Immunoprophylactic potential of wheat grass extract on benzene-induced leukemia: An in vivo study on murine model

Neelofar Khan, Aditya Ganeshpurkar, Nazneen Dubey, Divya Bansal

ABSTRACT

Objectives: Wheat grass (Triticum aestivum) is a gift of nature given to mankind. A number of scientific research on wheatgrass establishes its anticancer and antioxidant potential. Current work was focused to determine antileukemic effect of wheat grass.

Materials and Methods: The commercial wheatgrass powder was extracted with 95% of methanol. Methanol extract of wheat grass was studied for acute oral toxicity as per revised Organization for Economic Cooperation and Development Guidelines number 423. Leukemia was successfully induced in Wister rats by intravenous injection of benzene. The blood was collected and analyzed for hematological parameters. Phagocytotic activity of the extract was determined.

Results: Phytochemical screening revealed the presence of flavonoids, phenolics, carbohydrates, and amino acids. From acute toxicity studies, it was found that the methanol extract of wheatgrass was safe up to a dose level of 2000 mg/kg of body weight. Outcomes of hematological parameters in various experimental groups of murine model demonstrated antileukemic effect of extract. Methanol extract of wheatgrass aroused the process of phagocytosis of killed Candida albicans and also demonstrated a significant chemotactic activity at all tested concentrations.

Conclusion: In the current work, methanol extract of wheat grass demonstrated antileukemic potential that might be due to the presence of flavonoids and polyphenolics in it. Further isolation, structural characterization of active constituents is necessary to extrapolate the mechanism of action.

KEY WORDS: Benzene, flavonoids, leukemia, polyphenols, wheat grass

Introduction

From almost last century, benzene is regarded as “bone marrow poison.”[1] This “leukemogenic” property of benzene is due to the clastogenic effect that leads to produce chromosome aberrations, sister chromatid exchange, and micronuclei deformation. Trans-transmuconaldehyde, a metabolic product of benzene plays a key role in benzene mediated toxicity. The toxicity is further observed by DNA strand breakage, inhibition of topoisomerase II, and mitotic spindle damage. These consequences predispose to commencement of leukemia. Thus, the jeopardy of leukemia is allied with cumulative benzene exposures.[2-4] Thus, it becomes necessary to search for novel remedies against such terrified condition.

Nature serves to be a vital spring of medicinal herbs. Plants and macrofungi are considered to be a fundamental source of medicines and medicinal agents. Wheat grass from Triticum aestivum is a gift of nature given to mankind. The systematic viewpoint was engrossed to it whilst Schnabel, a food scientist established health promoting prospective of wheatgrass on chicks and hens.[5-7] A number of scientific research on wheatgrass establishes it’s anticancer[7,8] and antioxidant[9] potential. Current work was focused to determine antileukemic effect of wheat grass.
potential. Wheat grass is one of the richest sources of proteins, vitamins and minerals.

Recently, Alitheen et al., reported cytotoxic effects of wheat grass toward human acute promyelocytic leukemia cells (HL60)[10] demonstrating anti-proliferative and anti-apoptotic effect. Hence, we aimed to determine antileukemic potential of wheat grass on benzene-induced leukemia on Wistar rats.

Materials and Methods

The commercial wheat grass powder was used for extraction. As previously reported by Alitheen et al.,[10] methanol was used for extraction of wheat grass. Wheat grass was extracted with 95% of methanol in soxhlet apparatus. The extract was filtered using a muslin cloth and then with filter paper (Whatman number 4) and concentrated at rotary vacuum evaporator. Dried extract was stored at 4°C until use. The phytochemical analysis of the plant was carried out by the standard methods.[11]

Total soluble phenolic compounds in the extract were determined with Folin–Ciocalteu reagent according to the method of Slinkard and Singleton[12] using pyrocatechol as a standard phenolic compound. The total concentration of phenolic compounds in the extract determined as microgram of pyrocatechol equivalent (PE) by using an equation that was obtained from standard pyrocatechol graph:

\[
\text{Absorbance} = 0.0054 \times \text{total phenols (PE (μg))} - 0.0058.
\]

Total flavonoid content was determined using the method given elsewhere.[13] The concentrations of flavonoid compounds were calculated according to the following equation that was obtained from the standard quercetin graph:

\[
\text{Absorbance} = 0.0338 \times \text{quercetin (μg)} - 0.0002; R^2 = 0.9998
\]

Wistar rats of either sex (150–200 g) were used for the study. The animals were maintained in plastic cages at 22 ± 2°C with free access to pellet food and water. The experimental protocol was approved by the Institutional Animal Ethical Committee constituted as per the rules of the Committee for the Purpose of Control and Supervision of Experiments on Animals, India. After completion of the study, all the animals were euthanized by an overdose of anesthetic ether and the carcasses were disposed in accordance with institute regulations.

Methanol extract of wheat grass was studied for acute oral toxicity as per revised Organization for Economic Cooperation and Development Guidelines number 423.[14] The extract was devoid of any toxicity in rats when given in doses up to 2000 mg/kg by oral route. Hence, 200 and 400 mg/kg doses of extract were used for the study.

Leukemia was successfully induced in Wister rats by intravenous injection of 0.2 ml of a 1:10 diluted benzene solution (chloroform in water/2-propanol [50/50] v/v), given every 2 days for 3 consecutive weeks.[15] The methanol extract of wheat grass (200 mg/kg and 400 mg/kg; 0.2 ml; orally) was administered before, during, and after leukemia induction. Leukemia burden was assessed by comparing the hematological parameters at baseline and after leukemia induction in various experimental groups.

After 3 weeks of benzene injection and extract (as designed in the experimental protocol), animals in the respective groups were bled by cardiac puncture. The blood was collected into ethylenediaminetetraacetic acid vials, gently mixed, labeled, and analyzed. Samples were analyzed for packed cell volume, white blood cells (WBCs), red blood cells (RBCs), hemoglobin, platelets, lymphocytes, RBC distribution width, and mean platelet volume.

*Candida albicans* culture was incubated in Sabouraud broth overnight and then centrifuged to form a cell button and the supernatant was discarded. The cell button was washed with sterile Hank’s balanced salt solution (HBSS) and centrifuged again. This was done 3–4 times. The final cell button was mixed with a mixture of sterile HBSS and human serum in a proportion of 4:1. The final cell suspension of concentration 1 × 10^6 was used for the experiment.[16]

Goat blood (nonheparinized; 0.2 ml) freshly obtained from slaughterhouse was placed on a sterile glass slide and incubated at 37°C for 25 min to allow clotting. The blood clot was removed very gently, and the slide was drained slowly with sterile normal saline, taking care not to wash the adhered neutrophils (invisible). The slide consisting of polymorphonuclear neutrophils (PMNs) was flooded with a concentration of ferulic acid and incubated at 37°C for 15 min. The PMNs were covered with *C. albicans* suspension and incubated at 37°C for 1 h. The slide was drained, fixed with methanol, and stained with Giemsa stain.

The mean number of *Candida* cells phagocyted by PMNs on the slide was determined microscopically for 100 granulocytes using morphological criteria. This number was taken as phagocytic index (PI) and was compared with basal PI of control. This procedure was repeated for different concentrations (10 μg/ml, 20 μg/ml, 40 μg/ml, 100 μg/ml, and 1000 μg/ml) of the isolated compound.[17] Immunostimulation in percentage was calculated by using the following equation:

\[
\text{Stimulation (％) = [PI test – PI control] × 100/PI (control)}
\]

The results are expressed as mean ± standard error of mean. Experiments were always performed in triplicates. Statistical comparison was performed using analysis of variance followed by post-hoc Dunnet’s test (for in vivo studies) and Bonferroni’s test (for neutrophil locomotion and chemotaxis).

Results

Methanol extract of wheat grass was green-brown in color. Phytochemical screening revealed the presence of flavonoids, phenolics, carbohydrates, and amino acids. The total amount of phenolic content present in extract was found to be 626.4 ± 8.48 mg PE/100 g. By using the standard curve of quercetin (R^2 = 0.9998), the total flavonoid content of extract was found to be 217.54 ± 6.41 mg Quercetin equivalent/100 g.

From acute toxicity studies, it was found that the methanol extract of wheat grass was safe up to a dose level of 2000 mg/kg of body weight. No lethality or any toxic reactions were found up to the end of the study period. Hence, 200 and 400 mg/kg doses of extract were used for the study.

Outcomes of hematological parameters in various experimental groups of the murine model are demonstrated in Table 1. Remarkable increase in the posttreatment in Group II animals demonstrated the leukemogenic effect.

5-fluorouracil significantly inhibited the growth of leucocytes. Methanol extract of wheat grass significantly (P < 0.01) reduced leukocyte count.
which demonstrates success of wheat grass extract in treating leukemia when their baseline value was compared with the posttreatment values.

RBC counts were found to be increased in group IV and V animals when their baseline value was compared with the post-treatment values. This effect demonstrated nonhemolytic and nontoxic effect of wheat grass extract. Treatment of animals with benzene and wheat grass extract significantly altered levels of hemoglobin and platelets. Significant reduction in hemoglobin and increase in platelets was observed in extract treated group [Table 1].

**Phagocytotic Studies and Chemotaxis**

Methanol extract of wheat grass aroused the process of phagocytosis of killed *C. albicans*. The mean particle numbers were found to be 4–5, 4 and 4 for ferulic acid at concentrations of 1000 µg/ml, 100 µg/ml, and 40 µg/ml, respectively, when compared to positive control-pooled serum at the same concentrations [Table 2].

Methanol extract of wheat grass demonstrated a significant \( P < 0.05 \) chemotactic activity at all tested concentrations. Mean number of neutrophils per field for extract was found to be 138.02, 129.9, 122.3 at concentrations of 1000

\[ \mu g/ml \]

and 40

\[ \mu g/ml \]

respectively, when compared to the standard-casein [Figure 1].

**Discussion**

Leukemia is a hematological cancer that results from inhibition of differentiation of hematopoietic stem cells that arise due to a range of epigenetic faults. This pathological situation is exemplified by hysterical propagation of myeloid blasts. At present, chemotherapy with anthracyclines and cytarabine is customary loom. However, remission is observed with these drugs. Benzene is an important industrial solvent that is one of the reason for aplastic anemia and leukemia in humans and experimental animals. Biotransformation of benzene results in the formation of phenol, catechol, hydroquinone which are "hematoxic and genotoxic."[14] Current testimony point toward the formation of benzene oxide from benzene *in vitro* and *in vivo* and reaches bone marrow leading to leukemia.[13]

The current work was focused to evaluate anti leukemic effect of wheat grass extract on murine model. Previous report on *in vitro* antileukemic effect of methanol extract of wheat grass was the scientific basis of this study.[10] Leukemia is observed by an increase in WBC. Such cancerous cells check healthy red cells and platelets from being made creating a life-threatening condition. As discussed previously, benzene was chosen to induce leukemia in murine model. Administration of methanol extract of wheat grass significantly reduced the number of free circulating WBCs. Such a significant similar effect was also observed in 5-flourouracil treated animal group.

Platelet number is also altered during leukemia. There is also impairment in release of platelets during leukemia.[21] However in the current work, administration of methanol extract significantly increased platelets in postanalytical/posttreatment group.

Environment around neoplasm is often ignored while the therapeutic response to chemotherapeutic agent is taken into account. Such surrounding often comprise of extracellular matrix components along with soluble factors. Influence of chemotherapeutic environment along with alterations in cellular environment results in inhibition of apoptosis of cancer cells leading to drug resistance. Prevention of cell adhesion with a number of factors could be an important strategy for prevention of “failure of chemotherapy.” At this moment defense system of body plays a crucial role.

PMNs have been related with competent innate defense mechanisms. PMNs activated by endotoxic/phagocytic stimuli, which play an important role in “host defense.” In the current studies, extract caused a reduction in the percentage of reduced neutrophils after treatment.

"Engulfment of microorganism by leukocyte” is amongst foremost “defense mechanism” that is inherited in organism.[17]
In the present work, extract caused enhanced “neutrophil chemotactic movement” and it establishes the fact that methanol extract of wheat grass act as “chemo-attractant.”

Chemo-attractants induce “attraction of neutrophils toward certain chemicals.” Neutrophil locomotion and chemotaxis are the course of action for chemoattraction.[22][23] In this procedure, extract was examined for chemo attractant potential and mean number of neutrophils attracted per field was noted against positive control-casein. Methanol extract of wheat grass caused increase in number of neutrophils attraction per field.[23]

Previously, some medicinal agents such as *Coriolus versicolor* [24] and *Ganoderma lucidum*, [25] which are rich in flavonoids, are known for antileukemic potential. These herbal medicines control leukemia by diverse mechanisms.

Flavonoids and polyphenols are important class of phytoconstituents, which are found in fruits and vegetables. In the present study, wheatgrass extract was found to be rich in flavonoids and polyphenols. Flavonoids are known to inhibit the activity of “angiogenic mediators” and provoke “apoptosis,” they also exert “antiproliferative” effect on leukemic cells.[26] Quercetin and flavopiridol have demonstrated antiproliferative effects on leukemic cells. [27]

Some other flavonoids such as genistein, honokiol, machilin A, matairesinol, and arctigenin have also shown lethal effects on leukemia cell lines.[28] In the current work, methanol extract of wheat grass demonstrated antileukemic potential that might be due to the presence of flavonoids and polyphenolics in it. Further isolation, structural characterization of active constituents is necessary to extrapolate the mechanism of action.

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Conflicts of Interest
There are no conflicts of interest.

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