06	3.37	96.63	0.0005
Solvent	2.40	2.33	2.36	96.47	93.38	2.19	94.92	5.08	NA
10µg	2.45	2.64	2.54	98.31	105.98	5.43	102.15	-2.15	0.6777
100µg	2.49	2.40	2.44	100.00	96.19	2.70	98.06	1.91	0.4933
1mg	1.76	1.63	1.69	70.93	65.40	3.63	67.96	32.04	* 0.0502
10mg	0.35	0.32	0.33	14.01	12.73	0.91	13.37	86.63	*** 0.0014

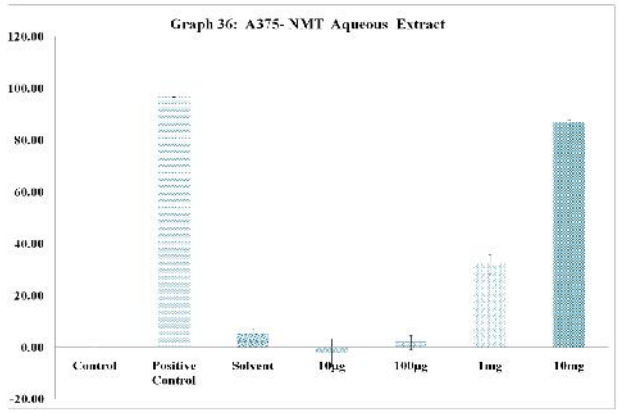

Table 82: HCT 116 (p53 $^{+/+}$) WMT Extract (14/8/2015; 2:50pm)

	Aqueous [x] (nm)		Average [y]	Xa	Xb	STDEV [Xa: Xb]	Cell Survival (100%), [z]	Cell Death [100-z]	t- Test
Control	0.999	1.373	1.14	76.67	130.33	38.76	100.00	0.00	NA
Positive Control	1.001	1.196	1.10	87.73	108.88	14.95	96.28	3.72	0.8904
Solvent	0.971	1.304	1.14	85.10	114.64	20.85	98.19	0.31	NA
10µg	1.562	1.265	1.42	136.90	89.65	33.11	121.05	24.06	0.8133
100µg	1.843	1.091	1.47	161.52	74.37	61.63	128.67	-28.67	0.8257
1mg	1.658	1.182	1.51	153.09	78.28	58.55	132.34	-32.34	0.8035
10mg	0.191	0.743	0.47	16.74	139.10	100.56	40.63	59.07	0.8513

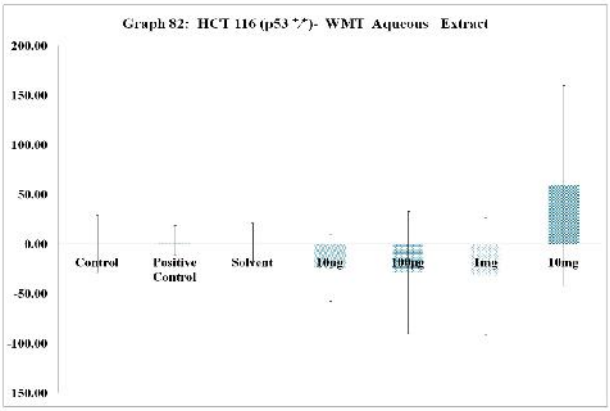

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Table 86: HCT 116 (p53 +/+)- NMT Extract (14/8/2015; 1:50pm)

	Aqueous [x] (nm)		Average [y]	Xa	Xb	STDEV [Xa: Xb]	Cell Survival (100%), [z]	Cell Death [100 z]	t- Test
Control	0.909	1.373	1.14	66.21	130.33	38.37	100.00	0.00	NA
Positive Control	1.001	1.196	1.10	72.61	104.82	22.57	96.28	3.72	0.7596
Solvent	0.971	1.301	1.14	70.73	114.29	30.80	99.69	0.31	NA
10µg	1.146	1.534	1.34	83.47	133.57	35.43	117.00	-17.00	0.0836
100µg	1.253	1.656	1.45	91.26	145.11	38.10	127.48	-27.48	*** 0.0032
1mg	1.868	1.595	1.73	136.09	139.61	2.52	151.67	-51.67	0.5282
10mg	0.465	0.447	0.46	33.87	39.18	3.75	39.96	60.04	0.2386

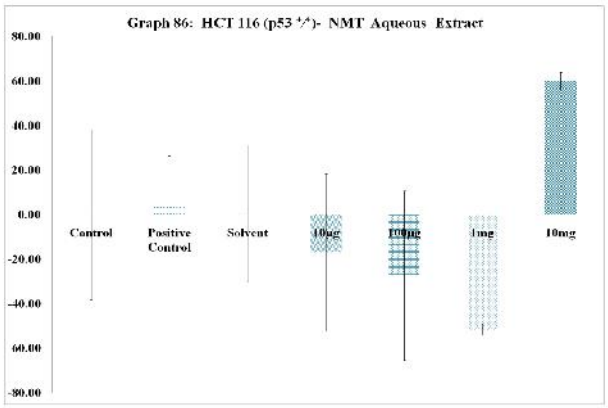

Table 94: HeLa- WMT Extract (15/8/2015; 3:00pm)

	Aqueous [x] (nm)		Average [y]	Xa	Xb	STDEV [Xa: Xb]	Cell Survival (100%), [z]	Cell Death [100 z]	t- Test
Control	0.429	0.373	0.40	106.98	93.02	9.87	100.00	0.00	NA
Positive Control	0.013	0.012	0.01	3.24	2.99	0.18	3.12	96.88	0.0150
Solvent	0.314	0.253	0.28	78.30	63.09	10.75	70.70	29.30	NA
10µg	0.338	0.243	0.29	84.29	60.60	16.75	72.44	27.56	0.1112
100µg	0.118	0.16	0.11	29.43	39.90	7.41	31.66	68.34	0.1177
1mg	0.314	0.469	0.40	78.30	121.95	30.83	120.12	-20.12	0.9972
10mg	0.194	0.217	0.21	48.38	54.11	4.86	51.25	48.75	0.1269

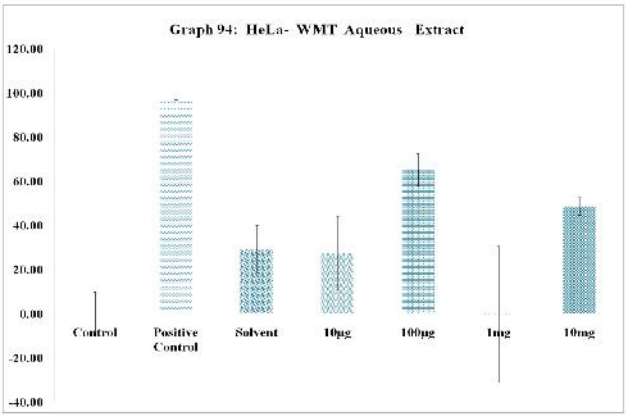

Table 98: HeLa- NMT Extract (15/8/2015; 3:00pm)

	Aqueous [x] (nm)		Average [y]	Xa	Xb	STDEV [Xa: Xb]	Cell Survival (100%), [z]	Cell Death [100 z]	t- Test
Control	0.429	0.373	0.40	106.98	93.02	9.87	100.00	0.00	NA
Positive Control	0.013	0.013	0.01	3.24	2.99	0.18	3.12	96.88	0.0450
Solvent	0.314	0.253	0.28	78.30	63.09	10.75	70.70	29.30	NA
10µg	0.38	0.419	0.40	94.76	104.49	6.85	99.63	0.37	0.9799
100µg	0.599	0.606	0.60	149.38	151.12	1.23	150.25	50.25	0.0987
1mg	0.448	0.434	0.44	111.72	108.23	2.47	109.98	-9.98	0.3078
10mg	0.116	0.112	0.11	28.93	27.93	0.71	28.43	71.57	* 0.0575

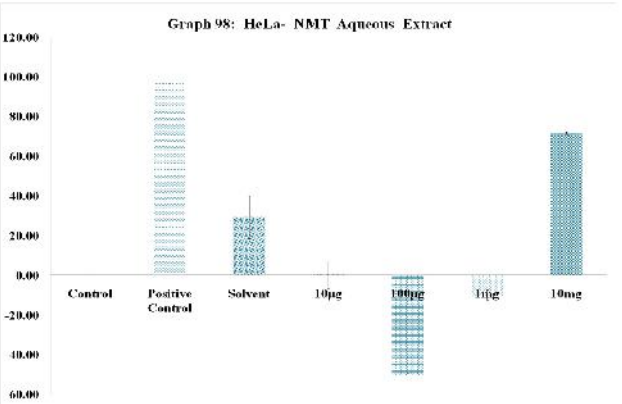

Table 114: HCT 116 (p53 +/+) WM1 Cold Extract (15/8/2015; 3:30pm)

	Aqueous [x] (nm)		Average [y]	Xa	Xb	STDEV [Xa: Xb]	Cell Survival (100%), [z]	Cell Death [100 z]	t- Test
Control	0.795	0.822	0.81	98.20	101.80	2.54	100.00	0.00	NA
Positive Control	0.024	0.031	0.03	2.97	3.60	0.36	3.29	97.21	0.0130
Solvent	0.791	0.824	0.81	97.69	102.01	3.09	97.52	2.48	NA
10µg	0.931	1.399	1.16	114.96	173.25	41.86	143.65	-43.65	0.3610
100µg	1.155	2.239	1.69	143.03	276.91	94.09	209.54	-109.54	0.3597
1mg	0.637	0.774	0.71	78.89	95.85	12.00	87.37	12.63	0.3100
10mg	0.889	0.149	0.52	110.09	18.45	64.80	64.27	35.73	0.5902

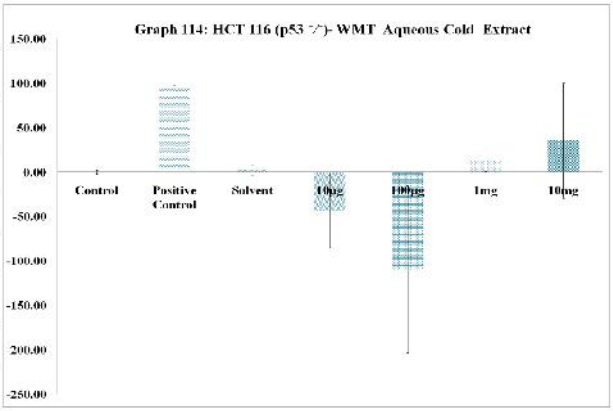

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Table 116: HCT 116 (p53 -/-)- NMT Cold Extract (15/8/2015; 3:30pm)

	Aqueous [x] (nm)		Average [y]	Xa	Xb	STDEV [Xa: Xb]	Cell Survival (100%), [z]	Cell Death [100-z]	t- Test
Control	0.793	0.822	0.81	98.20	101.80	2.54	100.00	0.00	NA
Positive Control	0.021	0.021	0.02	2.97	2.60	0.26	2.79	97.21	0.0130
Solvent	0.791	0.824	0.79	97.99	103.01	6.39	97.52	2.48	NA
10µg	1.117	0.921	1.02	138.33	111.43	26.90	126.38	-26.38	0.3058
100µg	1.069	1.637	1.36	132.38	205.20	51.49	168.79	-68.79	0.2958
1mg	1.799	2.12	1.66	148.48	262.54	80.65	205.51	-105.51	0.3070
10mg	0.166	1.122	0.64	20.56	138.95	83.91	79.75	20.25	0.7871

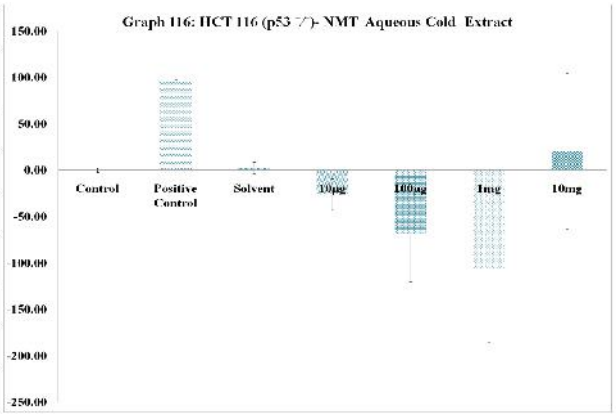

Table 138: Hep G2- WMT Cold Extract (24 8 2015; 9:40am)

	Aqueous [x] (nm)		Average [y]	Xa	Xb	STDEV [Xa: Xb]	Cell Survival (100%), [z]	Cell Death [100-z]	t Test
Control	2.584	2.249	2.42	106.93	93.07	9.80	100.00	0.00	NA
Positive Control	0.02	0.02	0.02	0.79	0.95	0.12	0.87	99.13	0.0150
Solvent	1.861	2.111	1.99	77.01	87.36	7.33	82.18	17.82	NA
10µg	1.998	2.22	2.11	82.68	91.87	6.50	87.27	12.73	0.4685
100µg	2.728	2.574	2.65	112.89	106.52	4.31	109.70	9.70	0.2345
1mg	2.587	1.518	2.05	107.06	62.82	31.28	84.94	15.06	0.5026
10mg	0.802	1.405	1.10	33.19	58.14	17.61	45.67	54.33	0.2184

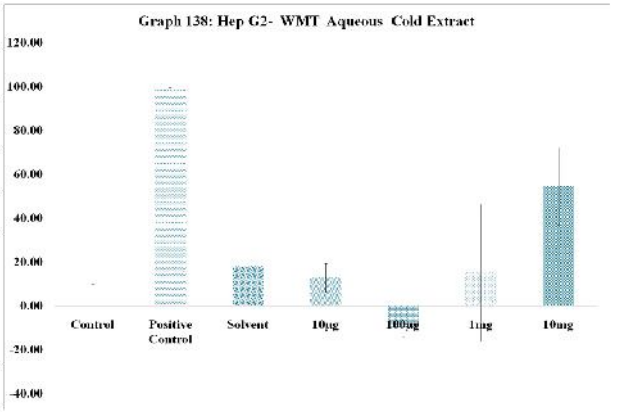

Table 140: Hep G2- NM1 Cold Extract (24 8 2015; 9:40am)

	Aqueous [x] (nm)		Average [y]	Xa	Xb	STDEV [Xa: Xb]	Cell Survival (100%), [z]	Cell Death [100 z]	t- Test
Control	2.584	2.249	2.42	106.93	93.07	9.80	100.00	0.00	NA
Positive Control	0.02	0.02	0.02	0.79	0.95	0.12	0.87	99.13	0.0450
Solvent	1.861	2.111	1.99	77.01	87.36	7.33	82.18	17.82	NA
10µg	2.17	2.038	2.10	89.80	121.58	22.47	105.69	-5.69	0.8441
100µg	2.543	2.186	2.36	105.19	90.46	10.42	97.83	2.17	0.1257
1mg	2.534	2.475	2.52	106.53	102.42	2.90	104.47	-4.47	0.5282
10mg	2.375	2.348	2.36	98.38	97.37	0.64	97.83	2.17	0.7939

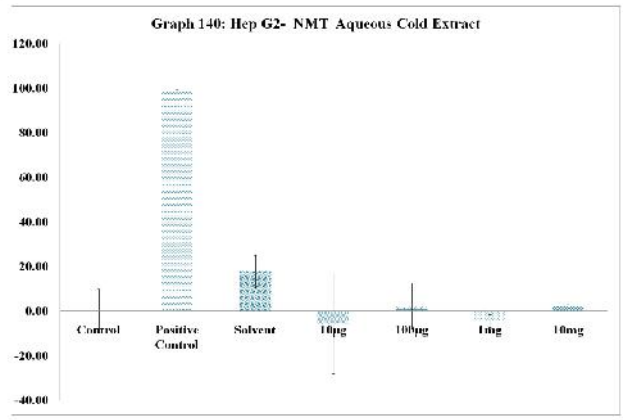

Table 142: U937- WMT Extract (24 8 2015; 10:20am)

	Aqueous [x] (nm)		Average [y]	Xa	Xb	STDEV [Xa: Xb]	Cell Survival (100%), [z]	Cell Death [100 z]	t- Test
Control	1.357	1.296	1.33	103.59	96.61	4.80	100.00	0.00	NA
Positive Control	0.02	0.03	0.03	1.71	2.01	0.21	1.86	98.14	0.0230
Solvent	1.23	1.078	1.15	91.89	80.36	8.01	86.02	13.98	NA
10µg	0.502	0.582	0.54	37.62	43.57	4.12	40.59	59.41	0.1388
100µg	0.411	0.385	0.37	30.64	24.97	4.01	27.80	72.20	*** 0.0049
1mg	0.878	0.606	0.71	65.45	45.17	14.34	55.31	44.69	0.0954
10mg	0.658	0.541	0.60	49.05	40.33	6.17	44.69	55.31	** 0.0112

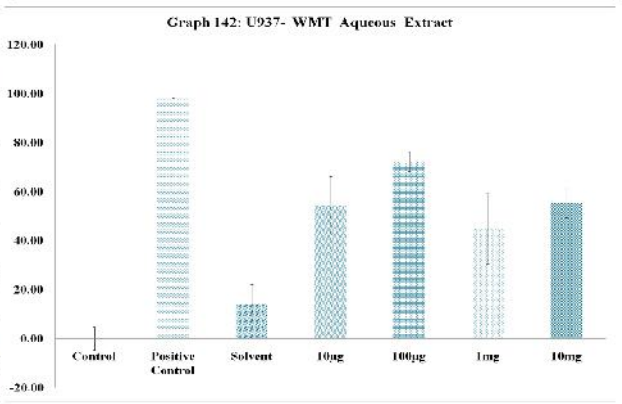

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Table 146: U937- NMT Extract (24 8 2015; 10:20am)

	Aqueous [x] (mm)		Average [X]	Xa	Xb	STDEV [Xa: Xb]	Cell Survival (100%), [z]	Cell Death [100 z]	t Test
Control	1.387	1.296	1.34	103.39	96.61	4.80	100.00	0.00	NA
Positive Control	0.02	0.03	0.03	1.91	2.01	0.21	1.86	98.14	0.0250
Solvent	1.23	1.078	1.15	91.69	80.36	8.01	86.02	13.98	NA
10µg	1.109	1.056	1.08	82.67	78.72	2.79	80.69	19.31	0.0166 **
100µg	1.573	1.674	1.62	117.26	124.79	5.32	121.02	-21.03	0.3089
1mg	0.849	0.859	0.85	63.29	64.03	0.53	63.66	36.34	0.0657
10mg	0.931	1.062	1.00	69.40	79.17	6.91	74.28	25.72	0.1982

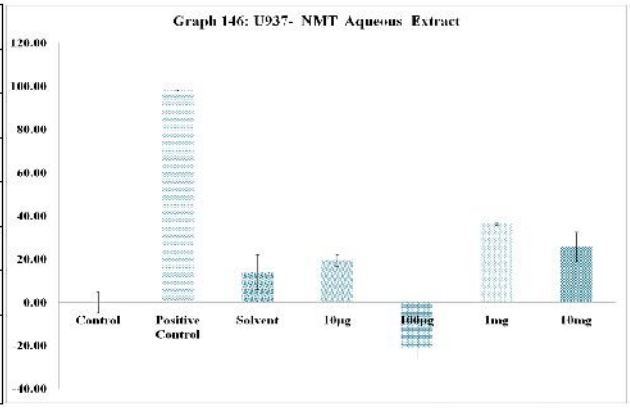


Figure 1 HPLC- WMT Aqueous Extract (270-380 nm)

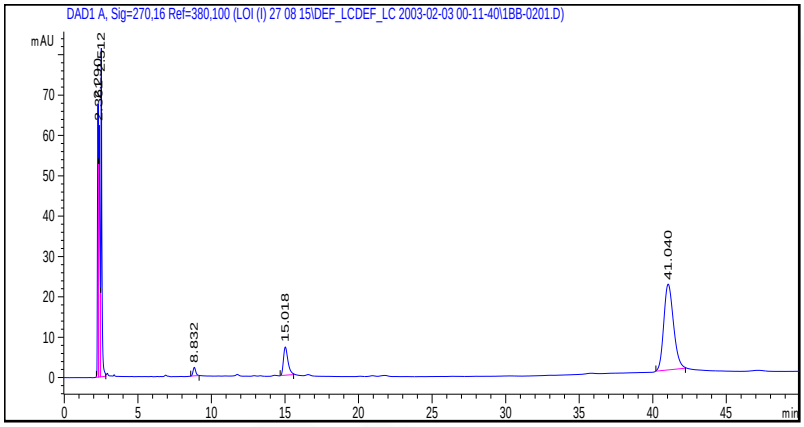
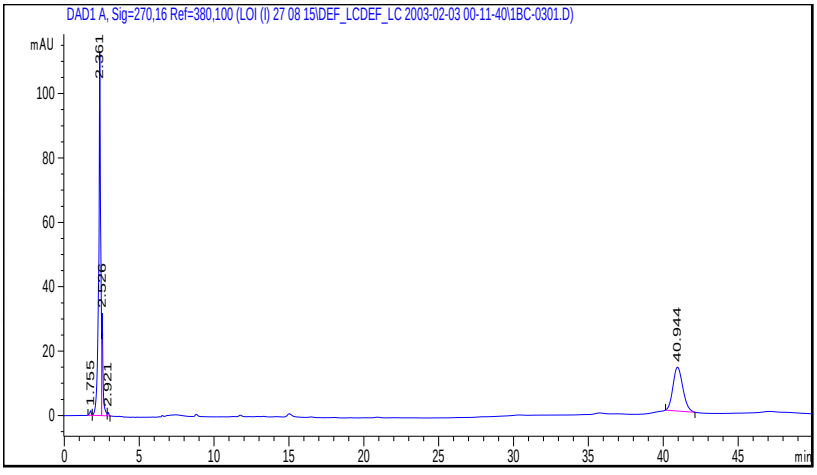


Figure 2 HPLC- NMT Aqueous Extract (270- 380 nm)



4. DISCUSSION

Nature is the world's best chemist: Many naturally occurring compounds have very complicated structures that present great challenges to chemists wishing to determine their structures or replicate them. The plant derived herbal compounds have a long history of clinical use, better patient tolerance and acceptance. Their high ligand binding affinities to the target introduce the prospect of their use in chemo preventive applications; in addition they are freely available natural compounds that can be safely

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used to prevent various ailments. Plants became the basis of traditional medicine system throughout the world for thousands of years and continue to provide mankind with new remedies. Plants used in folklore medicine continue to be an important source of discovery and development of novel therapeutic agents (Paul J *et.al*, 2013). Here, we present a research study on effects of crude aqueous extracts of walking mango tree and normal mango tree on the growth and invasion of cancer cells. Our results showed that the aqueous extracts are showing a better apoptosis than the other solvents used (Acetone, Chloroform, Methanol and Petroleum Ether).

The graphs and tables mentioned in the results determine the MTT assay/ cytotoxicity of the extracts upon the cell lines. With the data, we also calculated the standard deviation and also the student t-test to determine the significance value. The stars in the t-test resemble the significance. More starts, better the results. Hence we have included only the necessary MTT tables & graphs. Due to more number of tables & graphs in MTT, we have shown only aqueous extract results since the toxicity is highly less, whereas the rest of the solvents themselves have a higher toxicity level upon the cell lines.

The HPLC analysis for WMT & NMT are shown in Figure 1 & 2. We have seen that in WMT we have 3 strong peaks at the 2.2 – 2.6 min, whereas only 2 partial peaks in NMT data. Though we have a common peak at 40th min in both the spectrums, but we have an unknown peaks at 10th & 15th min in WMT, which is absent in NMT. This signifies that the WMT has different molecules/compounds than the NMT. Now these molecules may be responsible for apoptosis or for walking of the tree.

MALDI-TOF was performed to detect the molecular weight/mass of the molecule, where HCCA method was used to detect. We found out that our molecules had m/z value below 10,000 Da, which signifies that our desired molecule is not a protein; since proteins contain higher m/z values. Hence our molecule is below 10,000 Da (Daltons) and is not a protein. We do not consider the m/z values below 1000 Da, since these spectra belongs to HCCA salt. So the spectra between 1000 Da – 2400 Da shows our desired molecules.

With the above data; we can have proved that aqueous extract of WMT have better apoptosis and also we have identified different compounds that are absent in the NMT, but present in WMT. MALDI- TOF exhibited that our molecules are not proteins and have m/z value below 10,000 Da.

5. CONCLUSION

We conclude our report by stating the aqueous extracts of WMT have shown a better apoptosis upon the cancer cell lines. We have also identified a wide new range of molecules/ compounds that are absent in NMT, but present in WMT by HPLC- UV/DAD analysis. The relevant compounds were collected and further analyzed by MALDI-TOF, which resembled that our desired molecules are not proteins and have m/z values less than 10,000Da. Due to lack of helium gas, we were unable to perform GC-MS at the given schedule, but it will be performed once the gas in available, which will identify all the molecules along with the structures in these both the aqueous extracts of WMT & NMT. Hence we conclude by saying that WMT aqueous extract has a better apoptosis and has very negligible toxicity value and a greater significance (t-test).

SUMMARY OF RESEARCH

From the present screening, it could be concluded that the WMTBE (Aqueous) is more potent than NMTBE (Aqueous). Further, the detailed phytochemical research is required to identify the active principal responsible for activities and testing wider range of organisms would be encouraged.

FUTURE ISSUES

More trials need to perform upon NF-κB signaling. Also keeping in mind that we only have one tree available, resources has to utilized very carefully, without causing more harm to the tree.

DISCLOSURE STATEMENT

This work was supported through INSA-NASI-INSc fellowship, under the supervision of Dr.SK Manna, CDFD, HYD, IND.

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