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IN VITRO CYTOTOXICITY - TEST REPORT

- Sample A: 1,8 Cineol
- Sample B: Recovereez (Cardamom extract)
- Sample C: Memoreez (Cardamom extract and Virgin coconut oil)
- CYTOTOXICITY by MTT assay in L929 cells showed sufficient cell viability confirming the non-cytotoxic nature of 1,8-Cineol, Recovereez and Memoreez capsules.
- When the L929 cells were exposed with 1,8-Cineol, Recovereez and Memoreez capsule extracts, more than 60% cell viability was observed indicating that the tested compounds are non-cytotoxic in nature.
- All the samples in the tested concentrations as given in the detailed report which is attached below showed dose dependent anti-inflammatory action in the selected assays.

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NB: The experiments are carried out in *invitro* cultured cells and Intended only for research purposes not for diagnostic uses. All experiments are carried out only in triplicates

INVITRO CYTOTOXIC EFFECT DETERMINATION BY MTT ASSAY

L929 (Fibroblast) cells was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained in Dulbecco's modified Eagles medium, DMEM (Sigma aldrich, USA).

The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany) and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Cells seeding in 96 well plate:

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension (5x10^3 cells/well) wasseeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

Preparation of compound stock:

1mg of sample was weighed and dissolved in 1mL DMEM using a cyclomixer. The sample solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility.

Cytotoxicity Evaluation:

After 24 hours the growth medium was removed, freshly prepared each compounds in DMEM were five times serially diluted by two fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of DMEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. Non treated control cells were also maintained.

Cytotoxicity Assay by Direct Microscopic observation:

Entire plate was observed after 24 hours of treatment in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Cytotoxicity Assay by MTT Method:

Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization.

After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (Dimethyl sulphoxide: DMSO, Sigma Aldrich, USA) was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm (Laura B. Talarico et al., 2004).

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples} \times 100}{\text{Mean OD of control group}}$$

Sample Concentration (µg/mL)	OD value I	OD value II	OD value III	Average OD	Percentage Viability
Control	0.7129	0.7183	0.7114	0.7142	100.00
Sample code: A					
6.25	0.7033	0.7084	0.7027	0.7048	98.68
12.5	0.6505	0.6536	0.6552	0.6531	91.44
25	0.6276	0.6214	0.6235	0.6242	87.39
50	0.5891	0.5895	0.5863	0.5883	82.37
100	0.5688	0.5639	0.5614	0.5647	79.07
200	0.4805	0.4869	0.4834	0.4836	67.71
Sample code: B					

6.25	0.6952	0.7083	0.7062	0.7032	98.46
12.5	0.6735	0.6728	0.6713	0.6725	94.17
25	0.6587	0.6522	0.6519	0.6543	91.61
50	0.592	0.5921	0.5934	0.5925	82.96
100	0.5401	0.5437	0.5416	0.5418	75.86
200	0.487	0.4865	0.4693	0.4809	67.34
Sample code: C					
6.25	0.7004	0.7039	0.7084	0.7042	98.60
12.5	0.6656	0.6652	0.6611	0.6640	92.97
25	0.6413	0.6496	0.6435	0.6448	90.28
50	0.5834	0.5841	0.5823	0.5833	81.67
100	0.5632	0.5617	0.5672	0.5640	78.97
200	0.4954	0.4972	0.4961	0.4962	69.48

	Percentage viability 1	Percentage viability 2	Percentage viability 3	Average	Std dev	std error
SAMPLE A						
Control	100	100	100	100	0	0
6.25	98.65339	98.62175	98.77706	98.68406	0.082075	0.027358
12.5	91.24702	90.99262	92.10008	91.44658	0.580074	0.193358
25	88.03479	86.50981	87.64408	87.39623	0.792123	0.264041
50	82.63431	82.06877	82.41496	82.37268	0.285129	0.095043
100	79.78679	78.5048	78.91482	79.0688	0.654717	0.218239
200	67.40076	67.78505	67.95052	67.71211	0.282046	0.094015
SAMPLE B						
Control	100	100	100	100	0	0
6.25	97.51718	98.60782	99.26905	98.46468	0.88466	0.294887
12.5	94.47328	93.6656	94.36323	94.16737	0.438015	0.146005
25	92.39725	90.79772	91.63621	91.61039	0.800079	0.266693

50	83.0411	82.43074	83.41299	82.96161	0.495926	0.165309
100	75.76098	75.69261	76.13157	75.86172	0.236187	0.078729
200	68.31253	67.72936	65.96851	67.3368	1.220319	0.406773
SAMPLE C						
Control	100	100	100	100	0	0
6.25	98.2466	97.99527	99.5783	98.60672	0.850742	0.283581
12.5	93.36513	92.60755	92.92943	92.96737	0.380213	0.126738
25	89.95652	90.43575	90.45544	90.28257	0.282542	0.094181
50	81.83476	81.317	81.85268	81.66815	0.304236	0.101412
100	79.00126	78.19852	79.73011	78.97663	0.76609	0.255363
200	69.49081	69.21899	69.73573	69.48184	0.258488	0.086163

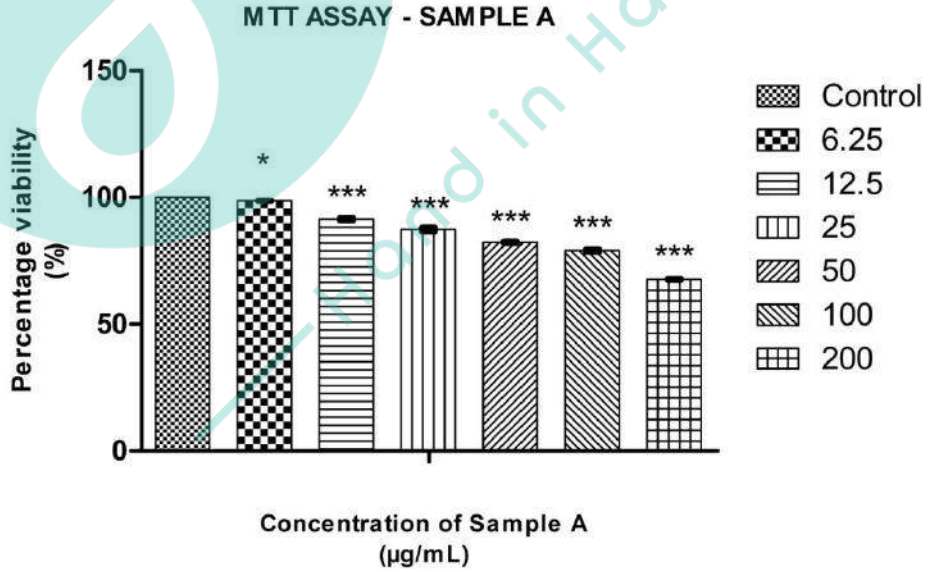
Table Analyzed	**Sample A**				
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	7				
F	1752				
R squared	0.9987				
ANOVA Table	SS	df	MS		
Treatment (between columns)	2343	6	390.5		
Residual (within columns)	3.120	14	0.2229		
Total	2346	20			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs 6.25	1.316	3.414	Yes	*	0.1932 to 2.439
Control vs 12.5	8.553	22.19	Yes	***	7.431 to 9.676
Control vs 25	12.60	32.70	Yes	***	11.48 to 13.73

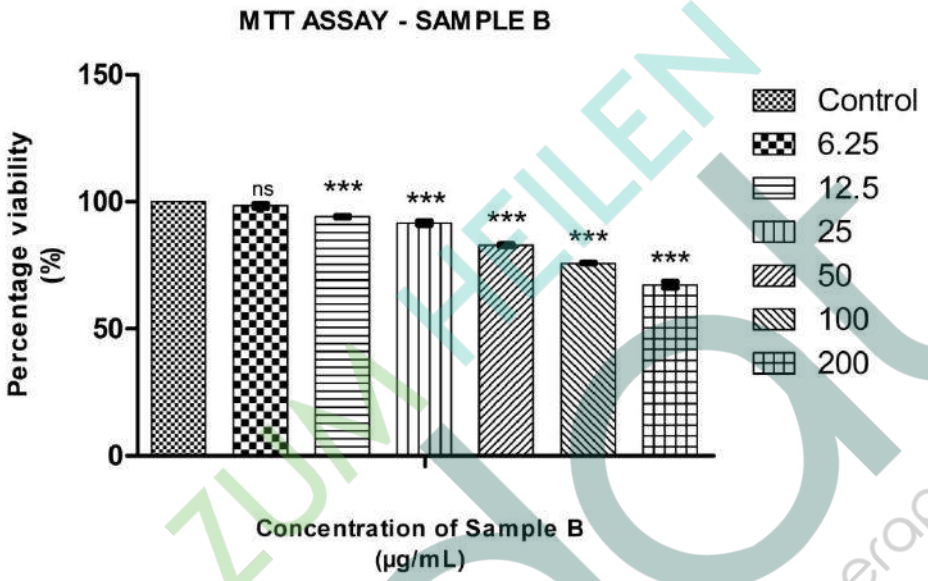
Control vs 50	17.63	45.73	Yes	***	16.50 to 18.75
Control vs 100	20.93	54.30	Yes	***	19.81 to 22.05
Control vs 200	32.29	83.76	Yes	***	31.17 to 33.41

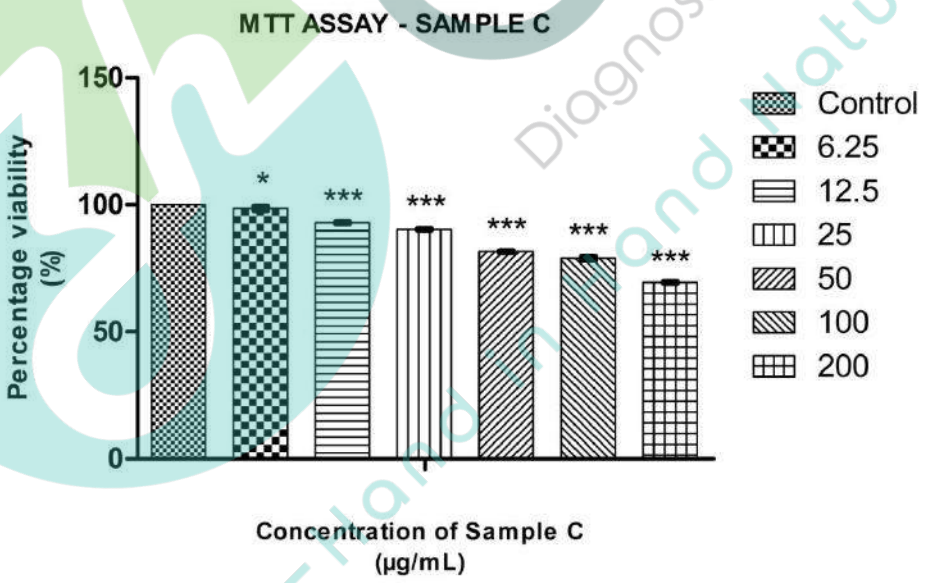
Table Analyzed	**Sample B**				
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	7				
F	924.8				
R squared	0.9975				
ANOVA Table	SS	df	MS		
Treatment (between columns)	2699	6	449.9		
Residual (within columns)	6.811	14	0.4865		
Total	2706	20			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs 6.25	1.535	2.696	No	ns	-0.1234 to 3.194
Control vs 12.5	5.833	10.24	Yes	***	4.174 to 7.491
Control vs 25	8.390	14.73	Yes	***	6.731 to 10.05
Control vs 50	17.04	29.92	Yes	***	15.38 to 18.70
Control vs 100	24.14	42.38	Yes	***	22.48 to 25.80
Control vs 200	32.66	57.35	Yes	***	31.00 to 34.32

Table Analyzed	**Sample C**				
One-way analysis of variance					
P value	< 0.0001				

P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	7				
F	1546				
R squared	0.9985				
ANOVA Table	SS	df	MS		
Treatment (between columns)	2246	6	374.3		
Residual (within columns)	3.389	14	0.2421		
Total	2249	20			
Dunnett's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
Control vs 6.25	1.393	3.468	Yes	*	0.2233 to 2.563
Control vs 12.5	7.033	17.51	Yes	***	5.863 to 8.203
Control vs 25	9.717	24.19	Yes	***	8.547 to 10.89
Control vs 50	18.33	45.63	Yes	***	17.16 to 19.50
Control vs 100	21.02	52.33	Yes	***	19.85 to 22.19
Control vs 200	30.52	75.97	Yes	***	29.35 to 31.69







LC50 Value : Sample (A)-318.87 µg/mL (Calculated using ED50 PLUS V1.0 Software)

Sample (B)- 294.507µg/mL (Calculated using ED50 PLUS V1.0 Software)

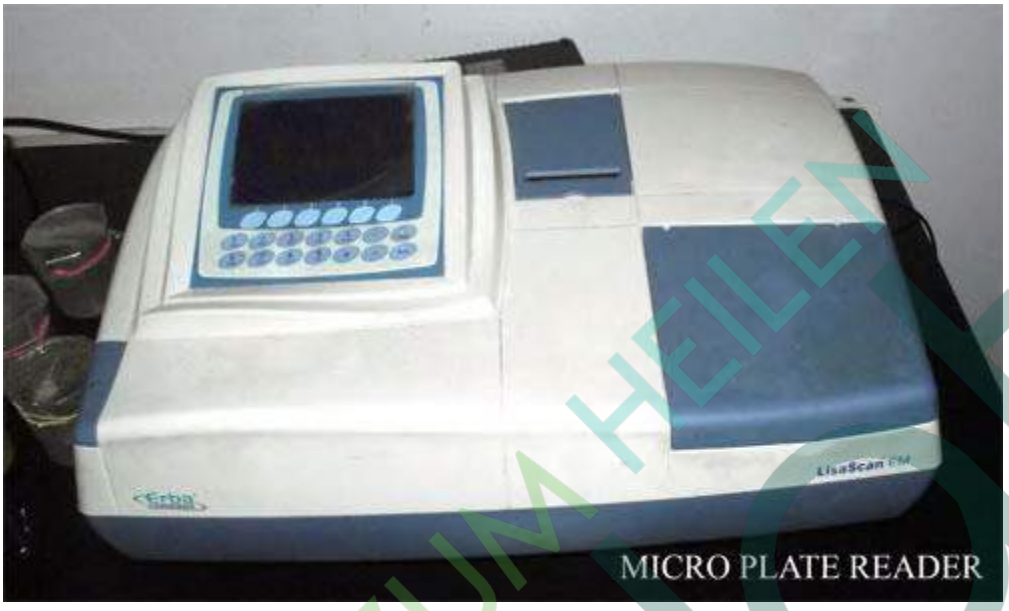
Sample (C)-328.999 µg/mL (Calculated using ED50 PLUS V1.0 Software)

APPENDIX

Instruments and reagents used:

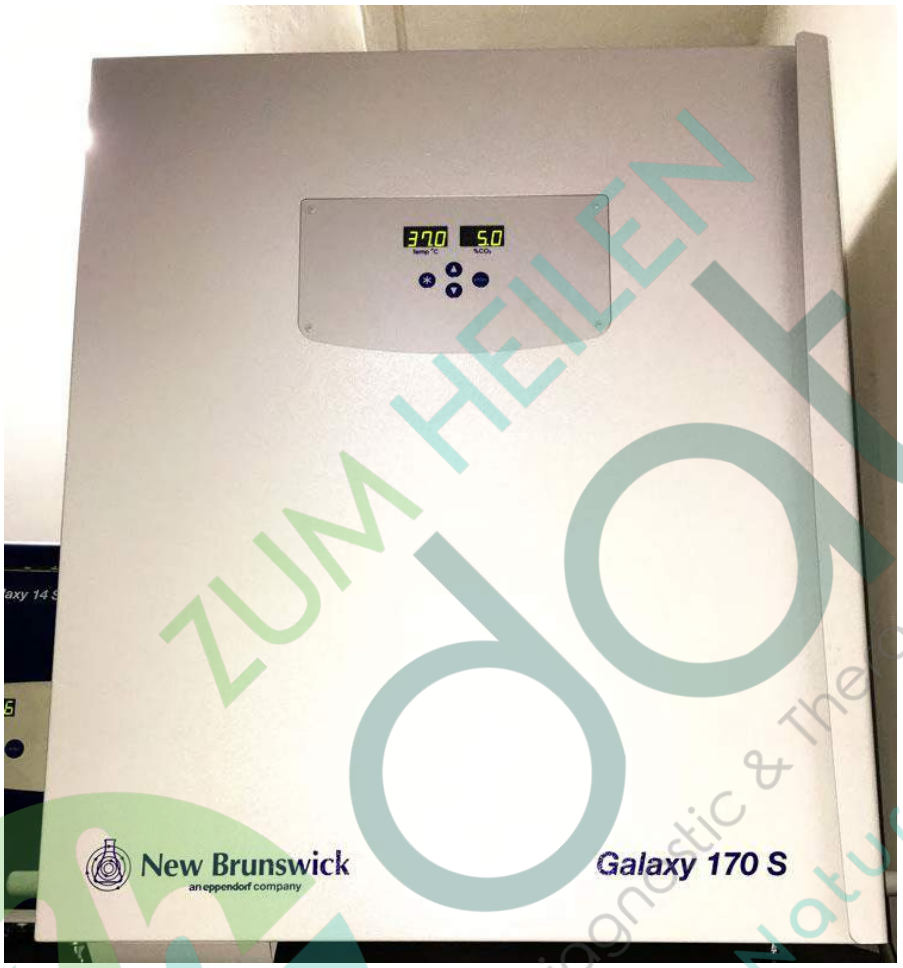
DMEM media	-Sigma Aldrich, USA D5648
Fetal Bovine Serum	-Gibco, US orgin-
0.25% Trypsin	- Invitrogen, USA 25200-056
Micropipettes	- F1 Thermoscientific USA
CO_2 Incubator	- Eppendorf, GERMANY
Phase Contrast Microscope	- Olympus, JAPAN with Optika Pro 5 Camera
MTT	- Sigma Aldrich M5655
ELISA Reader	- ERBA, GERMANY
Culture Plates and Flasks	- NUNC, Thermoscientific USA
Image Magnification	- 10X

Microplate Reader



Phase Contrast Microscope

CO_2 Incubator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5.0
New Brunswick
an eppendorf company
Galaxy 170 S