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Anti-Neoplastic and Cytotoxicity Potency Measuring of Five *Medicago sativa* L. (*Alfalfa*) Leaf Extracts Towards Melanoma (UACC62), Breast (MCF7), Prostate (PC3), and Colon (HCT116) Cancer Cells

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ABSTRACT

The National Cancer Registry of the National Department of Health of South Africa confirms that in females, the highest incidence of cancers diagnosed in the year 2019 was breast (10 172 cases), cervical (6 945 cases) and colorectal (1 952 cases) cancers, while in males the most diagnosed cancers were prostate, lung, and colorectal neoplasms. The risk factors that promote cancer development, and metastasis or spreading includes excessive alcohol and narcotic usage, carcinogenic infections (oncoviruses) and genetical predispositions. The global incidence of cancer is much likely to increase because of the increased exposure to risk factors (processed fast foods, industrial toxins in air and water) that promote oxidative stress, low grade chronic inflammation, diabetes mellitus, hyperglycemia, and insulin resistance. Because the ancient healer or doctor relied on plants and other natural resources for therapeutic compounds, we wanted to measure the anti-cancer actions induced by our *Alfac-facah* leaf extracts towards four major cancer cells. The growth inhibitory and cytotoxicity activity that was induced by our extracts was measured using the reliable Sulforhodamine B Assay as per the collaborative research program between the CSIR's Biosciences Pharmacology Group and the NCI. Our *Alfac-facah* leaf test material was extracted using five solvents: Ethanol, Methanol, Diethyl-ether, Acetone, and Water. Medicinal Plants persist to play a key role in medicine, whereby they not only help with treating or preventing diseases, but they also contribute to the general wellbeing of the patient. For this reason, natural resources remain a pivotal ingredient of novel drug development compounds.

Key words: NCI (National Cancer Institute, U.S Department of Health and Human Services), CSIR (The Council for Scientific and Industrial Research, South Africa), *Medicago sativa L.* (Alfalfa), Sulforhodamine B Assay (SRB), ECACC (European Collection of Authenticated Cell Cultures, UK), Cancer (malignancy, neoplasm, carcinoma, tumour, sarcoma, leukemia, lymphoma), AICR (American Institute for Cancer Research), International Agency for Research on Cancer (IARC), THC (*delta-9 tetrahydrocannabinol*). Rick Simpson oil (RSO).

INTRODUCTION

The American antique collector and dealer Edwin Smith, in 1862 procured the ancient medical scroll (the manual for military surgery also known as "*the secret book of the Physician*") from Egypt, the scroll contained earlier cases of injuries, fractures, wounds, dislocations, and tumours and means to cure/treat them. The authorship of this text is debated but it has been assumed to be written by Imhotep, who was the ancient Egyptian architect that was later (2000 years after his death) venerated as the God of medicine and healing. The ancient medical treatise *Edwin Smith Papyrus* indicates how far back in time humans have been facing the burden of cancer and other medical conditions. The fossilised bone tumours observed on ancient Egyptian mummies also confirms that cancer is an old problem that has now become the second leading cause of death according to the WHO.[1]

According to the World Health Organization (WHO) cancer is among the leading causes of death in the world, accounting for 8.2 million deaths in 2012.[2] International Agency for Research on Cancer (IARC) estimates that more than 60% of the world's new annual cases occur in Africa, Asia, Central and South America.[3] These regions account for approximately 70% of the world's cancer deaths. Even though 30% of cancers can be prevented according to WHO, the IARC estimates that there were 32.6 million people living with cancer in 2012, and the number of new cancer cases is expected to rise from 14 million in 2012 to 22 million cases over the next two decades.[3] The current cancer treatment's efficacy is threatened by physical and emotional side effects experienced by hundreds of cancer patients worldwide. Cancer treatment side effects include chemo brain (memory lapse), peripheral neuropathy, pain, fatigue, lymphoedema, infections, infertility and reduced sex drive, nausea, vomiting and many more. This rationalizes the basis for need for rapid development of novel, non-cytotoxic and non-genotoxic anti-cancer agents. Carcinogenesis is documented as a multistep process in which distinct molecular and cellular alterations occur. From the studies of experimentally induced carcinogenesis in rodents, tumour development is considered to consist of several separate, but closely linked, stages: tumour initiation, promotion, and progression.[4-6] Blocking agents inhibit carcinogens

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from reaching the target sites, from undergoing metabolic activation or from subsequently interacting with crucial cellular- macromolecules (for example, DNA, RNA, and proteins). While Suppressing agents on the other hand, inhibit the malignant transformation of initiated cells.[7,8] Chemo-preventive phytochemicals can block or reverse the premalignant stage (initiation and promotion) of the multistep carcinogenesis. They can also halt or at least retard the development and progression of precancerous cells into malignant ones. But plants can also deal with factors that promote oxidative stress, inflammation, and hyperglycemia thereby making conditions unconducive for malignant cells to grow and spread.[9-12]

Numerous intracellular signal-transduction pathways converge with the activation of the transcription factors NF-κB and AP1, which act independently or coordinately to regulate target-gene expression.[7,8] Atypical activation of NF-κB has been associated with protection against apoptosis and stimulation of proliferation in malignant cells,[7,8] and overexpression of NF-κB is causally linked to the phenotypic changes that are characteristic of neoplastic transformations.[8] Many chemo preventive phytochemicals that are derived from the diet have been shown to suppress constitutive NF-κB activation in malignant cells or NF-κB activation induced by the external tumour promoter phorbol 12-myristate 13-acetate (PMA) or tumour-necrosis factor-α (TNF-α).[9,13] The conventional approach to cancer treatment is based on drugs that retard the proliferation of abnormal cells (cytostatic) or that can kill the abnormal cells (cytotoxic). The mechanisms of actions employed by these drugs includes the inhibition of cell division or by the killing of neoplastic cells by the induction of apoptosis (suicide by cells). Current anti-cancer drugs like chemotherapy primarily target cell division components, consequently their toxicity is not restricted to cancer cells but healthy cells as well such hair cells, hence alopecia. Many conventional drugs also induce genetic damages that can turn out to be carcinogenic at times. A segment of the drug development research community is focusing on identifying novel chemotherapeutic agents/compounds from plants and other natural resources that do not only induce the untoward side effects of conventional therapeutic chemicals, but that are also cheap and easily accessible or that can be grown at home.[10,13-16]

The use of plants and their extracts in treatment of diseases dates back before 460-370 BC when Hippocrates practiced the art of healing by the use of plant-based drugs. The medicinal value of plants lies in the contained phytochemical (secondary metabolites) substances that can induce a definite physiological action in the human body. Natural products from natural resources (plants, etc) continue to be used in pharmaceutical preparations either as crude extracts, fractions, pure compounds, or analogous compounds.[17] Traditional medical system of time past still possesses great therapeutic value and also many medicinal plants have been used thus far to synthesize novel therapeutic compounds based on indigenous pharmacopoeias. The most bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds.[18] Many clinical trials on the use of nutritional supplements and modified diets to prevent cancer are also continuing. It is plausible that in the future people might only need to take specially synthesized pills that contain substances derived from edible plants to prevent cancer or delay its onset. More than 250 population-based studies, including case–control and cohort studies, indicate that people who eat about five servings of fruit and vegetables a day have approximately half the risk of developing cancer — particularly cancers of the digestive and respiratory tracts.[4,5,19]

The therapeutic potential of merging the traditional/indigenous/alternative/homeopathic herbal medicine and the modern conventional clinical medicine is evident in Japan where cancer patients now are also prescribed medicines from the *Kampo* traditional pharmacopeia that is said to originate from ancient China. The Kampo medical care system is easily accessible through the ancient treatise titled *Shang Han Lun* by Zhang Zhong Jing, and out of that ancient scroll, one potent anticancer medicine is listed therein as *Juzentaihoto* (JTT) or the so-called *Shi-Quan-Da-Bu-Tang* in Chinese medicine. Shintaro Ishikawa and others, demonstrated the B16 melanoma metastasis inhibition *in-vivo* that was induced by the JTT extract.[20] Ishikawa *et al* demonstrated the activity by injecting (intravenously) C57BL/6 male mice with B16 melanoma cells (2 x 10^5 cells) and feeding them food containing 3.0 % JTT. The mice were then sacrificed after 3 weeks (21 days) following treatment, and tumour cell colonies on the surface of the lung were counted to measure pharmacological potency of JTT. The experimental results demonstrated the therapeutic actions of JTT being to block B16 Melanoma cancer cells: because mice that was not treated with JTT had more than 50 malignant cell colonies, while those that were treated with JTT had less than 20 colonies of metastatic tumour cells.[20] This experiment supports the known fact that many plants can help with preventing cancer development and spreading, but they can also help with the effects of cancer treatment. The therapeutic potency of compounds from plants cannot be denied, evidence has been and is still being published on how medicinal plants continue to provide therapeutic metabolites that are antidiabetic, antimicrobial, hepatoprotective, chemo-preventive, antioxidant, anti-inflammatory, and anti-cancer among many others. Docetaxel (DTX) is a semisynthetic analog of Paclitaxel which is isolated from European yew (*Taxus baccata*) that is well used in clinical chemotherapy.[21] DTX is approved and used in clinical care against breast cancer, ovarian cancer, and non-small lung cancer. DTX was demonstrated to cause cell cycle arrest by interfering with microtubules functions.[21] The aim of this paper was to examine the anti-cancer potential of *Medicago sativa L.* leaf extracts towards clinical cancer cells. We report here the anti-cancer and cytotoxicity activity of five *Medicago sativa* L extracts towards melanoma (UACC62), breast (MCF7), prostate (PC3), and colon (HCT116) cancer cells for the first time.

MATERIALS AND METHODS

The Plant material, the extraction and the preparation for *in-vitro* anti-cancer and cytotoxicity activity measuring

The plant materials were collected from a nursery and medicinal plant farm located few degrees west of North of Pretoria (South Africa) with the Batch Number MH 71(10kg). The plant material was cultivated by Zizameleni Farming based in Mamogalieskraal, Northwest Province of South Africa. The test material was cultivated using regenerative natural farming principles and the fertilizers used were all natural and certified organics. The material was air-dried and stored in a cool dry area away from light and heat. The dry, grassy, and pale green leaves were extracted using five solvents: Ethanol, Methanol, Diethyl-ether, Acetone, and water. Sixty (60) grams of powdered plant material was extracted in 1 litre (1000 mL) of every solvent. The extracts were filtered using the Buchner funnel and Whatman no.1 filter paper. The extracts were frozen at -40 °C and freeze-dried for 48 hours at a yield of 9 g of dried extracts. The dried extracts were stored at -4 °C until analysis.

Materials (Cell lines) and maintenance

The human cancer cell lines UACC62, MCF7 and PC3 were acquired from the NCI as part of the framework collaborative research program between CSIR and NCI. HCT116 and WI-38 cell line (normal Human Fetal Lung Fibroblast) were obtained from ECACC (European Collection of Authenticated Cell Cultures, UK). Cell lines were routinely maintained as a monolayer cell culture at 37°C, 5% CO2, 95% air and 100% relative humidity in RPMI containing 5% fetal bovine serum, 2 mM L-glutamine and 50μg/ml gentamicin. Emetine, Parthenolide, glassware, apparatus and other materials used for our experiments were sourced from reputable supplies that include Merck, United Scientific, Minema, B&M Scientific, and Lasec).

Protocol for the growth inhibitory activity measuring of five *Medicago sativa* L extracts

The SRB Assay was developed by Philip Skehan and others that was chosen for its rapidity, sensitivity, and low cost of materials.[22] For the anti-cancer screening experiment, the cells (3-19 passages) were inoculated in a 96-well microtiter plates at plating densities of 7-10 000 cells/well. The plates were then incubated for 24 h. After the 24 h incubation, the cells were treated with the experimental drugs/extracts which were previously dissolved in DMSO and diluted in medium to produce 5 concentrations. Cells without drug addiction served as control. The blank contains complete medium without cells. Parthenolide was used as a standard. The plates were incubated for 48 h after the addition of the compounds. Viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried, and dyed by SRB. Unbound dye was removed, and protein-bound dye was extracted with 10mM Tri's base for optical density determination at the wavelength 540 nm using a multi-well spectrophotometer. Data analysis was performed using GraphPad Prism software. 50% of cell growth inhibition (IC50) was determined by non-linear regression.

Protocol for the cytotoxicity activity measuring of five *Medicago sativa* L extracts

For screening experiment, the cells (21-50 passages) were inoculated in a 96-well microtiter plates at plating densities of 10 000 cells/well and were incubated for 24 h. After 24 h the cells were treated with the experimental drugs which were previously dissolved in DMSO and diluted in medium to produce 5 concentrations. Cells without drug addiction served as control. The blank contains complete medium without cells. Emetine was used as a standard. The plates were incubated for 48 h after addition of the compounds. Viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried, and dyed by SRB. The unbound dye was removed, and protein-bound dye was extracted with 10mM Tri's base for optical density determination at the wavelength 540 nm using a multi-well spectrophotometer. Data analysis was performed using GraphPad Prism software. 50% of cell growth inhibition (IC50) was determined by non-linear regression.

RESULTS

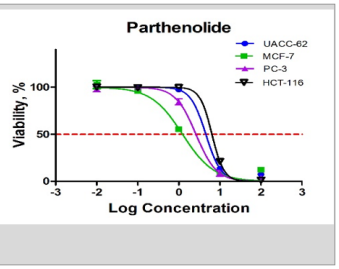
Results are shown in figures 1 to 12.

DISCUSSION AND CONCLUSION

Again, Cancer describes an anthology of diseases of higher multicellular organisms, characterized by mutations in the expression of multiple genes, leading to dysregulation of the normal cellular processes of cell division and cell differentiation.[23] This results in an imbalance of cell replication and cell death that favours growth of a tumour cell population. Cancer cells replicate fast and invade other tissue sites causing significant morbidity and if untreated or diagnosed late, mortality. The cost of living is already high, with consumers struggling to wrestle with the effects of inflation especially now after the COVID-19 pandemic. Despite modern advances in clinical care, the cost of treatments has been reported by the AICR to be costing consumers a staggering US$895 billion per year. The cost of treating cancer is not only from the hospitals/clinics (diagnosis, drugs, treatment, and admission fees) but also there are non-health care costs related to being a cancer patient. It is a well document fact that many cancer drugs causes for example alopecia and many other physio-psychological side effects that include weight loss and pain/fatigue, factors that can promote mental health issues in those who are living with cancer and their families. There are also costs related to the logistics of going to treatment sessions (chemotherapy, radiotherapy and so on), to this cost you can add mental health support, specialised foods and supplements, bras, or breast prostheses (following mastectomy), wigs (to cover head due to alopecia) and in some cases unpaid leave from work.

The SRB Assay was developed by Philip Skehan and others and was chosen for our experiments for its rapidity, sensitivity, and low cost of materials.[22] The method development and design were motivated by the high throughput of experimental needs of the NCI *in-vitro* anti-cancer drug development screening project, which required millions of culture wells per year. SRB is a reliable bright pink amino xanthene dye with two sulfonic groups. The histochemistry of SRB is similar to that of related protein dyes such as bromophenol blue, naphthol yellow and so on. Skehan *et al* compared SRB to other dyes based on its sensitivity, staining intensity and signal-to-noise ratio, and found that SRB was the same to some fluorescent dyes and superior to many other conventional dyes. The other advantages of the SRB assay is that the 100-fold range of linearity far exceeds that of the Lowry and Braford assays, as thus it eliminates the need for time-consuming dilutions of samples with high protein contents. The SRB Assay also allows a sensitive measuring of drug cytotoxicity in culture. The SRB assay is also comparatively

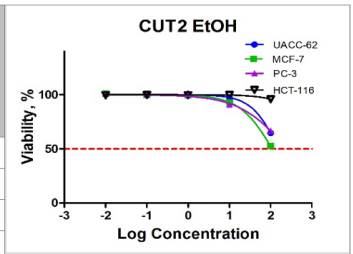
Conc., µg/ml	Log Conc.	%Viability UACC-62	SD	%Viability MCF-7	SD	%Viability PC-3	SD	%Viability HCT-116	SD
100	2.0	7.44	1.5	12.45	0.3	3.85	0.0	1.33	0.4
10	1.0	13.58	2.7	7.68	0.2	8.54	3.1	21.01	1.3
1	0.0	97.37	1.2	55.45	0.8	84.41	3.2	99.70	0.1
0.1	-1.0	98.89	0.3	96.04	0.7	99.75	1.2	99.45	0.2
0.01	-2.0	99.92	0.1	102.83	4.2	98.01	2.9	100.21	0.8



	UACC-62	MCF-7	PC-3	HCT-116
IC50, µg/ml	4.542	1.231	2.615	6.411

Figure 1: The experimental results of parthenolide (standard) and graphical representation of readings
Z' factor: 0.9

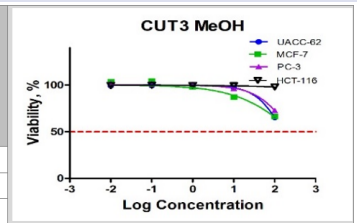
Conc., µg/ml	Log Conc.	%Viability UACC-62	SD	%Viability MCF-7	SD	%Viability PC-3	SD	%Viability HCT-116	SD
100	2.0	64.57	0.10	52.53	0.97	66.65	0.94	95.88	0.69
10	1.0	97.30	1.52	92.98	0.01	90.87	1.63	99.73	0.55
1	0.0	100.77	0.98	99.56	0.03	100.67	0.11	99.48	0.08
0.1	-1.0	100.04	0.03	100.00	0.09	100.45	1.12	99.70	0.35
0.01	-2.0	100.63	1.11	101.37	0.59	100.82	1.57	99.61	0.32



	UACC-62	MCF-7	PC-3	HCT-116
IC50, µg/ml	>100	>100	>100	>100

Figure 2: The experimental results of the ethanol extract (CUT2 EtOH) and graphical representation of readings
Z′ factor: 0.9

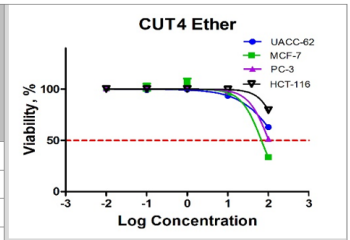
Conc., µg/ml	Log Conc.	%Viability UACC-62	SD	%Viability MCF-7	SD	%Viability PC-3	SD	%Viability HCT-116	SD
100	2.0	65.20	0.55	66.39	0.13	72.69	1.39	97.90	0.47
10	1.0	97.30	0.21	86.94	0.05	96.46	1.51	99.36	0.47
1	0.0	98.86	0.40	97.89	0.85	100.78	0.15	99.53	0.14
0.1	-1.0	99.49	0.76	104.41	0.05	101.82	0.30	99.77	0.25
0.01	-2.0	99.29	0.11	103.56	1.39	102.14	0.09	99.57	0.06



	UACC-62	MCF-7	PC-3	HCT-116
IC50, µg/ml	>100	>100	>100	>100

Figure 3: The experimental results of the methanol extract (CUT3 MeOH) and graphical representation of readings
Z′ factor: 0.9

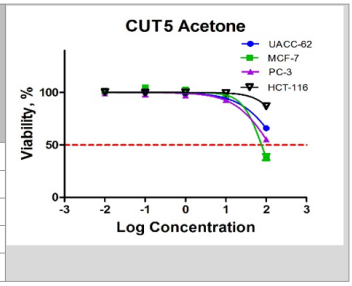
Conc., µg/ml	Log Conc.	%Viability UACC-62	SD	%Viability MCF-7	SD	%Viability PC-3	SD	%Viability HCT-116	SD
100	2.0	62.98	1.2	33.79	0.6	51.45	0.4	79.51	1.0
10	1.0	93.51	0.1	96.23	0.5	98.14	1.0	99.64	0.1
1	0.0	99.71	1.8	106.32	3.9	101.37	2.5	100.25	0.1
0.1	-1.0	99.09	1.5	102.06	3.3	101.59	0.0	100.15	0.2
0.01	-2.0	100.87	0.8	101.34	0.9	100.60	0.1	100.24	0.0



	UACC-62	MCF-7	PC-3	HCT-116
IC50 µg/ml	>100	67.77	>100	>100

Figure 4: The experimental results of the diethyl-ether extract (CUT4 Diethyl-Ether) and graphical representation of readings
Z′ factor: 0.9

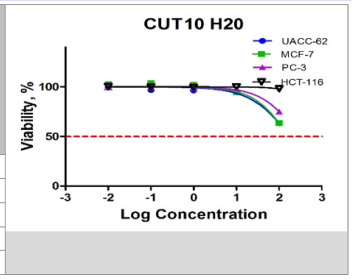
Conc., µg/ml	Log Conc.	%Viability UACC-62	SD	%Viability MCF-7	SD	%Viability PC-3	SD	%Viability HCT-116	SD
100	2.0	65.92	1.6	38.35	2.9	55.37	0.0	86.90	1.6
10	1.0	94.56	0.8	97.42	0.5	92.93	0.6	99.42	0.1
1	0.0	98.76	0.1	102.76	0.6	97.19	1.5	100.07	1.5
0.1	-1.0	100.26	1.8	104.86	1.1	98.21	2.2	99.81	0.1
0.01	-2.0	100.14	0.7	101.27	0.5	98.94	1.2	100.40	0.0



	UACC-62	MCF-7	PC-3	HCT-116
IC50 µg/ml	>100	76.81	>100	>100

Figure 5: The experimental results of the acetone extract (CUT5 Acetoner) and graphical representation of readings
Z′ factor: 0.9

Conc., µg/ml	Log Conc.	%Viability UACC-62	SD	%Viability MCF-7	SD	%Viability PC-3	SD	%Viability HCT-116	SD
100	2.0	63.33	1.5	63.58	0.8	75.05	1.4	98.05	0.8
10	1.0	94.42	0.7	94.46	0.0	97.03	0.9	99.95	0.5
1	0.0	96.41	1.4	102.07	0.2	98.91	0.5	99.50	0.5
0.1	-1.0	96.75	0.7	103.86	0.4	99.83	2.6	99.83	0.6
0.01	-2.0	99.86	0.3	102.86	2.5	100.39	3.1	100.32	1.8



	UACC-62	MCF-7	PC-3	HCT-116
IC50, µg/ml	>100	>100	>100	>100

Figure 6: The experimental results of the acetone extract (CUT10 H20) and graphical representation of readings
Z′ factor: 0.9

Concentration (µg/ml)	Log Conc.	%Viability	SD
100	2.000	15.24	0.38
10	1.000	25.25	0.09
1	0.000	83.31	0.42
0.1	-1.000	98.70	0.70
0.01	-2.000	99.29	1.49

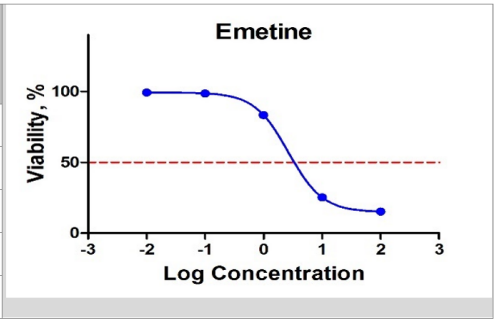


Figure 7: The experimental results of the standard (Emetine) and graphical representation of readings
Z′ factor: 0.9, IC50: **2.66** µg/ml

Concentration (µg/ml)	Log Conc.	%Viability	SD
100	2.0	90.55	2.49
10	1.0	92.66	0.28
1	0.0	94.57	1.54
0.1	-1.0	98.24	0.09
0.01	-2.0	100.86	0.33

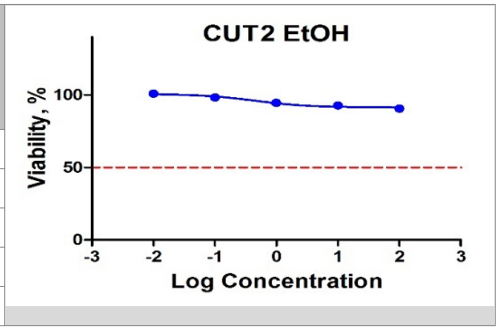


Figure 8: The experimental results of the ethanol extract (CUT2 EtOH) and graphical representation of readings
Z′ factor: 0.9, IC50: **>100** µg/ml

Concentration (µg/ml)	Log Conc.	%Viability	SD
100	2.0	76.87	0.58
10	1.0	82.57	0.39
1	0.0	95.51	0.47
0.1	-1.0	96.99	1.44
0.01	-2.0	103.76	0.59

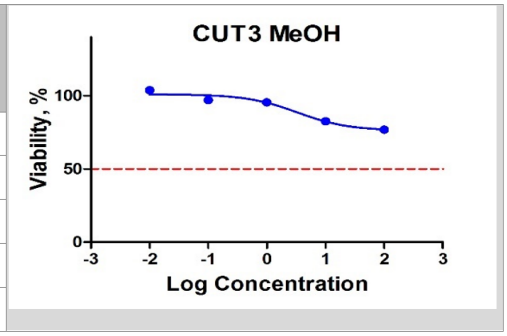


Figure 9: The experimental results of the methanol extract (CUT3 MeOH) and graphical representation of readings
Z′ factor: 0.9, IC50: **>100** µg/ml

Concentration (µg/ml)	Log Conc.	%Viability	SD
100	2.0	78.45	1.48
10	1.0	84.20	0.42
1	0.0	87.69	0.40
0.1	-1.0	88.24	0.05
0.01	-2.0	99.11	0.50

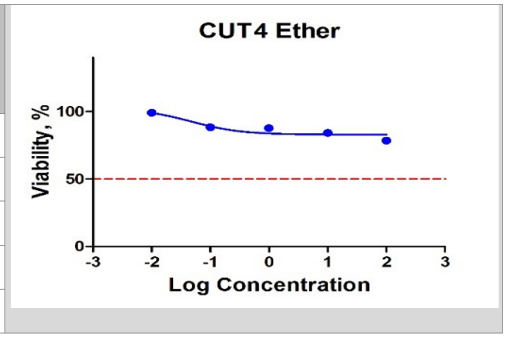


Figure 10: The experimental results of the diethyl-ether extract (CUT4 Diethyl-Ether) and graphical representation of readings
Z′ factor: 0.9, IC50: **>100** µg/ml

Concentration (µg/ml)	Log Conc.	%Viability	SD
100	2.0	88.38	1.38
10	1.0	95.81	0.02
1	0.0	97.17	1.18
0.1	-1.0	101.24	1.35
0.01	-2.0	103.45	1.76

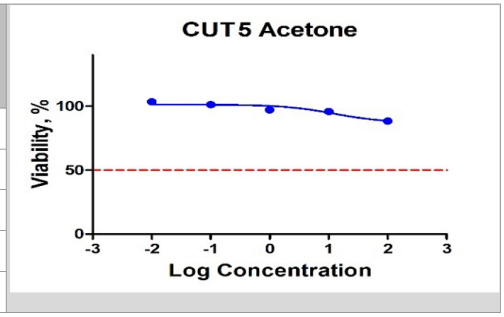


Figure 11: The experimental results of the acetone extract (CUT5 Acetoner) and graphical representation of readings
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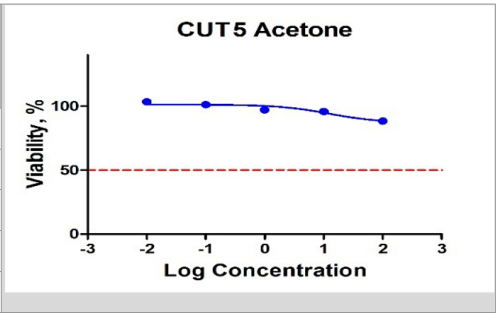

Figure 11: The experimental results of the acetone extract (CUT5 Acetoner) and graphical representation of readings Z' factor: 0.9, IC50: **>100** µg/ml

Table 1: The summary of inhibitory effects of *alfalfa* extracts and the standard towards four cancer cells.

No.	Sample	IC50 for UACC-62, µg/ml	IC50 for MCF-7, µg/ml	IC50 for PC-3 µg/ml	IC50 for HCT-116 µg/ml
1	CUT2 EtOH	>100	>100	>100	>100
2	CUT3 MeOH	>100	>100	>100	>100
3	CUT4 Ether	>100	67.77	>100	>100
4	CUT5 Acetone	>100	76.81	>100	>100
5	CUT10 H20	>100	>100	>100	>100
	Parthenolide	4.542	1.231	2.615	6.411

Table 2: The summary of cytotoxicity measuring of *alfalfa* extracts and the standard towards four cancer cells.

No.	Name of sample	IC50 (µg/ml)
1	CUT2 EtOH	>100
2	CUT3 MeOH	>100
3	CUT4 Ether	>100
4	CUT5 Acetone	>100
5	CUT10 H20	>100
	Emetine	2.66

simpler, faster, and more sensitive than the MTT assay.[22] We employed the SRB assay to measure the anti-cancer potency of five Alfalfa extracts at five different concentrations.

For our experiments we used plant-derived standards that are super potent: Parthenolide (PH) and Emetine. Parthenolide is a sesquiterpene lactone that occurs naturally in the leaves, flowers, and fruits of the feverfew plant (*Tanacetum parthenium*). PH has been demonstrated to inhibit nuclear factor kappa B signaling, induction of apoptosis and the reduction of subpopulation of cancer stem-like cells in various cancers. For our experiment, the plant material was extracted using five solvents: Ethanol (CUT2 EtOH), Methanol (CUT3 MeOH), Diethyl-ether (CUT4 ether), Acetone (CUT5 Acetone), and Water (CUT10 H2O). following treatments, optical density (OD) was determined at the wavelength of 540 nm using a multi-well spectrophotometer. Data analysis was performed using GraphPad Prism software. 50% of cell growth inhibition (IC50) was determined by non-linear regression. The readings and graphical readings of tests are recorded here as 3.1 to 3.12. for measuring pf anticancer and cytotoxicity actions we worked with samples/standard of 100 – 0.01 µg/ml (5 x 10-fold serial dilutions) concentration. The summary of the results of our anti-cancer potency testing are summarized in Table 1. According to criterion of the CSIR the sample is considered Weak if parameter IC50 for two or three cell lines is more than 15 µg/ml but less than 100µg/mL. Therefore, all tested samples can be said to be inactive (3.1 – 3.6). The diethyl-ether and acetone extracts displayed some weak activity towards the MCF-7 breast cancer cell line (3.4 and 3.5) with the 50% inhibitory concentration of 67.7 µg/mL and 76.81 µg/mL respectively.

Emetine is a pyridoisoquinoline that is used clinically as an emetic, antiprotozoal, antiviral, antimalarial, and antineoplastic agent. Emetine is a natural alkaloid that is found in European Ivy (*Hedera helix)*, *Alagium longiflorum* and others. Emetine is used clinical an amoebicidal drug, but it also possesses antineoplastic properties, where it induces apoptosis but also suppress the viability, migration, invasion, and sphere formation of breast cancer cells. The cytotoxic effects of the compounds were tested by Sulforhodamine B (SRB) assay on the WI38 cell line (normal Human Fetal Lung Fibroblast from ECACC). For our testing we worked with samples/standard of 100 – 0.01 µg/ml (5 x 10-fold serial dilutions) concentration. The summary of the results of our cytotoxicity potency testing are summarized in Table 2.

The CSIR criteria considers samples with IC50 of more than 100 µg/ml a low hazard/low potency, while samples that have IC50 of less than 5 µg/ml are regarded as high hazard / potent. The cytotoxicity dose dependant curves (3.7-3.12) clearly show the cytotoxicity potency of our standard: Emetine, and the experimental results confirms that none of our extracts exhibited notable bioactivity v (3.7-3.12). *Medicago sativa L* is a well-known plant around the world, with common names that includes Alfalfa, Lucerne, Buffalo Herb, Purple Medic/Medick/Medicle and many others.[15] Alfalfa is mostly used in agriculture as a fodder crop, where it provides beneficial nourishment to animals and humans. The herbaceous lucerne plant is indeed a multi-purpose crop, that benefits the farmer who grows it, the livestock rancher who uses it to feed animals during times of drought or winter, and for the human being who vows by the health and wellness enhancement (vitality) potency of alfalfa sprouts and tea from leaves and as well as part of commercial herbal concoctions. Lucerne is very high in proteins, vitamins, and phytoestrogens. The Purple Medic plant actually has environmental benefits as well, it has been used to prevent soil erosion for example by binding the soil particles together. Although the plant is safe to be consumed by animals and humans, we found in literature that it also possessed some risk, wherein the seeds are said to contain L-canavanine, an amino acid that is detrimental to patients with the autoimmune disease called lupus. Otherwise *Medicago sativa* has been regarded to be beneficial for health and wellness, where many experimental and ethnobotanical reports demonstrated therapeutic effects towards diabetes (lowering hyperglycemia), lowering cholesterol, acting as a diuretic, and to increase lactation and to promote menstruation just to mention a few. In the year 2003, Rick Simpson a Canadian Engineer was diagnosed with a form of skin cancer called basal cell carcinoma. As a general enthusiast of cultivating the age-old controversial medicinal plant of our ancestors: *Cannabis sativa* (called by many names: *matekoane,* weed, holy herb, *ganja*, hemp, *marijuana, Maryjane,* etc),

acting from hearsay evidence that marijuana is potent towards cancer, Rick decided to apply the oil he extracted from *matekoane* (*Cannabis sativa)* on the affected area and covering it with a bandage. Mr Simpson claims these actions were motivated by the scientific validation study he read from the National Cancer Institute (NCI) trial where THC (*delta-9 tetrahydrocannabinol*) was demonstrated to kill cancer in mice. Several days following the application of the oil, Rick removed the bandage to find the cancer gone and the area was healed. As a result of this "discovery", Rick started to produce the full cannabis extract that is now referred to Rick Simpson oil (RSO), but because it still contained the psychoactive ingredient THC, and it was (and still is) illegal to sell or buy in many parts of the world. The risk factors that promote cancer in humans have increased due to industrialization and the commercial use of toxic and carcinogenic compounds in food, toothpaste, wate, air and agriculture. The safety of radiofrequency waves such as 5th Generation Mobile Network (5G) implications on the human body, although the WHO assures that there are no envisioned health risks from 5G as consequent tissue heating caused by the exposure is below international guidelines of electromagnetic fields exposure (The International Commission on Non-ionizing Radiation Protection and The International Committee on Electromagnetic Safety). The burden of diseases such as cancer, diabetes, hypertension, and mental health disorders is evident everywhere we live, it is the moral obligation of the devotees of science to device the needed solutions. It was for this rationale that we found it necessary to measure the anti-neoplastic actions induced by the world-revered Medicago sativa and our experimental results as a whole clearly validate the ethnobotanical views that alfa-alfa has medicinal benefits to humans, animals, and the environment. We therefore recommend further scientific testing of the Purple Medic, and we rely on our experimental results published here and elsewhere that the religious drinking of alfalfa tea can be beneficial for health and wellness. Even though our extracts did not induce comparable potency of other compounds or plants, we still we recommend the combination of Alfalfa and *Sutherlandia frutescens* (otherwise known as cancer bush, *kankerbos)* for a potent non-drug-based supplement for health and wellness particularly towards the prevention of cancer, diabetes, inflammation, and oxidative stress. And for those with mental health issues we recommend the addition of *gingko biloba*.

CONFLICTS OF INTEREST

The authors declare no conflicts of interests regarding the publication of this paper. The views expressed in this text are of the Authors only and does not reflect the views of CUT.

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