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In-vitro anti-cancer and *in-vivo* immunomodulatory activity of two new compounds isolated from wheatgrass (*Triticum aestivum L.*)

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Phytotherapeutic agents, or plant-based drugs, are a new class of standardised medicinal agents. In recent years, plant-based therapeutics have been used for the prevention and management of cancer. Practitioners believe that wheatgrass is an effective agent because it contains chlorophyll-like molecules that increase haematopoiesis and strengthen the immune system. This study used wheatgrass juice and powder to substantiate the claim that wheatgrass is an effective anticancer agent. Researchers formulated a new extraction procedure to isolate compounds and subsequently evaluated their bioactivities, particularly their immunomodulatory and anti-cancer activities. This procedure successfully managed to isolate 2 new compounds whose structures were elucidated using Nuclear Magnetic Resonance (NMR), Liquid Chromatography – Mass Spectroscopy (LCMS), and Fourier Transform Infra Red (FTIR) techniques. Both compounds showed unique characteristics with respect to their melting points, colours, odours and solubilities. These 2 novel compounds, a ketone (WG1) and a polyphenol (YWG), exhibited strong *in vivo* immunomodulatory lymphocyte proliferation and potent *in vitro* cytotoxic activities against breast, pancreatic, colon, lung, and prostate cancer cell lines. This research concludes that wheatgrass juice and powder includes constituents with medicinal value that can be used for further research as an anti-cancer agent.

Keywords: Cytotoxicity assay, Immunomodulator, Lymphocyte proliferation, Structural elucidation, *Triticum aestivum*, Wheatgrass juice.

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Introduction

In 1938, German scientist Paul Seeger demonstrated that cancer is caused by a disturbance in the utilisation of oxygen[1] due to the destruction of respiratory enzymes, i.e. cytochrome oxidase, which is required for energy generation in mitochondria[2]. Since then, the modulation of immune responses to address the onset of severe diseases, such as cancer, Alzheimer's disease and diabetes, has become a point of interest for the research community. Researchers in the life sciences and in medicinal chemistry believe that medicinal plants have secondary metabolites that induce para-immunity, the non-specific immunomodulation of essential granulocytes, the activation of macrophages, and the generation of antibodies[3].

Numerous medicinal plants have been considered as remedies for human suffering, particularly for the treatment of inflammation, allergies, nutritional and metabolic disorders. Our interest in nature can be traced back thousands of years, and the use of natural agents for the benefit of humankind continues to this day. In the 1950s, scientists began to show great interest in the search for new medicinal agents from natural products; it was "the roaring decade of new drug discoveries". This era heralded the commercial development of drugs, such as reserpine for the treatment of hypertension, vincristine for use in chemotherapy, and paclitaxel as an anti-cancer agent[4].

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The 1980s brought medicinal plants to the forefront, and phytotherapeutic agents and plant-based drugs were used in standardised medicinal compositions. Such medicinal agents consisted of complex mixtures of one or more plant species and were introduced and used in most countries to cure various severe diseases[5]. The discovery of using chemotherapy has enhanced our ability to treat and manage cancer, the associated side effects have been widely criticised. One of the major adverse effects is haematological toxicity, which affects the patient's immune system and can lead to secondary conditions such as bleeding, lupus and coagulopathy[6].

The use of wheatgrass juice for the treatment of various nutritional and metabolic disorders has been recommended but has never been clinically assessed in a controlled clinical trial[7]. Although, a study confirmed that the use of orally consumed wheatgrass juice improved haemoglobin values in patients with intermediate thalassemia due to iron chelation, thus reducing the need for frequent transfusions[8,9]. In Israel, researchers studied the effects of wheatgrass on patients who were undergoing chemotherapy for breast cancer, they concluded that when wheatgrass juice was consumed in conjunction with chemotherapy administration, vital blood counts were maintained, thereby eliminating the need for any supplementary medications[10].

Our literature review came across a study performed by the researchers in Haifa, Israel, who studied the effects of wheatgrass on sixty patients that were undergoing chemotherapy for breast cancer. Of these sixty patients, thirty of them received wheatgrass, while the other thirty simply received routine care. In the group that received wheatgrass, the patients' haemoglobin levels were affected less than in the group that received routine chemotherapy care. This stabilisation of haemoglobin levels directly resulted in fewer cases of haematological side effects such as neutropenic fever and leucopaenia. These researchers concluded that when wheatgrass juice was consumed in conjunction with chemotherapy administration, vital blood counts were maintained, thereby eliminating the need for any supplementary medications[11].

Another study confirmed that the use of orally consumed wheatgrass juice improved haemoglobin values in patients with intermediate thalassemia due to iron chelation, thus reducing the need for frequent transfusions[12].

In yet another study, researchers selected two hundred thalassemia patients, among whom were 160 E-beta thalassemia patients, 30 E-thalassemia patients and 10 sickle cell thalassemia patients. These patients received a daily dosage of 30 mL of fresh wheatgrass juice extracted from 6-week-old wheatgrass plants. The study observed significant iron-chelating activity from the use of wheatgrass juice; the patients' mean haemoglobin levels showed a 26% increase, their serum ferritin levels decreased significantly, and 24 patients exhibited an increase in the intervals between transfusions. These results confirmed that wheatgrass juice was an effective alternative treatment to blood transfusion in thalassaemia intermedia patients, and it was highly recommended for continued use[13].

In all the above mentioned studies, the potential anti-cancer and immunomodulatory bioactivity of wheatgrass juice is evident.

In the preliminary study, researchers investigated and confirmed the molecular level ability of the wheatgrass plant in curing diseases. Researchers did so by isolating the compounds from the plant and studying their bioactivity. In our experiments, researchers designed the study to confirm the cytotoxicity and immunomodulatory activity of the compounds. The paper presents two novel compounds isolated from wheatgrass powder and assessed their *in-vivo* immunomodulatory and *in-vitro* anti-cancer bioactivities.

Materials and Methods

Reagents and solutions

Girmes brand wheatgrass powder was purchased from a medical store. All reagents and solvents used for the experiment i.e., Di-chloro methane (DCM), methanol, calcium chloride, petroleum ether (PET), and ethyl acetate (EtOAc) were of Merck analytical grade.

Sample preparation

Wheatgrass powder (50 g) was treated with 500 mL of a 1:1 DCM: methanol mixture, was soaked overnight and then filtered. The residue was subsequently treated three more times for 72 hours filtering the mixture every 24 h, with the same solvent system to further extract the compounds.

Extraction procedure

The experiment used fresh organic wheatgrass powder and followed a standardised extraction

protocol in which 50 g of dried wheatgrass powder was dissolved in 500 mL of a 1:1 DCM: methanol mixture. A magnetic stirrer was used to thoroughly and slowly mix the sample in the solvent system for 4 hrs, and the mixture was then left to soak overnight. The extraction was performed in 4 steps: 1- soaking, 2- stirring, 3- filtering, and 4- freeze-drying.

This four-step procedure was performed three times for maximal extraction of the constituents. The fourth and final extraction wash of the wheatgrass powder was performed using 500 mL of $CaCl_3$-dried methanol, and the sample was soaked for 24 h and filtered the following day. Merck Whatman paper no.1 was used for all filtration steps. A total of 500 mL of the eluate was collected from the four filtration processes and was subsequently freeze-dried in a lyophiliser at -75 ºC for 24 h. A total of 25 g of crude green colored powder was recovered after freeze-drying the eluate for 6 h. After conducting solubility test, researchers conducted TLC profiling on the crude green powder, to understand its composition (Fig. 1).

Column chromatography fractionation technique

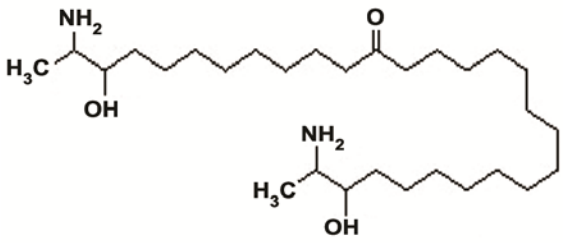
This crude green colored powder (15 g) was first dissolved in 15 mL of methanol, to prepare its solution form. This was later adsorbed on 35 g of Merck grade 60-120 mesh size silica gel, for preparing the slurry. This slurry was then packed into the column, for isolation of the compounds. Three different solvents systems were used to isolate the compounds. Starting from non-polar and moving to polar solvents, these solvents used were: distilled Petroleum Ether (PET), 3% ethyl acetate (EtOAc) in PET, and 1:1 PET:DCM. A Chemglass CG-1189-16 glass chromatography column with a fritted disc and a 2-mm PTFE stopcock (1-1/2" ID x 18" effective length, 24/40 outer joint) was used for the experiment.

The first isolated product was a white crystalline odourless compound whose melting point was 82.3 °C (Fig. 2). The second isolated extract was a yellow amorphous compound that had a fishy smell (Fig. 3). TLC profiling was conducted to identify the appropriate solvent systems to purify these extracts. After several rounds of trials, a 20% MeOH : 80% EtOAc solvent system was determined to be the best system for purification of these extracts.

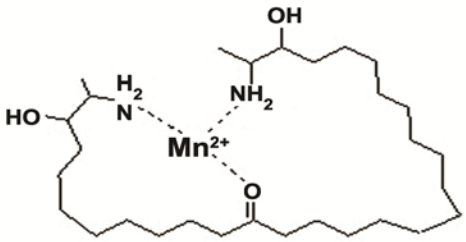
Analytical procedures

After completing the standardised extraction protocol, chemical characterisation and bioactivity

Fig. 1 — WG TLC profile in 1:1 DCM: PET



WG1:2,28- diamino-3,27- dihydroxyno -nacosan- 12- one



WG1 Mn- Ligand complex form

Fig. 2 — WG1 compound 1 structure

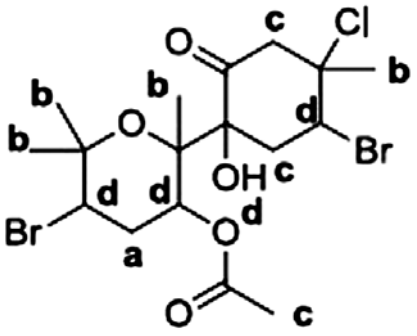


Fig. 3 — YWG compound 2 structure

studies were performed on the purified extracts to examine the medicinal value of wheatgrass. A FTIR run was done on the original crude and the purified wheatgrass powder, to understand if any changes took place to the composition of the compound (Fig. 4). The chemical characterisation experiments included elution of the compounds using preparative HPTLC, TLC and column chromatography, and structural elucidation was confirmed using analytical techniques such as HPLC, LCMS, FTIR, ^{13}C and ^{1}H 2D NMR, and HRMS. Bioactivity experiments such as immunomodulatory and anti-cancer activity assays were performed on the purified extracts to confirm their nutraceutical and medicinal values.

Experiment

Procedure for lymphocyte proliferation assay

To understand the immunomodulatory activity of biopolymeric fraction WG1 & YWG from wheatgrass powder, researchers investigated its effects on the proliferation of spleenic cells. Researchers observed the delayed-type hypersensitivity (DTH) response in these mice determined by measuring the footpad thickness after 4, 8, and 24 h.

To conduct this assay, healthy female Balb/c mice (18–22 g) were procured from the Animal House of the Institute. The mice were kept under standard laboratory conditions such as humidity, temperature (25 ± 2 ◦C) and photoperiod of 12 h. Commercial pellet diet and water were given at regular intervals. Animal experiments were approved by Institutional Animal Ethics Committee at (IAEC) Indian Institute of Integrative Medicine, Jammu.

For this assay researchers procured, MTT, RPMI-1640, bovine serum albumin, and LPS were purchased from Sigma Chemical Co, MO., USA. Assay kits were purchased from R&D systems, USA.

SRBC induced delayed type hypersensitivity (DTH) in mice

Delayed type hypersensitivity response was measured by the method of Doherty[14] (1981) with slight modifications. Briefly, the mice were sensitized by injecting 20 µL of 5×10^{9} SRBC/mL subcutaneously into the right hind foot paw[15] on day 0 and challenged by injecting the same volume of SRBC into the left hind foot paw on day +7. The thickness of the left hind foot paw of each mouse was measured using the sphiro-micrometer (reading to 0.01mm) 24 h after the challenge. Compounds WG1 & YWG were administered at three different doses i.e., 12.5, 25 and 50 mg/kg p.o. 2 h after SRBC injection (sensitization) on day 0 and once daily for next seven days. Similarly, Levamisole was taken as standard and given for the same period as the test drug at a dose of 2.5 mg/kg p.o. Results of these experiments are presented in Table 1 and 2.

Table 1 — DTH response of Compound-I WG1

Treatment	Dose mg/kg p.o.	DTH response {paw edema (mm)} (mean±SE) 24 h	% change
Control	-	1.87±0.07	-
Levamisole	2.5	2.60±0.06**	39.03
Comp-1 (WG1)	12.5	2.15±0.09*	14.97
Comp-1 (WG1)	25.0	2.45±0.07**	31.01
Comp-1(WG1)	50.0	2.88±0.05**	54.01

No of observations per group=5; P-value *= < 0.01 and **=< 0.001

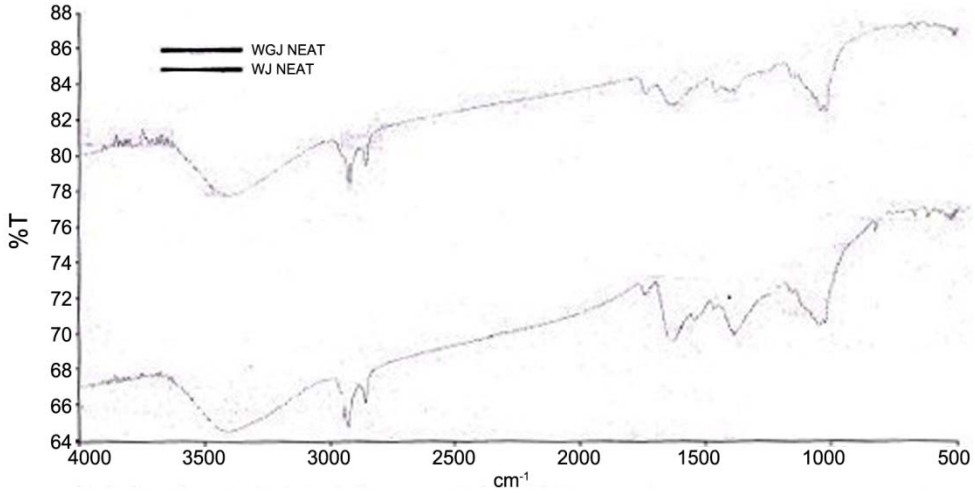


Fig. 4 — FTIR spectra overlap of wheatgrass juice crystals and wheatgrass powder

Splenocyte proliferation assay

Spleen collected under aseptic conditions in HBSS was minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension and the erythrocytes were lysed with ammonium chloride (0.8%, w/v). After centrifugation (380×g at 4°C for 10 min), the pelleted cells were washed three times with PBS and re-suspended in complete medium [RPMI 1640 supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2-mercaptoethanol, 100 IU/mL penicillin, 100 ug/mL streptomycin, and 10% FCS]. Cell number was counted with a haemo cytometer by the trypan blue dye exclusion technique. Cell viability exceeded 95%.

Table 2 — DTH response of Compound-II YWG

Treatment	Dose mg/kg p.o.	DTH response {paw edema (mm)} (mean±SE) 24hr	% change
Control	-	1.90±0.08	-
Levamisole	2.5	2.67±0.05**	40.52
Comp-ll (YWG)	12.5	2.34±0.08*	23.15
Comp-ll (YWG)	25.0	2.50±0.07**	31.57
Comp-ll (YWG)	50.0	2.72±0.06**	43.15

No of observations per group=5; P-value *= < 0.01 and **=< 0.001

Procedure: Delayed type hypersensitivity response was measurement by the method of Doherty (1981) with slight modifications. Briefly, the mice were sensitized by injecting 20 µl of 5×109 SRBC/ml subcutaneously into the right hind foot paw on day 0 and challenged by injecting the same volume of SRBC in to the left hind foot paw on day +7. The thickness of the left hind foot paw of each mouse was measured using the sphiro-micrometer (reading to 0.01mm) 24 hr after the challenge. Compounds WG1 & YWG were administered at three different doses i.e., 12.5, 25 and 50 mg/kg p.o. 2 h after SRBC injection (sensitization) on day 0 and once daily for next seven days. Similarly, Levamisole was taken as standard and given for the same period as the test drug at a dose of 2mg/kg p.o. (Doherty, N.S. (1981) Selective effect of immunosuppressive agents against the delayed hypersensitivity response and humoral response to sheep red blood cells in mice. Agents and Actions 11, 237-242.)

To evaluate the effect of WG1 and YWG on the proliferation of spleenic lymphocytes, spleen cell suspension (1×10^7 cell/mL) was pipetted into 96 well plates (200 µL/well) and cultured at 37°C for 72 h in a humid saturated atmosphere containing 5% CO_2 in the presence of Con-A (5 µg/mL) and LPS (10 µg/mL). After 72 h, 20 µL of MTT solution (5 mg/mL) were added to each well and incubated for 4 h. The plates were centrifuged (1400×g, 5 min) and the untransformed MTT was removed carefully by pipetting. To each well, 100 µL of a DMSO working solution (192 µL DMSO with 8 µL 1 M HCl) was added, and the absorbance was evaluated in an ELISA reader at 570 nm with a 630 nm reference after 15 min.

In the present lymphocyte assay, Table 3 and 4 (Lymphocyte proliferation assay) and Fig. 5 and 6 (Lymphocyte proliferation), presents the immunomodulatory potential of the isolated compounds from wheatgrass powder, and explored the modulation of T-cells & B-cells. It was observed that B-cells augmented more than the T-cells. The test doses significantly increased the proliferation of LPS splenetic cells directly proportional to the dose given, while the results for CON A weren't that significant. LPS stimulated spleenocyte proliferation was significantly enhanced by bio polymeric fraction

Table 3 — Compound WG1 Spleenocyte assay data – Percentage Cell Proliferation

	(B –cells antibodies regulation)	(T-cells antibodies production)
Drug dosage	LPS STIMULATED	CON A STIMULATED
Control	1.142333	0.213
BMS	2.065	2.034
0.01	2.242	0.319667
0.1	2.402333	0.329667
1	2.554	0.630667
10	2.484667	0.384
100	2.559667	0.945667

Table 4 — Compound YWG Spleenocyte assay data – Percentage Cell Proliferation

Concentration (mg/mL)	Percentage Cell Proliferation	
	LPS STIMULATED (B –cells antibodies regulation)	CON A STIMULATED (T-cells antibodies production)
CONTROL	1.142333	0.213
BMS	1.807704058	0.9549295775
0.01	2.344333	0.637
0.1	2.560667	0.339
1.0	2.258	0.305333
10	2.111	0.284667
100	2.042333	0.958

WG1 at 0.1 mg/kg. These results also indicates that these compounds are potential antigens, exhibiting the capability of inducing an immune response.

In-vitro anti-cancer - cytotoxicity activity

A sulphorhodamine B (SRB) colorimetric assay was conducted on WG1 & YWG test samples for cytotoxicity screening. PC3 (prostate cancer cell line), MCF-7 (breast cancer cell line), HCT-116 (colon cancer cell line), A549 (lung cancer cell line) and MIAPACA (pancreatic cancer cell line) were seeded into the wells of a 96-well plate. After an incubation period of 24 h in a CO_2 incubator, the extract or drug was added, and the plates were incubated for an additional 48 hrs in RPMI-1640 media. Cell monolayers were fixed by addition of 50% (w/vol) trichloroacetic acid and incubation for 1 h at 4ºC. After 1 h, the plates were washed and then dried for 1 h.

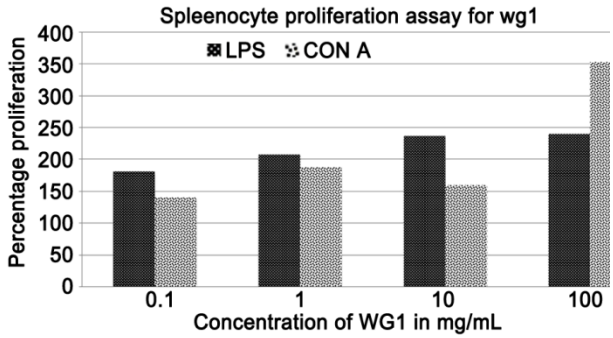


Fig. 5 — Spleenocyte proliferation graph WG1

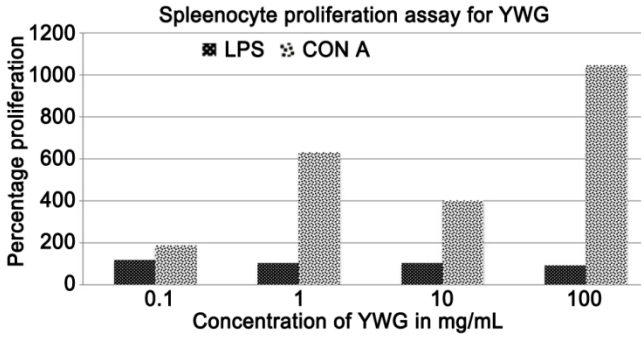


Fig. 6 — Spleenocyte proliferation graph YWG

The 96 well-plates were stained by incubating the cells with 100 µL of SRB for 30 min. After 30 min, excess dye was removed by washing the wells repeatedly with 1% (vol/vol) glacial acetic acid. The protein-bound dye was dissolved by adding 100 µL of a 10 mM Tris buffer solution to the wells and shaking the plates for 15 minutes. The Optical Density of the plate wells was recorded at 540 nm, and was determined using a micro plate reader. The drug 5-fluorouracil was used as the standard throughout the experiment.

For the experiment, the blank wells contained medium but no cells and the control wells contained cells but no test samples. Growth inhibition was calculated as the percent survival of treated cells over control cells × 100 (T/C %). The percent inhibition is exhibited in Table 5. The percent inhibitions of the test compounds were compared to the inhibitory activity of 5 Fluoro Uracil (5FU) standard (Fig. 7).

Results

In the present study, researchers have successfully demonstrated the medicinal value of wheatgrass juice/powder, by studying its bioactivities. Using column chromatography technique, the researchers were able to successfully isolate two compounds from wheatgrass powder and were able to elucidate their complete structures by applying analytical techniques such as NMR, FTIR, LCMS, and High Resolution Mass Spectrometry (HRMS). Furthermore, researchers have been able to successfully assign the peaks with proper references, for both the molecules using 1H NMR spectral chromatographs (Fig. 8 and 9). After identifying the predicted structures, researchers then conducted an extensive literature review for these compounds, which has confirmed them to be new moieties.

Table 5 — Anti-Cancer Bioactivity experiment results

Optical Density measurements @ 540nm, grown in RPMI – 1640 media,

	Cell line type		PC3	MCF	HCT-116	A549	MIAPACA
	TISSUE TYPE		PROSTATE	BREAST	COLON	LUNG	PANCREATIC
Nature	Code	Conc	Percentage inhibition				
Wheatgrass	WG1	24uM	17	8	14	29	5
Wheatgrass	YWG	26uM	23.5	5.5	18.5	44	33.5
Wheatgrass	WG 3% E:PET	23uM	17.5	0	52	64	14
Wheatgrass	WG 1:1 PET:DCM	24uM	30.5	37	73	61.5	53.5
Wheatgrass	WG PET	21uM	18.5	46	82	34.5	3
Std	5FU	20uM	32	53	41	66	50

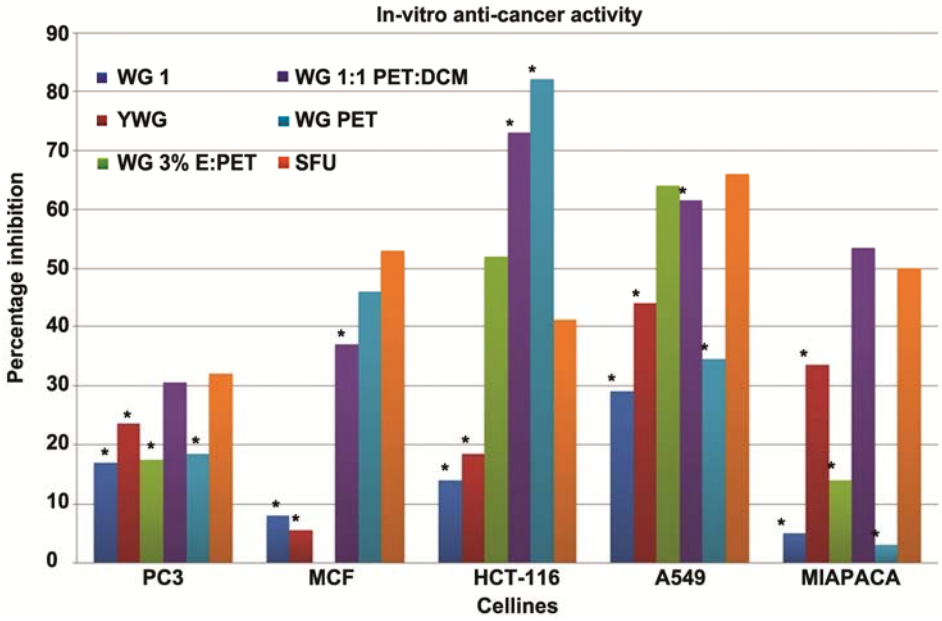


Fig. 7 — Graph of cytotoxicity assay of all cancer cell lines

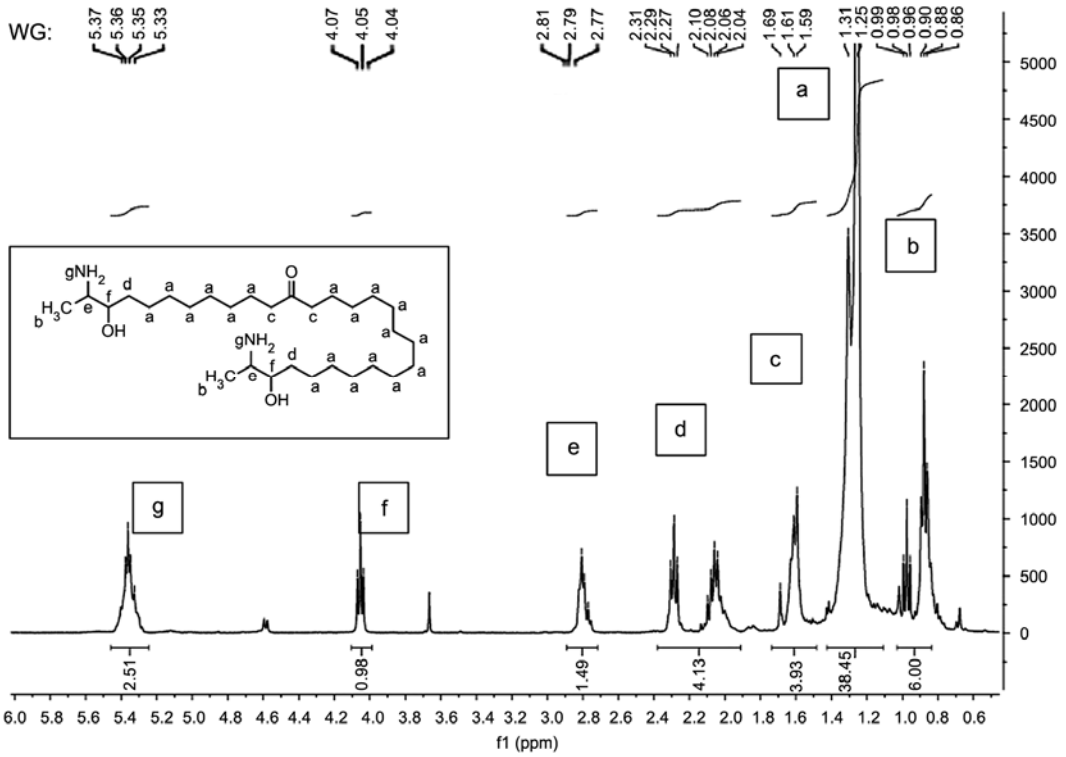


Fig. 8 — WG1 1H NMR peaks assignment

In this study, researchers confirmed and elucidated structures for both the compounds, a) white crystalline compound-1 (WG1)* and the b) yellow amorphous compound-2 (YWG)* as shown in Fig 2 and 3, respectively (patent applications filed). Researchers conducted a literature review of these 2 compounds, using PubChem and ChemSpider searches to confirm that they represent new moieties. Researchers reviewed each molecule for its origin and novelty; this review confirmed that the 2 identified compounds were indeed new and that their bioactivities had not previously been detected, studied

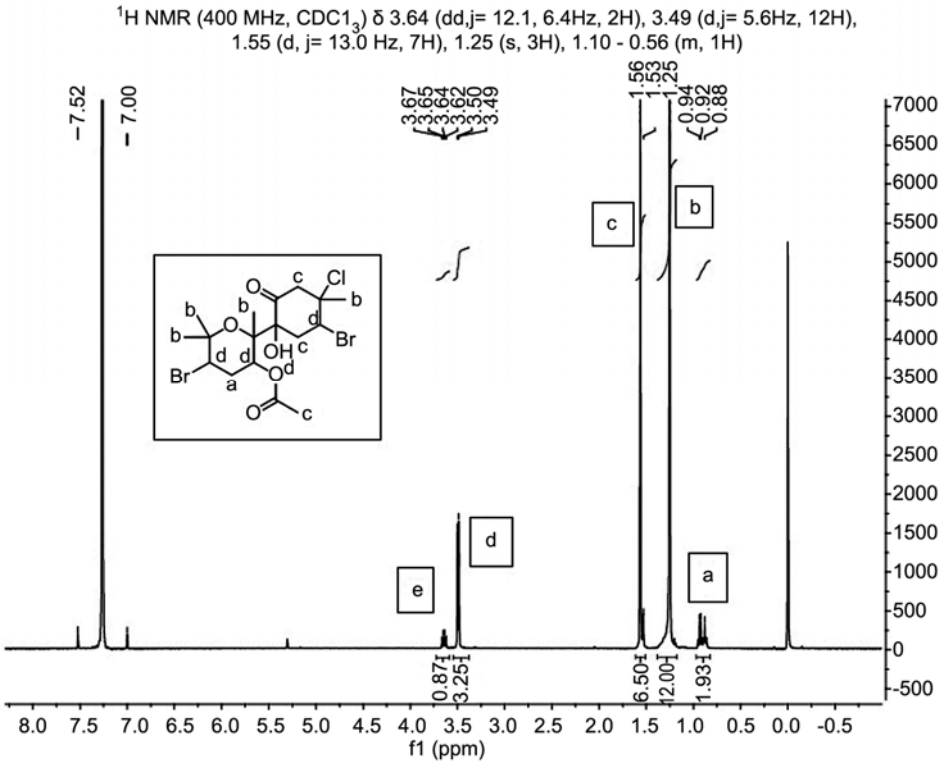


Fig. 9 — YWG 1H NMR peaks assignment

or compared to any other moieties of similar molecular weight. In this literature study, researchers used the IUPAC names as search strings and found that the white crystalline compound WG1 had 30 isomers, 0 tautomers and no identically structured moieties. For the yellow amorphous compound YWG, the search identified 14 isomers, 0 tautomers and no identical moieties.

Subsequently, after the researchers completed the literature review and reported the elucidated structures of these two new molecules, that were code named WG1 and YWG respectively, they performed three bioactivity assay's; 1) delayed type of hypersensitivity assay, 2) splenocyte proliferation assay and 3) cytotoxicity assay against five cancer cell lines- PC3 cell line (prostrate cancer), MCF cell line (breast cancer), HCT-116 cell line (colon cancer), A549 cell line (lung cancer), MIAPACA cell line (pancreatic cancer) on these newly identified molecules WG1 and YWG, using different solvent fractions of wheatgrass powder.

Researchers conducted the Delayed Type of Hypersensitivity (DTH) response experiment, a classical TH1 mediated response which is classified as Type IV Hypersensitivity. This response has been implicated in many as an inflammatory disease and an autoimmune condition. However, it is critical for the clearance of certain intracellular parasites, and is therefore a desirable immuno-modulatory activity.

In this study, WG1 & YWG exhibited significant DTH response at all three doses used in the experiment. The researchers found that the DTH response of WG1 & YWG compounds at 50 mg/kg body weight were better i.e. 54.01 and 43.13% respectively; than that compared to the Levamisole standard i.e. 39 to 40%.

The researchers further performed, the splenocyte proliferation assay, to assess the immune activating potential of both the compounds on immune T & B cells. To evaluate the effect of WG1 and YWG on the proliferation of spleenic lymphocytes, spleen cell suspension (1×10^7 cell/mL) was pipetted into 96 well plates (200 µL/well) and cultured at 37°C for 72 h in a humid saturated atmosphere containing 5% CO2 in the presence of Con-A (5 µg/mL) and LPS (10 µg/mL). Researchers observed that both the compounds at all concentrations exhibited significant cell proliferation – both T & B cells showed stimulated effect. In human physiology, regulated stimulation of T & B cells is pivotal to the management of many immune suppressive disorders. Researchers therefore inferred that, both these

compounds i.e. WG1 & YWG, are potential candidates for immune stimulating activities.

The researchers confirmed that splenocyte modulations assay conducted on both the compounds, exhibited potent immunomodualtion, at all dosages. Compound WG1 exhibited significant proliferation of CON A stimulated cells, whereas LPS stimulated cells also exhibited marginal growth. Compound YWG too exhibited significant proliferation of CON A stimulated cells whereas it marginally inhibited LPS stimulated cells w.r.t. BMS standard.

In this study, researchers isolated 2 compounds from wheatgrass powder and characterised both of them. The confirmed and elucidated structures for the a) white crystalline compound-1 (WG1)* and the b) yellow amorphous compound-2 (YWG)* are shown in Fig 2 and Fig 3, respectively (patent applications filed). Researchers conducted a literature review of these 2 compounds, using PubChem and ChemSpider searches to confirm that they represented new moieties. Researchers reviewed each molecule for its origin and novelty, and conducted bioactivity assays; this review confirmed that the 2 identified compounds were indeed new and that their bioactivities had not previously been detected, studied or compared to any other moieties of similar molecular weight.

After checking for structurally identical moieties in ChemSpider, researchers conducted a PubChem BioActivity search, focusing on both compound-and assay-centric searches. Researchers used IUPAC names to obtain the data and found that there were no available data related to these compounds. In literature review, researchers used the IUPAC names as search strings and found that the white crystalline compound WG1 had 30 isomers, 0 tautomers and no identically structured moieties. For the yellow amorphous compound YWG, the search identified 14 isomers, 0 tautomers and no identical moieties.

With respect to the origins of the compounds, researchers found that moieties of similar molecular weight were primarily synthesised in laboratories and did not have similar origins i.e., a plant or wheatgrass. Researchers also found that products with these molecular weights were primarily used in research and were by-products of the synthesis of a T-epitope, threonyl. Meanwhile, a thymidine analogue with a similar molecular weight to the second molecule (YWG) was shown to have been synthetically prepared and to exhibit anti-cancer properties. The literature review conducted on both these compounds confirmed that the molecules were new and have novel medicinal value.

Description of compounds

In this section, researchers are presenting two new compounds i.e. WG1 and YWG that are exhibiting medicinal properties.

Compound WG1 details **Patent* Application No.201721033651 A

WG1: A new compound, white and crystalline in nature, melting point 83.2ºC, fishy odour, soluble in DCM and MeOH, with an IUPAC name of 2,28-diamino-3,27-dihydroxynonacosan-12-one. It is a super ligand, a secondary amine and a plant nutrient that is important because it contributes to the medicinal activity of a plant by chelating important functional groups that influence certain bioactivity patterns. The presence of metal ions & in particular manganese is confirmed by TLC spot analysis & LCMS m/z ion values. Metal ions such as Na, Fe, and Cu have the ability to catalyse free radicals.

C (71.85%); H (12.4%); N (5.78%); O (9.90%); MF:$C_{29}H_{60}N_2O_3$, M.Wt.484.7883; m.p: 83.2ºC; white crystals, Rf value: 1.487 ± 0.02; solubility: MeOH; FTIR (chloroform neat) *U*max: 3305, 2984, 2954, 2858, 1597, 1472, 1121, 1057, 831, 729; δ^1H(400 MHz, $CDCl_3$) (ppm) 0.8 – 0.95 (s, 1H), 1.25 (s, 3H), 1.56 (s, 2H), 3.5 (d, 1H), 7.25 (s, 6H); δ^{13}C, (400 MHz, $CDCl_3$) 14 (-CH_3), 22.71 – 32.83 (8 –CH_2), 63.14 (-CH_2), 113 (-CH_3); m/z (EI): 567, 525, 507, 485, 307.

1H NMR peak assignment : (400 MHz, CDCl3) δ 5.35 (dd, *J* = 12.2, 6.9 Hz, 3H), 4.05 (t, *J* = 6.7 Hz, 1H), 2.89 – 2.72 (m, 2H), 2.39 – 1.91 (m, 4H), 1.75 – 1.48 (m, 4H), 1.28 (d, *J* = 21.3 Hz, 38H), 0.93 (dt, *J* = 13.5, 7.3 Hz, 6H) (Fig. 8)

Because of the close structural relationship between chlorophyll and haem, it has been suggested that there may be common biosynthetic pathways for these 2 molecules. Chlorophyll and Haem are also associated functionally and structurally, and their relationship is shown in the following equation:

$$2H_2O \xleftarrow{+n\ hv,\ chlorophyll} O_2 + 4H + 4\ (e)^8$$

Within this context, researchers have proposed a ligand structure for the WG1 moiety, which had showed yellowish brown spots on TLC plate indicating presence of Mn-ion during the reagent test.

These studies indicate that there might be molecules within wheatgrass that have medicinal value having ability to cure cancer[16]. WG1 moiety

might be one of them, which is showing cytotoxity as well as spleenocyte – lymphocyte proliferation.

Compound YWG1 details

(Patent Application in process)

A new compound, yellow colored and amorphous in nature, fishy odour, soluble in DCM and MeOH, with an IUPAC name of 5-bromo-2-(5-bromo-4-chloro-1,2-dihydroxy-4-methylcyclohexyl)-tetrahydro-2,6,6-trimethyl-2H-pran-3-yl-acetate.This is a new compound that falls into the iso-flavan category and is a secondary metabolite that is known to contribute to the antioxidant effects of plants based on its ability to chelate metal ions.

In support of this theory of chelating metal ions in plants, researchers reviewed a paper in which scientists undertook a 6-month pilot study to understand the role of the iron-chelating activity in wheatgrass juice in comparison to that of a standard chelator (i.e.desferrioxamine) .

Molecule YWG details

(Patent application filed)

C: (38.98%), H: (56.30 %), O: (15.27%), N: (2.67 %), Br: (30.51%), Cl: (6.78 %), MF: $C_{17}H_{30}O_5NBr_2Cl$,

M. Wt.: 523.24, yellow amphorpous, Rf: 0.4 – 0.9, solubility: MeOH, ^1H values (400 MHz, $CDCl_3$) (ppm): 0.84 – 0.99, 1.25 – 1.31, 1.59 – 1.63, 2.08 – 2.04, 2.27 – 2.231, 2.77 – 2.81, 4.04 – 4.07, 5.33 – 5.40,^{13}C values (400 MHz, $CDCl_3$) (ppm): 132 – 138,101.15, 127 – 131, 174, 214.60, 76 – 82, 22- 64, FTIR values (chloroform neat) *U*max: 3453.98, 3011.89, 2954.46, 2849, 2917, 1736, 1472, 1463, 1376, 1215, 1173, 959, 729, 759, 720, LCMS: m/z: 293, 358, 429, 485, 507, (M+NH3) 523; HRMS: m/z: 523.

1H NMR peak assignment: 1H NMR (400 MHz, CDCl3) δ 3.64 (dd, *J* = 12.1, 6.4 Hz, 2H), 3.49 (d, *J* = 5.6 Hz, 12H), 1.55 (d, *J* = 13.0 Hz, 7H), 1.25 (s, 3H), 1.10 – 0.56 (m, 1H) (Fig. 9).

Bioactivity assays

Immunomodulation proliferation assay

Immunomodulation is a strategy for overcoming incurable autoimmune diseases, including cancer, AIDS, arthritis, and allergies. Immunomodulation is defined as a process that can alter the immune system of an organism by interfering with its function[17]. Such interference results in either immune stimulation, enhancement of immune reactions or immune suppression; the latter corresponds with a reduction in resistance against infections and stress, which may be a result of environmental or chemotherapeutic factors.

Researchers conducted immunomodulatory bioactivity assays on the isolated compounds that were extracted using three solvent systems: distilled petroleum ether (PET), 3% ethyl acetate (EtOAc) in PET, and 1:1 PET:DCM. Researchers observed the delayed-type hypersensitivity (DTH) response and Spleenocyte Proliferation Assay on these compounds.

The induction of B cell proliferation by the isolated compounds was comparable to or higher than the BMS standard. In contrast, these compounds showed moderate T-cell proliferative activity, as shown in Table 3-6. Immunomodulatory activity experiments confirmed that wheatgrass juice/powder is indeed a strong immuno-stimulant, supporting the claim that it is an immunomodulator or an immune booster.

The cytotoxicity assay

Anticancer experiments were conducted to investigate the cytotoxic activity of the compounds (Fig. 7). The PC3 cell line was used to test for activity against prostate cancer; the MCF cell line was used to test for activity against breast cancer; the HCT-116 cell line was used to test for activity against colon cancer; the A549 cell line was used to test for activity against lung cancer; and the MIAPACA cell line was used to test for activity against pancreatic cancer.

The human cancer cell lines used in the *in-vitro* experiment were obtained from the National Cancer Institute, Frederick, USA. The cells were grown and maintained in RPMI-1640 medium, at pH-7.4, supplemented with 10% fetal calf serum, glutamine (2mM), penicillin (100 units/mL) and streptomycin (100 µg/mL). The cell cultures were grown in a carbon dioxide incubator (Heraeus, GmbH, Germany) at 37 ^oC with 90% humidity and 5% carbon dioxide. (Fig. 7).

The cytotoxicity results showed the following, WG 3% E:PET extract exhibited significantly higher *in-vitro* anticancer activity on HCT-116 (colon) (Fig. 8) and comparable activities on A549 (lung) cells (Fig. 9) w.r.t 5FU. While, WG 1:1 PET: DCM extracts exhibited better activity w.r.t. 5 FU standard or significantly higher in-vitro anti-cancer activities on HCT-116 (colon) (Fig. 10), A549 (lung) (Fig. 11), PC3 (prostrate) (Fig. 12), and MIAPACA (pancreatic) cells (Fig. 13). These may therefore be taken ahead for identification of potential leads for anti-cancer agents especially in Lung, Colon and Prostate cancers. All other compounds exhibited significantly reduced activities compared to 5 FU standard.

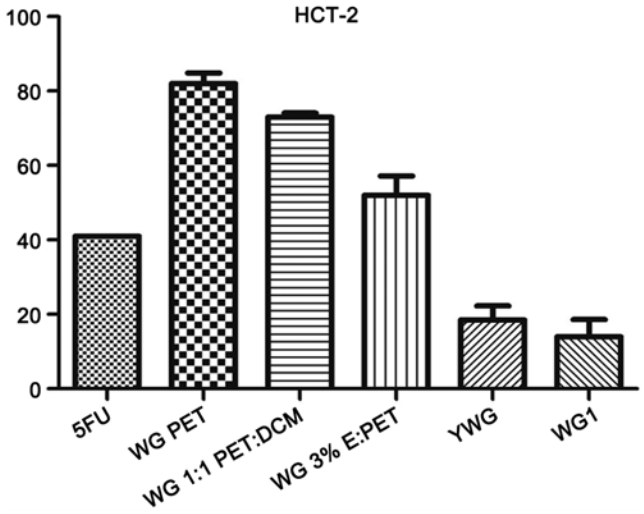


Fig. 10 — HCT-116 cytotoxicity activity on cells

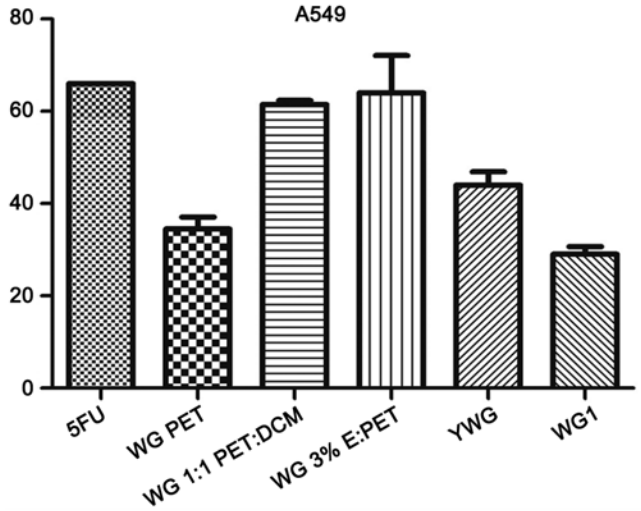


Fig. 11 — A549 cytotoxicity activity on cells

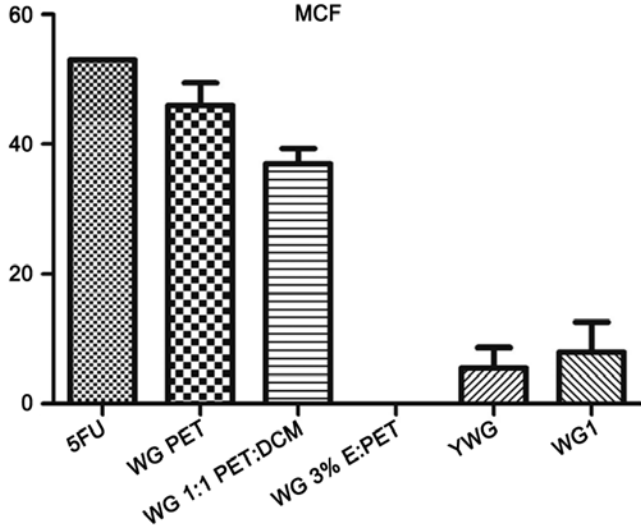


Fig. 12 — PC3 Cytotoxicity Activity on cells

Table 5 and 7 shows the effects of the various isolated compounds on the viability of the 5 different cancer cell lines. The experiment concluded that wheatgrass powder extracts using 3% EtOAc in PET, 1:1 PET in DCM and PET alone exhibited strong anti-cancer activities against breast (Fig. 14), colon (Fig. 10), lung (Fig. 11) and pancreatic cancer cell lines (Fig. 13) and that it exhibited stronger activity than the experimental standard 5-fluorouracil. These findings support the claim that wheatgrass and the isolated compounds are indeed a potential cure for cancer if administered appropriately under supervision.

Discussion

In 1950, biochemist Dr. Thelma Arthur's research demonstrated that wheatgrass is one of the most

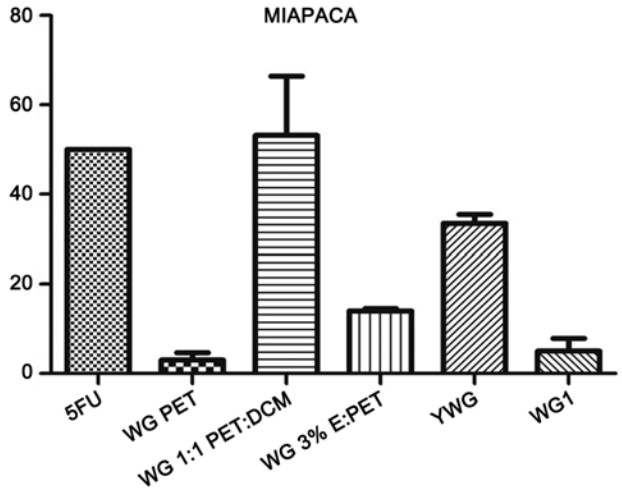


Fig. 13 — MIAPACA cytotoxicity activity on cells

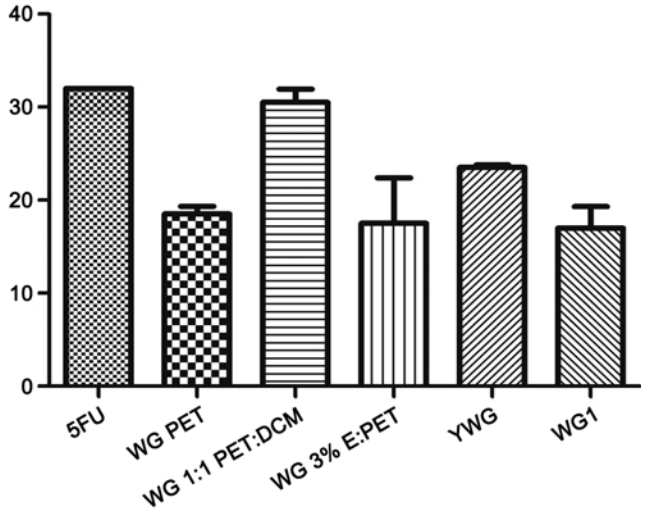


Fig. 14 — MCF Cytotoxicity activity on cells

potent alkaline foods known to mankind and that it can help maintain bodily pH and prevent diseases; in particular, cancer cells were shown to disintegrate under alkaline pH conditions. Her research concluded that both cytochrome oxidase enzymes and alkaline pH play pivotal roles in cell biochemistry and are indicators of a healthy immunological system. It is known that inflammation causes an increase in free radicals, such as hydroxyl ($^{\bullet}OH$) and peroxy nitrite ($ONOO^{-}$) radicals, which play important roles in the progression of inflammation. Thus, the containment of such free radicals could reduce the severity of inflammation[18].

Practitioners have found that wheatgrass contains valuable compounds and advantageous enzymes that help protect humans from carcinogens. Such enzymes include superoxide dismutase (SOD), which reduces the effects of radiation and facilitates metabolic detoxification. Powerful nutrients in wheatgrass prevent the DNA degradation and cellular breakdown that are responsible for the onset of degenerative diseases[19]. Literature shows the presence of secondary metabolites in the wheatgrass extracts, exhibiting antidiabetic[20], anti-oxidants[21] and anti-cancer properties; like alkaloids, flavonoids, saponin, tannins, amino acids and protein, carbohydrates, coumarin, phenols, alkaloids, terpenoids and cardiac glycosides[22].

In this study, researchers used wheatgrass juice and powder to substantiate the claim that wheatgrass is an effective anticancer agent as well as an immunomodulator. Researchers have formulated a new extraction procedure to isolate compounds i.e. secondary metabolites and subsequently evaluated their bioactivities, particularly their immuno-modulatory and anti-cancer activities, using three solvent systems i.e. distilled Petroleum Ether (PET), 3% ethyl acetate (EtOAc) in PET, and 1:1 PET:DCM. Following is the discussion of the experiments that were conducted, to confirm the bioactivity as well as structural elucidation of compounds isolated from wheatgrass juice.

The Bhabha Atomic Research Center (BARC) has studied wheatgrass powder elemental content, physicochemical characteristics, compositional details, contaminants, and storage stability. Their quality analysis study identified that wheatgrass contains 13 vitamins, 16 minerals and all 20 essential amino acids. Wheatgrass is mostly 70% chlorophyll and the rest 30% is composition of proteins, carbohydrates, and amino acids[23]. The researchers from Girand Singh Memorial Degree College, Uttar Pradesh conducted a pilot study on wheat grass juice for its phytochemical, nutritional and therapeutic potential on chronic diseases and their experiments showed promising results towards anti-cancer activity, anti-ulcer activity, anti-inflammatory, antioxidant activity, anti-arthritic activity, and blood building activity in Thalassemia[24].

Before researchers performed analytical tests on the extracts, they conducted solubility experiments, followed by Thin Layer chromatography (TLC) and High performance thin layer chromatography (HPTLC) experiments respectively, on the test samples. The solubility tests indicated that the constituents of wheatgrass were highly polar and were clearly resolved in a methanol-and-hexane solvent system.

The rank order of solubility of wheatgrass powder i.e. according to eluting power of the solvent, i.e. according to its dielectric constant "ε" is as follows:

hexane (1.8)>chloroform (4.7) >ethyl acetate (6.0) >dichloromethane (8.9) >acetone (20.7) >methanol (32.6) > water (78.5)

After conducting the solubility test based on its outcome, researchers selected a TLC solvent system to generate the profile for wheatgrass powder. This test run was conducted in a development chamber using 9:1 DCM:methanol, and it showed the presence of 22 compounds as seen in Fig. 1. TLC of Wheatgrass powder in 9:1 DCM:methanol solvent system. These compounds were detected using four different types of reagents, i.e. Dragendroffs reagent, an iodine chamber, permanganate stain and 1% ninhydrin reagent. Spot screening of the dried TLC plate showed the presence of 22 spots that were green, pink, reddish-orange, greenish brown, and brown coloured, which indicated the presence of active biological compounds such as aldopentose, aldohexose, alkaloids, phenols, amino acids, amines, amino sugars, and flavonoids[25]. Trace metals such as Na, Fe, and Mn, were also observed on TLC plate as yellowish brown spots, using permanganate stain.

The TLC spectrum matched the HPTLC profile that was obtained with a 9:1 DCM:methanol (vol/vol) solvent system on a Camag™ HPTLC system, manufactured in Muttenz, Switzerland containing a Camag™ ATS 4 automatic TLC sample applicator and using a Hamilton syringe (100 µL). Densitometric identification of the spots was conducted at 235 nm

using winCATS Planar Chromatography Manager software and HPLC grade reagents.

After completing the solubility test, the TLC and HPTLC analysis on wheatgrass powder, researchers performed analytical testing on the isolated compounds, which were extracted using column chromatography technique followed by elucidating their structures. An FTIR experiment was conducted on the wheatgrass powder and on wheatgrass juice crystals; by comparison of their spectra seen in Fig. 4 i.e. the overlap of wheatgrass juice crystals spectra on the top & wheatgrass powder spectra at the bottom showed complete overlap of the functional group regions.

This spectra clearly indicated the presence of –OH, -CO, primary –NH, and –COOH groups in the wheatgrass powder.

The FTIR spectra also complemented the TLC spot analysis results, confirming the presence of phenol, amino acids, and flavonoids. LCMS and NMR analysis further confirmed the presence of different moieties, which assisted towards building the compound structure. M/z ion fragment values at 155, 293.5, 316, 475.5, 484.4 from LC MS data assisted towards elucidating and predicting the structure. From NMR analysis researchers observed peak values for proton NMR chemical shifts at 0.95δ (C=C), 1.25δ (C-NH2), 1.56δ(C-R_2), 3.50δ(C-OH), 7.2δ(C=O) that confirms the structural elucidation of the new compound.

Researchers concurred from their extensive literature review, that there have been no published studies reporting the extraction of compounds from wheatgrass powder or juice and their corresponding bioactivity studies.

The bioassay studies were conducted, that included, DTH assay, Immunomodulatory and Anti-Cancer experiments, on the two new isolated compounds WG1 IUPAC name: 2,28-diamino-3, 27-dihydroxynonacosan-12-one & YWG IUPAC name:5-bromo-2-(5-bromo-4-chloro-1,2-dihydroxy-4-methylcyclohexyl)-tetrahydro-2,6,6-trimethyl-2H-pran-3-yl-acetate.

The DTH assay showed that both compounds were having significant delayed immuno response. The immunomodualtion, lymphocyte proliferation experiment indicated that at different dose concentrations, the induction of B cell proliferation by the isolated compounds was comparable to or higher than the BMS standard. In contrast, these compounds showed moderate T cell proliferative activity. The anti-cancer, cytotoxic assay indicates both compounds are expressing anti-cancer activity.

In bioassay studies, these isolated compounds showed better bioactivities in comparison to the standard drugs researchers used i.e. Betamethasone (BMS) and 5-Fluorouracil (5 FU) in splenocyte proliferation and anti-cancer assay respectively.

Conclusion

This study scientifically confirms that wheatgrass is an effective, economical, easily available alternative or adjuvant treatment for cancer and an immunomodulator. Our body is mainly composed of specific and non-specific immune response system. The specific immune response includes humoral and cellular immunity. Humoral immunity, via the antibody response, is regulated by B cells and T immune cells are involved in antibody production. The WG1 fraction stimulated the humoral response against SRBCs, which was evidence by the increase in LPS value in mice, whereas the fraction YWG was effective on stimulating the T cells.

The two molecules, WG1: 2,28-diamino-3,27-dihydroxynonacosan-12-one and YWG: 5-bromo-2-(5-bromo-4-chloro-1, 2-dihydroxy-4-methylcycloh exyl)-tetrahydro-2, 6,6-trimethyl-2H-pran-3-yl-acetate extracted from wheatgrass exhibited anti-cancer and immunomodulatory activities.

Researchers, therefore propose that there is a need for an immunomodulator to normalise cellular degeneration in cancer patients, and propose the use of wheat grass juice (WGJ) for this purpose, to modulate defence mechanisms in cellular systems.

Conflict of Interest

The authors declare no competing financial interests.

Acknowledgement

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