IJPSR (2024), Volume 15, Issue 8 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 09 January 2024; received in revised form, 03 July 2024; accepted, 06 July 2024; published 01 August 2024

EX-VIVO STUDIES OF THE EFFECT OF WHEATGRASS ON GLUCOSE RELEASE AND GLUCONEOGENESIS USING CORTISOL-INDUCED HEPATOCYTES

Kavitha G. Singh *, S. Saleema Sultana, Sonali Angela D. Almeida and Sanjana Amarnath Kadur

Department of Biochemistry, Mount Carmel College, Palace Road, Bangalore - 560052, Karnataka, India.

Keywords:

Wheatgrass, *Triticum aestivum*, Cortisol, Cell-viability, *Ex-vivo* studies, Glucose release, Gluconeogenesis

Correspondence to Author:
Kavitha G. Singh

Department of Biochemistry, Mount Carmel College, Palace Road, Bangalore - 560052, Karnataka, India.

E-mail: kavi182@yahoo.co.in

ABSTRACT: Cortisol is one of the vital glucocorticoids secreted by the adrenal glands and is also referred to as the "stress hormone" as the synthesis and release of cortisol by the adrenal glands is directly proportional to the stress levels in an individual. It is a catabolic hormone that stimulates gluconeogenesis and has an indirect role in liver and muscle glycogenolysis. It facilitates lipid, protein and carbohydrate metabolism. Over the years medicinal grasses have been in the highlight for their potent medicinal value. Hence, Wheatgrass was chosen for study. Wheatgrass is derived from the common wheat plant. It is the grass of the common wheat plant. It belongs to the Poaceae family. Wheatgrass is the freshly sprouted first leaves of the wheat plant (*Triticum aestivum*). The Wheatgrass extract was obtained from methanol. Phytochemical screening was conducted which showed the presence of carbohydrates, phenols, flavonoids, terpenoids, tannins, alkaloids, glucosides, proteins, steroids, saponins and fixed oils. Wheatgrass was taken and cell viability assay was done using MTT dye. This assay indicated that methanolic extract of wheatgrass decreased cell viability. It showed cytotoxic activity and inhibited cell propagation. *Ex-vivo* studies were also carried out using Wheatgrass and cortisol. In these ex-vivo studies hepatocytes were used to determine the glucose release and gluconeogenesis. In these experiments the methanolic extracts of wheatgrass lowered cortisol activity which led to the significant reduction in liberation of glucose in condition of stress, starvation, as well as normal conditions. The glucose released was measured at regular time intervals in terms of percentage reduction.

INTRODUCTION: Cortisol is a steroid hormone synthesized by the zona fasciculate of the adrenal cortex. It is one of the major glucocorticoids natively secreted by the adrenal glands. Cortisol is a catabolic hormone. There are various hormones involved in the secretion of cortisol.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.15(8).2296-04

This article can be accessed online on www.ijpsr.com

DOI link: https://doi.org/10.13040/IJPSR.0975-8232.15 (8). 2296-04

Its production is modulated by pituitary hormone, hypothalamic hormone, corticotrophin-releasing hormone (CRH), adrenocorticotropic releasing hormone (ACTH) [1]. There are two forms of cortisol existing in blood. A large quantity of cortisol is bound to carrier proteins and the soluble free form of cortisol is found in a small portion [2].

About 90% of cortisol is bound to albumin and cortisol binding globulin (CBG) [3]. The secretion of cortisol is majorly dependent on the circadian rhythms and on behavioural patterns to a lesser extent [4]. It induces lipolysis, as well as disintegration of body tissues such as bone, muscle

and skin. It has a major role in fat redistribution in the body [5]. Elevation and demotion of cortisol has various effects on the human body. Cortisol levels are increased in type 2 diabetic patients [6]. Hypercortisolism is majorly responsible for the occurrence of insulin resistance, diabetes mellitus, cardio vascular disease *etc* [7]. This influences insulin to store glucose as glycogen in adipose tissues and muscle tissues. This inhibits glycogenolysis and hepatic gluconeogenesis [8]. Insulin resistance caused due to cortisol has an adverse effect on protein and lipid metabolism [9]. Local cortisol excess is responsible for hypertension. Cortisol induced hypertension is due sodium retention and volume expansion [10].

In hypertension the blood pressure in the arteries is persistently elevated [11]. Amenorrhoea has been observed in women with elevated levels of cortisol [12]. Concerns regarding elevated cortisol levels and early pregnancy termination have also been raised [13]. Several studies carried out suggest that offspring of mothers with high levels of glucocorticoids face an increased risk of developing metabolic, cardiovascular and neurobiological pathophysiologies [14]. Long term exposure to stress results in consistently high levels of cortisol and corticosteroids, leading to developing resistance to cortisol and an impaired immune system. This can result in occurrence of chronic infections, chronic inflammatory diseases, or cancer [15].

Hypercortisolism causes increased levels of interlukin-1 (IL-1) interlukin-6 (IL-6) and tumour necrosis factor alpha (TNFα) and infiltration of adipose tissue by immune cells. Thus, persistent high levels of cortisol involve a complex combination of low grade, non-resolving inflammation, and selectively impaired immune response [16]. Cortisol promotes occurrence of CVD in many ways. It inhibits growth hormone, the deficiency of which is linked to premature cardiovascular diseases in adults. Inhibition of growth hormone and visceral fat promotes visceral fat accumulation. This excess fat can mead to insulin resistance, dyslipidaemia and other medical illnesses [17]. Wheatgrass is derived from the common wheat plant. The young grass shoots of the wheat berry are the wheatgrass plant. Wheatgrass bears semblance to normal grass. It may have a dark green or bright green colour to it. It has a very characteristic odour and an acrid taste. The culms of wheatgrass are simple, hollow and smooth [18]. It is extensively used as a food, drink, or dietary source. Wheatgrass can be served fresh or freeze dried. It contains chlorophyll, vitamins, minerals, amino acids and a wide range of enzymes [19]. Wheatgrass shows anti-inflammatory, anti-carcinogenic, diuretic, anti-ageing, anti-bacterial and immunomodulatory properties [20].

Wheatgrass has many benefits, including anti-diabetic, antioxidant, anti-cancer, anti-inflammatory, anti-cholesterol, cardioprotective and hepatoprotective properties [21]. It was shown to have prominent anti-diabetic activities. It lowers blood glucose levels effectively due to its high fibre content. It has also been proven that its high concentration of chlorophyll reduces blood sugar levels [22]. Wheatgrass is well-known to reduce cholesterol levels. It has been experimentally proven to reduce the levels of LDL while also increasing levels of HDL [23].

MATERIALS AND METHODS:

Sample Collection: Wheat seeds of Raksha 999 variety were obtained from Ballia district, Uttar Pradesh. The seeds were sowed after soaking them in water for 24 hours. The grass was exposed to indirect sunlight and allowed to grow for 8–10 days. Wheatgrass was then harvested, and sample extract was prepared. This process was repeated intermittently throughout the course of the project.

Sample Preparation: A 10% sample extract was prepared. 10g of fresh wheatgrass was homogenised in 100ml of methanol and centrifuged at 10,000rpm for 10 minutes. The methanolic extract of wheatgrass was stored in airtight containers at 4℃.

Phytochemical Screening:

Carbohydrates - Benedict's test: 1 mL of the sample extract was taken in a test tube, to which Benedict's reagent was added and heated gently. The formation of orange-red precipitate indicates the presence of carbohydrates [23].

Flavonoids: 1mL of the sample was taken in a test tube, and few drops of 20% sodium hydroxide was added to it. The appearance of a yellow colour indicated the presence of flavonoids [24].

Phenols: 1mL of the sample was taken in a test tube, to which 0.2mL of ferric chloride solution was added. The appearance of a violet colour indicated the presence of phenols [23].

Terpenoids: The sample was taken in a test tube, and 2mL of chloroform and 2mL of sulphuric acid were added. The formation of a white layer with a reddish-brown interface indicated the presence of terpenoids [23].

Alkaloids – Dragendorff's test: The sample was taken in a test tube, and concentrated hydrochloric acid was added to it, followed by the addition of Dragendorff's reagent. The formation of a reddish-brown colour indicated the presence of alkaloids [24].

Fixed oils: 1mL of the extract was mixed with 1mL of 0.5N potassium hydroxide. The appearance of a pink colour indicated the presence of fixed oils [25].

Proteins: 1mL of the sample was mixed with 5% sodium hydroxide and 1% copper sulphate solutions. The appearance of a purple colour indicates the presence of proteins [23].

Tannins: In a test tube containing 5.0 mL of plant extract, a few drops of 1% solution of lead acetate were added. Formation of yellow or red precipitate indicates the presence if tannins [24].

Steroids: To 2.0 mL of sample extract 1.0 mL of concentrated sulphuric acid was added carefully along the sides of the test tube. Formation of red colour chloroform layer indicates the presence of steroids [24].

Saponins: In a test tube containing 5 mL of sample extract, a few drops of sodium bicarbonate were added. The mixture was shaken vigorously for 3 minutes. Presence of a honeycomb like froth indicates the presence of saponins [24].

Glycosides: To a small volume of extract dissolved in water, aqueous solution of sodium hydroxide was added. Formation of a yellow colour indicates the indicates the presence of glycosides [24].

Study of Cell Viability - MTT Assay: The MTT assay is performed to determine the cellular metabolic activity, which indicates cell viability, proliferation and cytotoxicity [25]. The adipose tissue of *Sus scrofa domesticus* (obtained from Bangalore Ham Shop, M.G. Road, Bangalore) was used for the study of the effect of sample extract on cell viability.

In this assay, a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) or MTT is reduced to form purple formazan crystals by metabolically active cells. The viable cells contain the enzyme NAD(P)H–dependent oxidoreductase enzymes which reduces the MTT to formazan. The insoluble formazan crystals that are formed is then dissolved using a solubilization solution to yield a purple color. The intensity of the color formed is directly proportional to the number of cells that are viable

1g of adipose tissue was accurately weighed and cultured in DMEM medium. The cells were treated with increasing concentrations of methanolic extract of wheatgrass. The cells were then incubated for 24 hours at 37°C. This was followed by the addition of 20µL of 5mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (Sigma Aldrich) into each well and was kept for incubation for 4 hours. 0.04N HCl made in isopropanol was added to stop the reaction and solubilize the crystals. The color produced was observed and recorded.

***Ex-vivo* Studies on Glucose Release by Hepatocytes:**

Liver Tissue Source: Liver tissue of *Capra aegagrus hircus* was obtained from R T Nagar, Bangalore, Karnataka, India. Fresh liver tissue was rinsed with normal saline. Approximately 1g of the liver tissue was sliced and placed into a six-well culture plates. Into each well, 4 mL of saline was added. Under these conditions, the liver cells are viable for approximately six hours and the entire experiment was performed within 4 hours [26].

Cortisol (2 mg/mL) was obtained from Vishal Medical Pharmacy, R T Nagar, Bangalore. Using a micropipette, 40µL of cortisol was added to previously added liver and saline mixture.

Estimation of Glucose Release by Cortisol Activity: Cortisol is a glucocorticoid hormone, that is produced by the adrenal glands present on top of the kidneys. Cortisol levels in the body significantly influence carbohydrate, protein and fat

metabolism. Liver can utilize substrates such as sodium pyruvate, amino acids and glycerol, to produce glucose. The glucose released was estimated using di-nitro salicylic acid (DNS) method.

The standard reaction mixtures were prepared as follows:

A. Liver + Normal saline: 1g of the liver sample was weighed and placed in a cell culture plate. 4 mL of saline was added to these plates. The glucose released by the liver cells was estimated at zero minutes and after 30, 60, 90 and 120 minutes.

B. Liver + Normal saline + Cortisol: 1g of the liver sample was weighed and placed in a cell culture plate. To the liver, 4 mL of saline and 40 µL of cortisol was added. The glucose release was estimated at zero minutes and after 30, 60, 90 and 120 minutes.

C. Liver+ normal saline + Sodium pyruvate: 1g of the liver sample was weighed and placed in a cell culture plate. To the liver, 4mL of saline and 400µL of Sodium pyruvate was added and the glucose released by the liver cells was estimated at zero minute and after 30, 60, 90 and 120 minutes.

D. Liver + Normal Saline + Sodium pyruvate + Cortisol: 1g of the liver sample was weighed and placed in a cell culture plate. To the liver, 4mL of saline, 400µL of Sodium pyruvate and 40 µL of cortisol was added and the glucose released by the liver cells was estimated at zero minute and after 30, 60, 90 and 120 minutes.

0.5 mL of the reaction mixture was taken, and the volume was made up to 3 mL using distilled water. To this 3 mL of DNS Reagent was added and the solution was placed in boiling water bath for a period of 5 minutes. The absorbance was read at 575 nm.

***Ex-vivo* Study of Cortisol Activity in Presence of Sample Extract:** Inference of cortisol on glucose release: 1g of liver was weighed and placed in a cell culture plate. To the liver, 4mL of saline, 4mL of sample extract and 40µL of cortisol was added and the glucose released by the liver cells was estimated at zero minute and after 0, 30, 60, 90 and 120 minutes.

Inference of Cortisol on Gluconeogenesis: 1g of liver was weighed and placed in a cell culture plate. To the liver, 4mL of saline, 4mL of sample extract, 40µL of cortisol and 400µL of sodium pyruvate was added and the glucose released was estimated at zero minute and after 0, 30, 60, 90 and 120 minutes. The glucose released was estimated using di-nitro salicylic acid (DNS) method.

RESULTS AND DISCUSSION:

Qualitative Analysis of Phytochemicals:

TABLE 1: QUALITATIVE ANALYSIS OF PHYTOCHEMICALS. THE RESULTS ARE PRESENTED AS + FOR POSITIVE RESULT AND - FOR NEGATIVE RESULT

Parameters	Aqueous extract	Methanolic extract
Carbohydrates	+	+
Flavonoids	+	+
Phenols	+	+
Terpenoids	+	+
Alkaloids	+	+
Tannins	+	+
Glycosides	+	+
Proteins	+	+
Steroids	+	+
Saponins	+	-
Fixed Oils	-	-

In this study aqueous and methanolic extracts of wheatgrass were screen for the presence of bioactive compounds. Both aqueous and methanolic extracts were found to contain carbohydrates, flavonoids, phenols, terpenoids, alkaloids, tannins, glycosides, proteins, and steroids. Aqueous extracts contained saponins, but methanolic extract of wheatgrass tested negative for the presence of saponins. Both aqueous and methanolic extract of wheatgrass tested negative for the presence of fixed oils.

Cell Viability Assay: Incubating the adipose tissue with MTT, resulted in the formation of a dark violet colouration, indicating formazan precipitation. The amount of colour is directly proportional to the viability of the adipocytes. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the conversion of the water-soluble yellow dye MTT to an insoluble purple formazan by living cells which determines mitochondrial activity.

The mitochondrial activity, for most cell populations is related to the number of viable cells [27]. MTT assay, among various product by living cells, is one of the most versatile and popular assays [28]. It is rapid and highly reproducible. It can be used for large scale antitumor drug-screening program.

The treatment of adipocytes with increasing volumes of methanolic extract of wheatgrass under ex-vivo conditions rendered a decrease in purple colouration as the volume of extract increased in comparison to the control cells that were untreated as seen in **Fig. 1**. This essentially indicated that methanolic extracts of wheatgrass has cytotoxic activity and inhibits cell proliferation. Active constituents of wheatgrass can thus be effective candidate for the treatment of cancer.

Methanolic extracts of wheatgrass has been found to effectively reduce cell viability. In addition to this, studies involving cell cycle analysis showed that the extract treatment resulted in G_1 arrest. Cyclin D1 levels were found to decrease while p53 levels were increased [29]. The effect of wheatgrass on oral squamous cell carcinoma has been studied, and the results indicate the same effect, inhibition of oral cancer cell line proliferation[30]. Antiproliferative, apoptotic and antioxidant studies of wheatgrass extract on chronic myeloid leukaemia (CML) cell line were carried out and the results showed that both aqueous and alcoholic preparations of wheatgrass inhibited the growth of leukaemia cells. This finding represents a novel therapeutic approach for the treatment of CML[31].

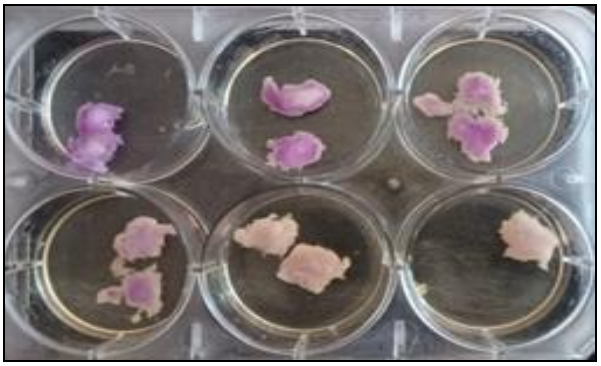

FIG. 1: ADIPOCYTES TREATED WITH MTT FOR CELL VIABILITY

***Ex-vivo* Studies on Glucose Release and Gluconeogenesis Study by Hepatocytes:**
Glucose Release and Gluconeogenesis Study:
Effect of Wheatgrass on Glucose Release by Cortisol-Induced Liver:

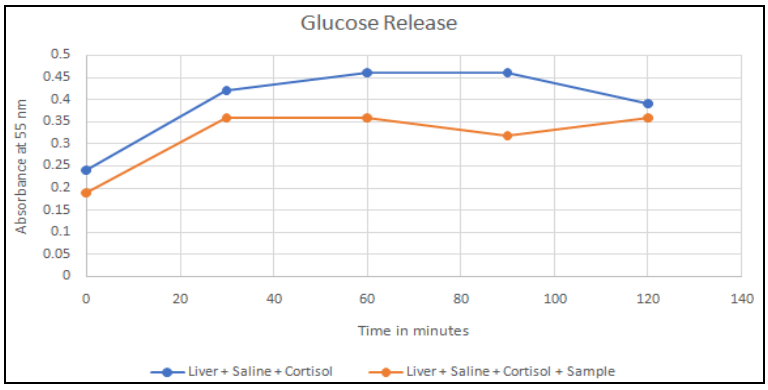

FIG. 2: EFFECT OF WHEATGRASS ON GLUCOSE RELEASE BY HEPATOCYTES (MEAN +/- SD)

	0	30	60	90	120
Liver + Saline + Cortisol	0.24	0.42	0.46	0.46	0.39
Liver + Saline + Cortisol + Sample	0.19	0.36	0.36	0.32	0.36

The effect of the sample (10% methanolic extract of wheatgrass) on hepatocytes treated with cortisol showed a significantly lower concentration of glucose released, when estimated using Miller's DNS method of glucose estimation.

- The control (liver + saline + cortisol) showed absorbance readings of 0.37, 0.6, 0.68, 0.56 and 0.63 when measured at time intervals of 0, 30, 60, 90 and 120 minutes.
- Cortisol-induced liver on treatment with the sample showed absorbance readings of 0.19, 0.36, 0.36, 0.32 and 0.36 when measured at time intervals of 0, 30, 60, 90 and 120 minutes.

Study of the Reduction (%) of Glucose Release by Wheatgrass Extract:

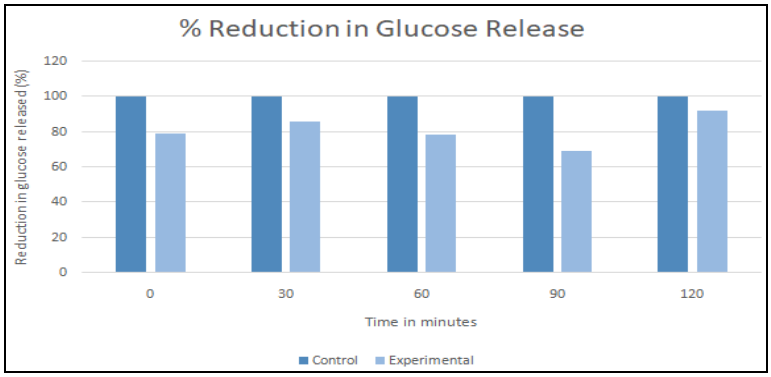

FIG. 3: REDUCTION OF GLUCOSE RELEASED BY WHEATGRASS EXTRACT (MEAN +/- SD)

The addition of 10% extract of wheatgrass showed significant reduction in the glucose release, as verified spectrophotometrically. In this experiment, the standard control, liver + saline + cortisol, was considered to show 100% glucose release, and the reduction in glucose release was measured at regular time intervals in terms of percentage reduction.

- At the 0th minute, the extract was found to have reduced the amount of glucose released by 20.83%, implying that the glucose released by the experimental sample was 79.17% that of the control.
- At the 30th minute, the extract was found to have reduced the amount of glucose released by 14.28%, implying that the glucose released by the experimental sample was 85.72% that of the control.
- At the 60th minute, the extract was found to have reduced the amount of glucose released by 21.73%, implying that the glucose released by the experimental sample was 78.27% that of the control.
- At the 90th minute, the extract was found to have reduced the amount of glucose released by 30.43%, implying that the glucose released by the experimental sample was 69.57% that of the control.
- At the 120th minute, the extract was found to have reduced the amount of glucose released by 7.69%, implying that the glucose released by the experimental sample was 92.31% that of the control.

This experiment has proven that 10% methanolic extracts of wheatgrass can significantly lower the amount of glucose released by hepatocytes under conditions of stress and starvation, as well as normal conditions.

On average, wheatgrass extract was found to have reduced the amount of glucose released by hepatocytes by 18.99%. This therefore implies the role of wheatgrass extracts in lowering cortisol activity, by lowering the amount of glucose released.

Gluconeogenesis Study:

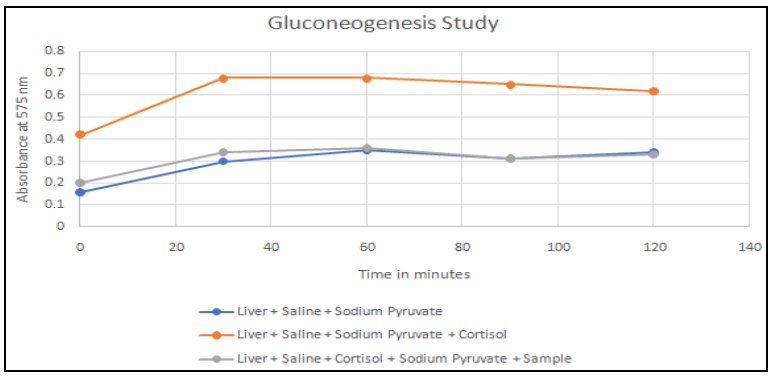

FIG. 4: EFFECT OF WHEATGRASS ON GLUCONEOGENESIS (MEAN +/- SD)

	0	30	60	90	120
Liver + Saline + Sodium pyruvate	0.16	0.3	0.35	0.31	0.34
Liver + Saline + Sodium pyruvate + Cortisol	0.42	0.68	0.68	0.65	0.62
Liver + Saline + Cortisol + Sodium pyruvate + Sample	0.2	0.34	0.36	0.31	0.33

The effect of the sample (10% methanolic extract of wheatgrass) along with sodium pyruvate on hepatocytes treated with cortisol showed a significantly lower concentration of glucose released, when estimated using Miller's DNS method of glucose estimation.

- The control (liver + saline + sodium pyruvate + cortisol) showed absorbance readings of 0.42, 0.68, 0.68, 0.65 and 0.62 when measured at time intervals of 0, 30, 60, 90 and 120 minutes.
- Cortisol-induced liver on treatment with the sample along with sodium pyruvate, showed absorbance readings of 0.2, 0.34, 0.36, 0.31 and 0.33 when measured at time intervals of 0, 30, 60, 90 and 120 minutes.

Study of the Reduction (%) of Glucose Release during Gluconeogenesis by Wheatgrass Extract: The addition of 10% extract of wheatgrass showed significant reduction in the glucose release, as verified spectrophotometrically. In this experiment, the standard control, liver + saline + sodium pyruvate + cortisol, was considered to show 100% glucose release, and the reduction in glucose release was measured at regular time intervals in terms of percentage reduction.

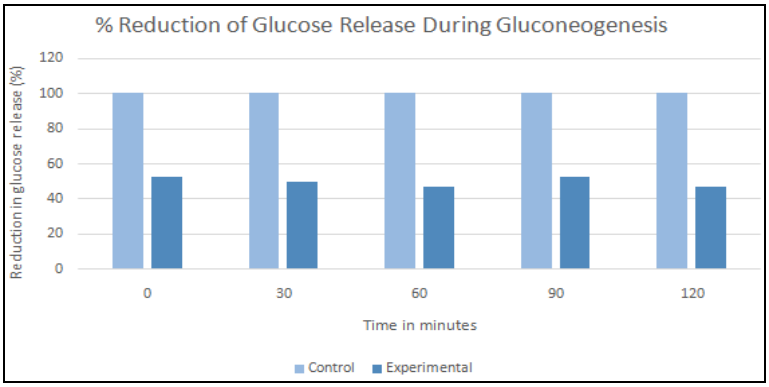

FIG. 5: REDUCTION OF GLUCOSE RELEASE DURING GLUCONEOGENESIS BY WHEATGRASS EXTRACT (MEAN +/- SD)

The addition of 10% extract of wheatgrass showed significant reduction in the glucose release, as verified spectrophotometrically. In this experiment, the standard control, liver + saline + sodium pyruvate + cortisol, was considered to show 100% glucose release, and the reduction in glucose release was measured at regular time intervals in terms of percentage reduction.

- At the 0th minute, the extract was found to have reduced the amount of glucose released by 47.61%, implying that the glucose released by the experimental sample was 52.38% that of the control.
- At the 30th minute, the extract was found to have reduced the amount of glucose released by 50%, implying that the glucose released by the experimental sample was 50% that of the control.
- At the 60th minute, the extract was found to have reduced the amount of glucose released by 52.94%, implying that the glucose released by the experimental sample was 47.05% that of the control.
- At the 90th minute, the extract was found to have reduced the amount of glucose released by 47.69%, implying that the glucose released by the experimental sample was 52.30% that of the control.
- At the 120th minute, the extract was found to have reduced the amount of glucose released by 53.22%, implying that the glucose released by

the experimental sample was 46.77% that of the control.

This experiment has proven that 10% methanolic extracts of wheatgrass can significantly lower the amount of glucose released during gluconeogenesis by hepatocytes under conditions of stress and starvation, as well as normal conditions. On average, wheatgrass extract was found to have reduced the amount of glucose released by hepatocytes by 50.29%. This therefore implies the role of wheatgrass extracts in lowering cortisol activity, by lowering the amount of glucose released.

ACKNOWLEDGMENT: The authors would like to acknowledge the Department of Biochemistry (PG Biochemistry) and the management of Mount Carmel College Autonomous, Bengaluru, for backing this project and providing their facilities for the study.

CONFLICTS OF INTEREST: Nil

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How to cite this article:
Singh KG, Sultana SS, Almeida SAD and Kadur SA: *Ex-vivo* studies of the effect of wheatgrass on glucose release and gluconeogenesis using cortisol-induced hepatocytes. Int J Pharm Sci & Res 2024; 15(8): 2296-04. doi: 10.13040/IJPSR.0975-8232.15(8).2296-04.