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The medicinal and pharmacological screening of wheatgrass juice (*Triticum aestivum* L.): an investigation into chlorophyll content and antimicrobial activity

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Abstract

Plant products are of increasing interest in the search for new drugs and medicines in the treatment of disease; and the discovery of new alternatives to existing antibiotics is an important area of this research due to pathogen resistance. One example of a plant product currently under pharmacological investigation is wheatgrass juice; a dietary supplement derived from *Triticum aestivum* L. There is both anecdotal and scientific evidence to suggest that the medicinal efficacy of this plant may be due to its high chlorophyll concentrations. In this study chlorophyll fresh weight was determined in mg/100g for organic field grown wheatgrass juice and samples grown hydroponically in a glasshouse. Broccoli and Kale samples were included for comparison. Fresh undiluted wheatgrass juice was then screened against bacteria: *Escherichia coli* NCTC 10418; *Staphylococcus aureus* NCTC 6571 and *Streptococcus mutans* NCIMB 702062, using the agar well diffusion method. Acetone extracts were also tested. Results showed field grown samples had the highest chlorophyll content, not significantly different to Kale (p =0.834) and significantly higher than Broccoli (p <0.001). Hydroponically grown samples contained 73.4% less chlorophyll than field grown wheatgrass (p <0.001). None of the extracts tested displayed any kind of antimicrobial activity against selected pathogens. There is growing evidence that dietary chlorophyll may have cancer preventing properties by limiting the bio-availability of carcinogens, and wheatgrass juice appears to be a good source of this phytopigment. Although this study reported no antimicrobial activity, previous studies have reported conflicting results, indicating further investigation is required in this area.

Keywords: Antimicrobial, Chlorophyll, Medicinal plants, Wheatgrass juice, Pharmacology.

Introduction

The use of and search for drugs and dietary supplements derived from plants has accelerated in recent years (Cowan, 1999). Plant products are of increasing interest as a source of safer alternatives to synthetically produced drugs, therefore pharmacological screening of plant extracts is an important area of medical research. The protocol when investigating an herbal extract for medicinal value is to pre-screen samples in the laboratory for biological activity.

Wheatgrass juice (WGJ) is a dietary supplement derived from common wheat; *Triticum aestivum* L. WGJ is an aqueous form of the plant which is produced by juicing the young shoots, just before the jointing stage. The supplement is available commercially in liquid, powdered or concentrated forms, depending on the supplier and can be consumed on its own, or mixed with fruit juices. WGJ has been shown to have some medicinal value; a review of the scientific literature found studies reporting high levels of antioxidants (Falcioni et al. 2002; Kulkarni et al. 2006; Rana et al. 2011). It has demonstrated anti-cancer properties both *in-vitro* and *in-vivo*; (Alitheen et al. 2011; Arya and Kumar 2011; Aydos et al. 2011; Bar-sela et al. 2007) and has been found to reduce the frequency of blood transfusions in thalassemia patients. (Marwaha et al. 2004; Singh et al. 2010)

The benefits of WGJ have been attributed to its high chlorophyll content in much of the anecdotal literature. Scientific studies regarding the health benefits of chlorophyll have shown anti-cancer effects in animal models, and studies have been extended to human subjects. Two studies published in 2005 by de Vogel et al. found that chlorophyll inhibited haem-induced cytotoxicity and reduced epithelial cell turnover (hypoproliferation) in rat colons; the first (2005a) found this effect to be specific to natural chlorophyll and cannot be mimicked by Sodium Copper Chlorophyllin. The second study (2005b) found spinach and chlorophyll caused cytotoxic inhibition and concluded that green vegetables in the diet may decrease colon cancer risk, as chlorophyll prevents the detrimental, cytotoxic and hyperproliferative effects of dietary haem in rats.

A further two studies investigating the cytotoxic inhibitory effects of chlorophyll were carried out in rainbow trout: Simonech et al. (2008) found that low-dose dietary chlorophyll inhibited multi-organ carcinogenesis. This study demonstrated significant and substantial chemopreventative effects of natural chlorophyll against liver and stomach carcinogenesis in trout when administered by dietary co-exposure with carcinogen. A later study by McQuistan et al. (2012) found that chlorophyll enriched spinach extract provided increasing and potent protection against dibenzo[*def,p*]chrysene induced tumour response. These studies have been partially extended to human volunteers in an attempt to validate these findings.

The association of diets rich in phytochemicals with the prevention of cancer has intensified interest in chlorophyll as a class of plant pigments with potential chemopreventative effects (Ferruzzi et al. 2006). In 2009 Jubert et al. investigated the effects of chlorophyll (Chla) and chlorophyllin (CHL) on low dose aflatoxin pharmacokinetics in human volunteers. Aflatoxin is a carcinogenic mycotoxin associated with the growth of parasitic mould; and in the study human volunteers were administered low doses of aflatoxin along with Chla and CHL extracts. The conclusion was Chla and CHL co-consumption may limit the bioavailability of ingested aflatoxin in humans.

Dietary chlorophyll has demonstrated antioxidant and antimutagenic activity. Following the assumption that chlorophyll is unable to be absorbed by humans and the strong data to indicate antimutagenic activity *in-vitro*, a central hypothesis has been formed around the notion that mutagen trapping in the gastro intestinal tract is the primary mode of action by which chlorophyll and its derivatives deliver cancer preventative activity (Ferruzzi et al. 2006); although there is evidence to suggest natural chlorophyll derivatives are absorbable by human intestinal cells (Ferruzzi et al. 2001). However, in light of these findings there appears to be a lack of studies attempting to quantify fresh weight chlorophyll concentrations of plant materials. According to a cohort study by Balder et al. (2006) publications reporting chlorophyll content in vegetables seemed to be scarce in published literature.

Another common claim amongst the anecdotal literature is that WGJ has antimicrobial properties, inhibiting the growth of microorganisms. There appears to be a lack of published scientific data on this topic, highlighting an area for continued research. Further studies testing a wide range of pathogens against wheatgrass extracts would provide useful information and assist in our understanding and knowledge of the efficacy and actions of antimicrobial plant extracts.

Pallavi et al. (2011) tested wheatgrass extracts against the Gram-positive bacteria; *Staphylococcus aureus*, *Bacillus subtilis* and Gram-negative *Eschericia coli*; using Amoxicillin as standard. The findings were that certain extracts showed considerable activity against *Bacillus subtilis* and moderate activity against *Staphylococcus aureus* and *Escherchia coli.* Ashok (2011) also reported antibacterial activity against *E. coli; Pseudomonas aeruginosa* and *S. aureus*. Antifungal activity was also reported against *Candida albicans*.

Das et al. (2012) found 80% acetone extracts of wheatgrass to be effective against five relevant food-borne microorganisms, including the fungus *Aspergiullus niger;* a cause of black mould, which is a common contaminant of food.

Due to cumulative resistance to antibiotics in many bacteria, the development of new antiseptics and antimicrobial agents is of growing interest (Weckesser et al. 2006). Plants contain thousands of constituents and are valuable sources of new and biologically active molecules possessing antimicrobial properties (Das et al. 2010); including a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids, and flavonoids, which have been found to have antimicrobial properties *in-vitro* (Cowan, 1999). These products are of interest as a source of safer or more effective substitutes to synthetically produced antimicrobial agents (Mullen et al. 2012). WGJ has a high content of bioflavonoids (Padalia et al. 2010) which may contribute towards antimicrobial effects of the supplement.

The aim of this paper is to determine the chlorophyll content and the anti-microbial actions of commercially available WGJ supplements, from LiveWheatgrass Ltd. Chlorophyll content will be compared to other dietary plants reputed to contain high levels of this pigment, to give an indication of the concentrations. Organic field grown WGJ samples will also be compared to commercial WGJ grown hydroponically in a glasshouse to investigate whether production methods influence chlorophyll concentrations. Hydroponic samples will be referred to as tray grown.

The antimicrobial properties will be investigated against strains of bacteria relevant for a food supplement. Fresh, undiluted samples will be tested in the commercially available form, in an attempt to provide insight into any anti-microbial activity these products may possess, as consumed.

Materials and Methods

Samples

Frozen WGJ samples were received from LiveWheatgrass Ltd. The crop was grown organically in a field and samples were harvested, juiced and blast frozen; then kept at - 30˚C prior to dispatch. Samples were processed and delivered as they are to customers; packaged in individual 28ml plastic pots with lids. Broccoli, Kale and tray grown WGJ were included in the delivery for comparisons.

Extracts

Samples arrived pre-juiced; in homogenised form. Fresh extracts were prepared by defrosting each sample and centrifuging at 2000 rpm for 2 minutes, then carefully removing the supernatant with a pipette. Solvent extracts were dissolved in 80% acetone before centrifuging and separation from the sediment was carried out, as described above. 6 replicates for each plant sample were prepared.

Microorganisms

Relevant bacterial organisms were selected, which were suitable for testing a food supplement: *Escherichia coli* NCTC 10418; *Staphylococcus aureus* NCTC 6571 and *Streptococcus mutans* NCIMB 702062. *E .coli* and *S .aureus* are associated with food poisoning and gastro-intestinal infections. *S. mutans* is the leading cause of dental caries (tooth decay) worldwide.

Chlorophyll analysis

Chlorophyll content was determined using the procedure by Arnon (1949). Individual 28ml WGJ pots were defrosted in separate glass beakers labelled 1-6. The labelled beakers were placed into a bowl of tepid water and the sample was stirred periodically using a glass rod to avoid separation of the homogenate. Once completely defrosted, 0.3g of extract was removed from each beaker using a pipette and placed into individually labelled Fisher*brand* ®15ml centrifuge tubes, using digital scales to obtain an exact weight. 80% acetone was then added to make each tube up to a volume of 10ml of solution. The tubes were then balanced and spun in an MSE Centrifuge, at 2000 rpm for 2 minutes. The supernatant was then carefully removed from each tube using a pipette and placed into a 1ml glass spectrophotometer cuvette. Zero was set on a Unicam UV/Vis Spectrophotometer using a blank cuvette containing 80% acetone and readings were taken for each sample at 645nm (Chlorophyll *b*) and 663nm (Chlorophyll *a*). Readings were recorded and using the equations from Arnon (1949), the fresh weight of total chlorophyll from each sample was determined as mg/100g.

For chlorophyll comparisons, this procedure was repeated using homogenised 28ml samples of the following plants: Broccoli, Kale and tray grown wheatgrass. 6 replicates of each were obtained.

Antimicrobial analysis

Fresh homogenate samples taken from each replicate were centrifuged at 2000 rpm for 2 minutes and the supernatant removed. These samples; along with the acetone extracted samples, were pre-screened for anti-microbial activity using the agar well diffusion method against Gram-positive bacteria: *Staphylococcus aureus* NCTC 6571*; Streptococcus mutans* NCIMB 702062 and Gram-negative *Escherichia coli* NCTC 10418.

A broth suspension of each pathogen was obtained by using a loop of each bacterium to inoculate10ml of Muller-Hinton Broth in individual polymer tubes, which were labelled and placed in a benchtop orbital shaking incubator for 24 hours prior to assaying. Diagnostic Sensitivity Test Agar (D.S.T.Agar) plates were then seeded with each pathogen suspension using individual swabs; and using a sterile cork borer, wells were cut from the agar. Each well was filled with 50µl of 100% concentration extract using a pipette. Control wells containing 80% acetone, and distilled water were included. Plates were labelled and incubated at 37˚C for 24 hours before being analysed by observation. Activity is determined by presence of a zone of inhibition around each well.

Using the methods described above, 6 fresh WGJ homogenate extracts were prepared; using samples from 2012, these replicates were then tested against each bacterium. A positive control plate containing 100% Tea Tree oil (*Melaluca alternifolia*) was also prepared for each pathogen for comparison.

Results

Chlorophyll analysis

Chlorophyll content was determined for each plant sample using the formula by Arnon (1949) as fresh weight in mg/100g. Mean weights plus Standard Deviation were calculated using Microsoft Excel 2010©. These figures are presented in Table 1.

Table 1: Mean fresh weights of chlorophyll for each plant sample including Standard Deviation

Plant sample *n*=6	**Mean Chlorophyll mg/100g**		
	Chl *a*	**Chl *b***	**Total chl**
Field grown WGJ	63.02 ±2.32	20.08 ±1.80	83.10 ±2.91
Tray grown WGJ	14.90 ±5.47	7.19 ±3.47	22.09 ±5.51
Broccoli	6.73 ±0.67	4.23 ±1.01	10.97 ±1.66
Kale	59.65 ± 14.9	21.60 ±5.40	81.26 ±20.30

Comparisons between samples were subject to two-sample T-Tests using Minitab®. Significance was determined as a value of $p < 0.05$. No significance was found between Organic WGJ and Kale (p=0.834). Significant differences were seen between organic WGJ and Broccoli ($p < 0.001$) and between organic versus tray grown wheatgrass. ($p < 0.001$)

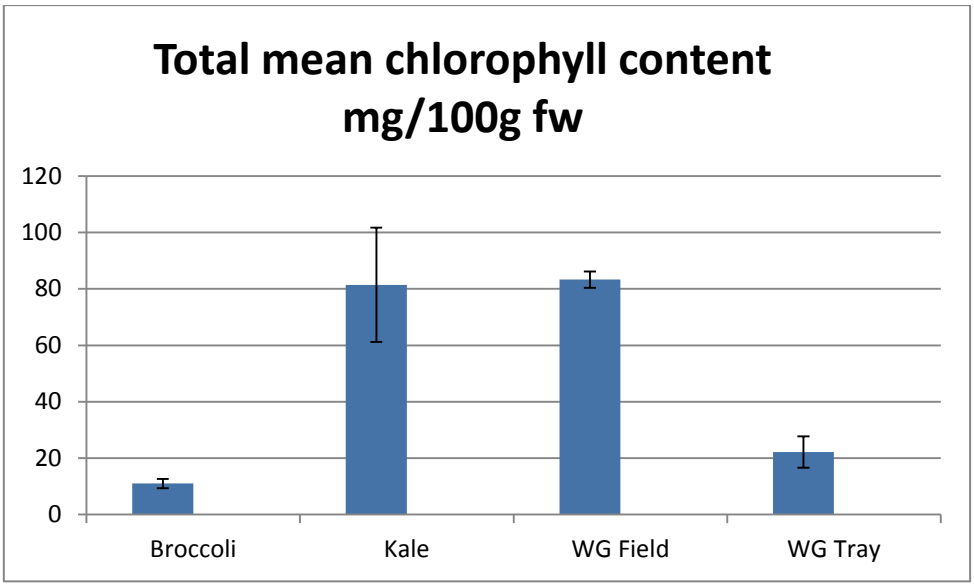

Figure 1: Chlorophyll content comparison between field grown WGJ, tray grown WGJ, Broccoli and Kale. 2xSD bars are included

Antimicrobial analysis

Initial pre-screened homogenate and acetone plant extracts; including organic and tray grown WGJ, broccoli and kale, taken from the chlorophyll analysis samples showed no antimicrobial activity against the selected pathogens. No inhibition was observed after 24 hrs.

Fresh, undiluted WGJ homogenate extracts from 2012 also showed no activity. However the tea tree oil positive control plates showed considerable activity against *E. coli*, moderate activity against *S. aureus* and no activity against *S. mutans.* See Table 2.

Table 2: Shows the zone of inhibition in millimetres of plant extracts against bacteria

Plant extract 50 µl at 100% concentration *n* =6	**Bacteria**		
	***E.coli* (NCTC 10418)**	***S. aureus* (NCTC 6571)**	***S.mutans* (NCIMB 702062)**
WGJ field grown	-	-	-
WGJ tray grown	-	-	-
Tea tree oil	50	12	-

Discussion

Organic field grown WGJ showed the highest fresh weight chlorophyll content, showing no significant difference to Kale (p =0.834) and a significantly higher content to Broccoli (p <0.001) and tray grown WGJ (p <0.001). It has been suggested that dietary chlorophyll-bound magnesium may play an important role in magnesium

nutrition. (Bohn et al. 2004) Dietary chlorophyll may also reduce the risk of cancer due to inhibiting cytotoxicity (Simonech et al. 2008; Jubert et al. 2009; de Vogel et al. 2005a & b; McQuistan et al. 2012). High intake of red meat is associated with increased colon cancer risk which may be due to the high haem content of red meat (Sesink et al. 2001; Balder et al. 2006). It has been suggested green vegetables in the diet may decrease colon cancer risk, as chlorophyll prevents the cytotoxic effects of dietary haem (de Vogel et al. 2005b).

Production methods appeared to influence WGJ chlorophyll concentrations. Tray grown wheatgrass: grown hydroponically in a glasshouse contained 73.4% less total chlorophyll fresh weight than the field grown samples. This could be due to the fact field grown plants are subject to higher levels of unfiltered sunlight. As plant development and physiology are strongly influenced by the light spectrum of the growing environment (Hogewoning, 2010), the light spectrum filtering effects of glass could influence pigment development. Research has shown that blue light is essential for chlorophyll synthesis in angiosperms (Kawabata et al. 2006).

None of the plant extracts in this study demonstrated antibacterial activity against the selected pathogens, except the tea tree oil control; which was included to compare samples against an extract previously shown to have efficacy. According to Carson et al. (2006), a wealth of *in-vitro* data now supports the long-held beliefs that tea tree oil has antimicrobial and anti-inflammatory properties. The aim was to test the samples with minimum extraction processes in order to obtain a true picture of any effects the extracts may have in the form in which they are consumed. Extracts in acetone were included but also showed no activity.

Pallavi et al. (2011) reported activity in acetone WGJ extracts against *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli*. Another paper reported activity in 80% acetone extracted samples against four bacteria: *Bacillus cereus*, *Staphylococcus aureus*, *Escherichia coli, Shigella flexneri* and one fungus: *Aspergillus niger,* all are food-related microorganisms (Das et al. 2012). However, Desai (2005) found acetone and methanolic extracts did not show any antibacterial activity, while fresh and undiluted wheatgrass juice exhibited mild antibacterial activity against *Staphylococcus aureus, Bacillus cereus, Salmonella typhimurium* and *Kleibsella pneumoneae.* It is interesting to note that in the study by Desai, the efficacy of fresh WGJ declined after 2 hours.

In this study the first set of extracts; which were taken from the chlorophyll assay samples, were defrosted and stored in a refrigerator for several days before antimicrobial testing took place. In light of findings regarding decreased efficacy over time, another set of samples were freshly defrosted in a tray of tepid water; as recommended by LiveWheatgrass Ltd. Extracts were then assayed immediately after centrifuging; but still showed no activity.

The main limitations of this study were the range of pathogens included only a small number of possible food-related bacteria and no fungal pathogens. Limited plant extraction methods were also employed. Further studies relating to WGJ extracts using a variety of solvent extraction methods, and screening against a wider range of both fungal and bacterial pathogens would provide a better picture of the antimicrobial potential of this supplement.

In light of the growing evidence for the chemopreventative effects of dietary chlorophyll; especially when co-consumed with potential carcinogens such as dietary haem, the lack of publications reporting chlorophyll content in vegetables is surprising. It would therefore be useful to continue investigations and compile a data base of foods high in this phytochemical.

Further investigations into the influence of production methods on chlorophyll concentrations would prove useful for both growers and companies wishing to exploit medicinally active phytochemicals.

Conclusion

The findings of his study are that organic field grown wheatgrass juice contains high amounts of chlorophyll: equal to kale, a dark green vegetable high in this phytochemical. There is growing evidence dietary chlorophyll may have cancer preventing properties by limiting the bio-availability of carcinogens and some evidence that co-consumption with red meat may reduce the incidence of haem induced cytotoxicity.

This particular study did not report any antimicrobial activity of WGJ extracts against the chosen bacteria. However there is evidence in the literature to suggest this supplement does possess some efficacy towards microbial pathogens. Therefore further screening against a range of different microorganisms, using different extraction methods would provide more data, thus contributing towards the knowledge on this topic.

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