IJPSR (2017), Volume 8, Issue 8 **(Research Article)**

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 18 January, 2017; received in revised form, 13 July, 2017; accepted, 27 July, 2017; published 01 August, 2017

THE REDUCTION IN CORTISOL INDUCED STRESS MARKERS BY THE AQUEOUS EXTRACT OF *CLERODENDRUM COLEBROOKIANUM*

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Keywords:

Clerodendrum colebrookianum, Phuihnam, Cortisol, Gluconeogenesis, *Ex-vivo* studies, *Sus scrofa domesticus* hepatocytes

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ABSTRACT: In the present competitive world, stress is a major cause for several health disorders– Hypertension, Cardiovascular disorders, Supressed Immunity and other conditions. Proper stress management takes on great importance given the wide range of bodily systems impacted by stress hormones. Cortisol – the "stress hormone" affects the liver by increasing several biochemical pathways. One of the prominent stress marker is increase in gluconeogenesis. Due to the growing importance of herbal medicines, *Clerodendrum colebrookianum* was chosen for the study, commonly called as *Phuihnam* by the Mizo tribe of north eastern India. Previously carried out qualitative studies indicated the presence of alkaloids, flavonoids, steroids, saponins, phenolscardiac-glycosides and anthraquinones in *Phuihnam*. *Ex-vivo* studies were carried out using the hepatocytes from *Sus scrofa domesticus*. Our study indicated a high glucose release having an absorbance about 0.335 ± 0.001 at 520 nm after one hour of incubation when cortisol was induced but on addition of the boiled extract the absorbance drastically reduced to 0.124 ± 0.010 at 520 nm. Similarly on addition of sodium pyruvate as substrate along with cortisol, the boiled extract proved to show more than 50% of reduction in glucose release indicating it as a potent source of reducing cortisol activity. The results of the present work suggests that the boiled form of the vegetable proves to be a good anti-stress source and could be used to derive a semi-synthetic drug to curb stress and stress-related disorders in future.

INTRODUCTION: In the present competitive world and changing lifestyle, stress is becoming a major cause for several health disorders.[1] Reducing stress in everyday life is vital for maintaining one's overall health, as it can improve mood, boost immune function, promote longevity and allow one to be more productive.[2]

QUICK RESPONSE CODE	
	DOI: 10.13040/IJPSR.0975-8232.8(8).3591-98
	Article can be accessed online on: www.ijpsr.com
DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.8 (8).3591-98	

Stress can be defined as a person's physiological response to a stimulus that triggers the fight-or-flight response. [3] For a short duration of time, stress prepares the body by making a person stronger and faster and ready for an action to achieve a particular goal. [4]

Often, however, prolonged stress when it reaches a deleterious and harmful level, chronic disorders and toxic insult consequences may follow, such as compromised immune function, weight gain and developmental impairment and so on.[5, 6, 7] The intensity of the stress response is governed largely by glucocorticoids, the primary molecules involved in the stress response. [8]

Proper stress management takes on great importance given the wide range of bodily systems impacted by stress hormones.[6, 7]

Cortisol is also known as the "stress hormone". It is a steroid hormone which belongs to a broader class of steroids called glucocorticoids. Cortisol basically affects the carbohydrate, protein and lipid metabolism. The most important biochemical metabolic pathway directed to counter stress is the Gluconeogenesis pathway.[8]

The synthesis of glucose from non-carbohydrate precursors is called gluconeogenesis.[8] Cortisol acts on muscle, liver and adipose tissue to supply the organism with fuel to withstand stress but prolonged elevated levels of Cortisol may lead to serious consequences causing Type 2 Diabetes and Insulin Resistance, [9] Cardiovascular disorders,[10] Hypertension, [11, 12] Hyperglycaemia,[13] Macro mineral Deficiencies and Acid-Base Disorders, Fertility problems,[14] Bone Loss, [15] Supressed Immunity and other issues such as insomnia, chronic fatigue syndrome, thyroid disorders, dementia, depression, decreased immune system, decreased metabolism, chronic fatigue, migraines, tunnel vision and other conditions.[16, 17]

The WHO estimated that about 80% of populations in developing countries rely on traditional medicine for their primary health care needs.[18] It is estimated that about 25% of all modern medicine are directly or indirectly derived from higher plants. Hence due to the growing importance of herbal medicines, *Clerodendrum colebrookianum* was chosen for the study.

Clerodendrum colebrookianum is known by more than 30 vernacular names among 20 different tribes and communities in the north eastern region of India.[19] The plant is used for the treatment and cure of more than 16 different diseases and ailments.[20] Various parts of the plant especially its leaf and root extracts have been used for the treatment of rheumatism,[21] asthma, gastrointestinal tract disorders, inflammatory diseases,[22] coughs, skin diseases, [23] vermifuge, febrifuge, malaria *etc*., the leaves being consumed as antihypertensive source is most common. [24] The use of the plant for cure and treatment of diseases is based on administration of the leaves either by boiling or as raw vegetable.[25, 26, 27] Previous experiments and studies carried out indicated the presence of potent phytochemicals [28] such as phenols, [29] alkaloids, [30] flavonoids, [31] cardiac glycosides [32], saponins, [33] steroids both in the raw and boiled extracts.[34, 35, 36]

The current study attempts to compare the effects of bioactive molecules present in raw and boiled aqueous leaf extracts in combating stress by studying its effects on cortisol stress markers (gluconeogenesis) in the liver using *ex-vivo* studies. Due to the high K_m of liver glucokinase, most glucose so formed in the liver will not be phosphorylated and will flow down its concentration gradient out of the hepatocytes into the blood. This concept is so utilized in our present project to estimate the glucose release during gluconeogenesis by the hepatocytes of *Sus scrofa domesticus* using *ex-vivo* studies.

Ex-vivo refers to experimentation or measurements done in or on tissue from an organism in an external environment with the minimum alteration of natural conditions within a given duration of time. *Ex-vivo* studies are advantageous compared to *in-vivo* studies since it is simpler to perform and *ex vivo* models are less expensive and easier to obtain.

MATERIALS AND METHODS

Plant Source: *Clerodendrum colebrookianum* is indigenous to the North – Eastern states of India. In Mizoram the plant grows as wild plant under temperate conditions and does not consume much water. It is popularly consumed as a vegetable delicacy usually along with meat and oily foodstuff. In the recent years, due to its known high medicinal value and properties the plant has become a commercialized product and is cultivated and grown by many farmers. The five year old plant *Clerodendrum colebrookianum* was transplanted by root transplantation from Mizoram in 2005 and was grown in United Theological College, Benson Town, Bengaluru.

The plant was identified and confirmed by the Horticulture Department of Mizoram as "*Phuihnam"* (*Clerodendrum colebrookianum*). The leaves were randomly selected and collected freshly from the plant for each and every trial carried out during the course of the experiment.

Preparation of Sample Extract: *Ex-vivo* study was carried out using 50% raw and boiled aqueous leaf extract. Raw extract was prepared by homogenizing the leaves using a pestle and mortar followed by filtration and centrifugation at high speed. Boiled extract was prepared by homogenizing the leaves using pestle and mortar followed by boiling in water (water is taken according to the extract percentage to be prepared). Boiling was carried out for 10 minutes and was cooled. This was followed by filtration and centrifugation at high speed. The raw and boiled extracts so prepared were kept in different air tight containers and was stored at 4 °C until use.

Chemicals: The chemicals used were Cortisol (Hydrocortisone), Ethanol, Glucose, Glucose Oxidase Reagent, Sodium Chloride, Sodium Pyruvate.

Equipment: The equipment used were Petri-dishes, Blades, Elisa reader, Micropipettes, Homogenizer and Weighing Balance.

Liver Tissue Source: The liver was obtained from the slaughter house of Karnataka Ham Shop, Frazer Town, Bengaluru. The liver tissue was washed using normal saline and approximately 250 mg of the tissue pieces were sliced and placed into 12 well-cell culture plate (Nest Company) into which 1 mL of normal saline was added. The liver cells are viable under these conditions for 6 hours and the entire experiment was carried out within 3 hours. [37]

***Ex-vivo* Studies:**

To Study the Effect of Cortisol activity: Cortisol (2mg/mL) was obtained from Jain hospital, Miller's road, Vasanthanagar, Bengaluru. About 5 µL (i.e. 100µg of cortisol) was taken using a micropipette and added to the cell culture well plate where the liver tissue along with normal saline was previously added.[38]

Study of Glucose Release: The glucose released by the hepatocytes are estimated with the help of glucose oxidase reagent 10µL of the reaction aliquot was taken into a 96 well elisa reader plate and was made up to 50 µL using millipore water and 50 µL of glucose oxidase reagent was added. A standard was also carried out simultaneously by taking 2µL, 4µL, 6µL, 8µL, 10µL, 15 µL and 20 µL of glucose (having a concentration of 900µg/mL) and was made up to 50µL using millipore water to which 50µL of glucose oxidase reagent was added. Pink colour developed after 10 minutes of incubation and the absorbance was read at 520 nm using elisa reader against a suitable blank (millipore water).Glucose is oxidized by glucose oxidase to produce gluconate and hydrogen peroxide. The hydrogen peroxide is then oxidatively coupled with 4 Amino-Antipyrene (4-AAP) and phenol in the presence of peroxidase to yield a red Quinoeimine dye that is measured at 520 nm. The absorbance at 520 nm is proportional to concentration of glucose in the sample.[39]

$$\text{Glucose} + 2H_2O + O_2 \rightarrow \text{Gluconate} + H_2O_2$$

$$2H_2O_2 + \text{4-AAP} + \text{Phenol} \rightarrow \text{Quinoeimine Dye}$$

Study of Gluconeogenesis: The stress hormone Cortisol is a Glucocorticoid which affects the carbohydrate, protein and fat metabolism. The pronounced effect can be seen in the liver, various substrates such as sodium pyruvate, glycerol and amino acids taken up and utilized to produce glucose. The glucose released is thus estimated by using glucose oxidase reagent. The percentage of glucose released was also estimated.[38]

The following standard reaction mixtures were prepared as follows: Reaction 1 mixture (Liver + Normal Saline) - To the liver placed in the cell culture well, 2 mL of normal saline was added and the basal glucose released by the liver tissue cells were estimated at zero minute after 30, 60, 90 and 120 minutes. Reaction 2 mixture (Liver + Normal Saline + Cortisol) - To the liver placed in the cell culture well 1 mL of normal saline was added to which 5 µL of cortisol was added and the glucose so released by the liver cells were estimated at zero minute and after 30, 60, 90 and 120 minutes. Reaction 3 mixture (Liver + Normal Saline + Sodium Pyruvate) - To the liver placed in the cell culture well 1 mL of normal saline was added to which 100 µL of sodium pyruvate was added and the glucose so released by the liver cells were estimated at zero minute and after 30, 60, 90 and 120 minutes. Reaction 4 mixture (Liver + Normal Saline + Cortisol + Sodium Pyruvate) - To the liver placed in the cell culture well 1 mL of normal saline was added to which 5 µL of cortisol was added followed by 100 µL sodium pyruvate and the glucose released by the liver cells were estimated at zero minute and 30, 60, 90 and 120 minutes. 10µL of the reaction mixtures were taken into elisa reader plate and made up to 50 µL using millipore water to which 50µL of glucose reagent was added and the color developed was read at 520nm.

Ex-vivo* Studies of the Reduction of Cortisol Activity by the Aqueous Extract of *Clerodendrum colebrookianum: Raw and boiled aqueous extracts of 50% were prepared freshly and used for the carrying out *ex-vivo* studies. 1000 µL of the extract was added to the reaction mixtures present in the cell- culture wells. A comparative study was thus carried out with the effects of the raw and boiled extracts and these effects (*i.e.*, glucose released) were compared to the standard parameters carried out.

To Study the Effect of Raw Extract on Glucose Release: Raw extract (1000µL) was added to the cell culture well containing liver (250g), normal saline (1000µL) and cortisol (5µL) and the glucose released was estimated at zero minute and after 30, 60, 90 and 120 minutes. 10µL of the reaction mixture was taken into elisa reader plate and made up to 50 µL using millipore water to which 50µL of glucose reagent was added and the color developed was read at 520nm.

To Study the Effect of Raw Extract on Gluconeogenesis: Raw extract (1000µL) was added to the cell culture well containing liver (250g), normal saline (1000µL), cortisol (5µL) and sodium pyruvate(100µL) and the glucose released was estimated at zero minute and after 30, 60, 90 and 120 minutes. 10µL of the reaction mixture was taken into elisa reader plate and made up to 50 µL using millipore water to which 50µL of glucose reagent was added and the color developed was read at 520nm.

To Study the Effect of Boiled Extract on Glucose Release: Boiled extract (1000µL) was added to the cell culture well containing liver (250g), normal saline (1000µL) and cortisol (5µL) and the glucose released was estimated at zero minute and after 30, 60, 90 and 120 minutes. 10µL of the reaction mixture was taken into elisa reader plate and made up to 50 µL using millipore water to which 50µL of glucose reagent was added and the color developed was read at 520nm.

To Study the Effect of Boiled Extract on Gluconeogenesis: Boiled extract (1000µL) was added to the cell culture well containing liver (250g), normal saline (1000µL), cortisol (5µL) and sodium pyruvate (100µL) and the glucose released was estimated at zero minute and after 30, 60, 90 and 120 minutes. 10µL of the reaction mixture was taken into elisa reader plate and made up to 50 µL using millipore water to which 50µL of glucose reagent was added and the color developed was read at 520nm.

RESULTS AND DISCUSSION:

Glucose Released by Hepatocytes Under *ex-vivo* Conditions:

TABLE 1: GLUCOSE RELEASED BY HEPATOCYTES UNDER *EX-VIVO* CONDITIONS

Time (minutes)	Normal Saline	Normal Saline + Sodium Pyruvate	Normal Saline + Cortisol	Normal Saline + Cortisol + Sodium Pyruvate
30	0.270 ± 0.120	0.254 ± 0.070	0.395 ± 0.004	0.314 ± 0.003
60	0.341 ± 0.045	0.338 ± 0.021	0.377 ± 0.024	0.335 ±0.001
90	0.343 ± 0.007	0.368 ± 0.001	0.359 ± 0.010	0.356 ± 0.034
120	0.353 ± 0.007	0.368 ± 0.001	0.343 ± 0.007	0.361 ± 0.001

Each experiment was carried out in triplicates and the values are the corresponding glucose absorbance at 520 nm which is represented as Mean + SD.

The liver tissue sliced into pieces and placed in normal saline undergoes some amount of stress which was estimated with the help of glucose released by the hepatocytes. The glucose released is estimated by measuring its absorbance at 520 nm as seen in **Table 1**. The absorbance value continues to increase in time under the given *ex-vivo* conditions indicating that there is glucose synthesis via gluconeogenesis pathway. On addition of the substrate – sodium pyruvate the absorbance value increases indicating that glucose is synthesized at a higher rate on addition of the substrate. When cortisol was induced a drastic increase in absorbance was noted indicating that the rate of gluconeogenesis was heightened in the presence of the stress hormone. Thus supporting that gluconeogenesis could be considered as a major stress marker and the percentage of glucose synthesized could be related to the amount of stress the tissue is subjected to. For fasting periods longer than one day, or during periods of intense exercise or stress- glucose must be synthesized from noncarbohydrate precursors in order to maintain the blood glucose levels.

The noncarbohydrate precursors are pyruvate, lactate, oxaloacetate, amino acids and glycerol. The noncarbohydrate precursors enter the gluconeogenic pathway in the forms of pyruvate, oxaloacetate and dihydroxyacetone phosphate. There are two major sites for gluconeogenesis, the liver and the kidneys. The liver accounts for 90% of gluconeogenesis in the body, the kidneys produce the other 10%. Very little gluconeogenesis occurs in the other tissues of the body.

Gluconeogenesis is a pathway consisting of eleven enzyme-catalyzed reactions. The pathway can begin in the mitochondria or cytoplasm, depending on the substrate being used. Many of the reactions are the reversible steps found in glycolysis. The liver and kidneys maintain the glucose level in the blood so that the brain, muscle and red blood cells have sufficient glucose to meet their metabolic demands.

A working standard was obtained to compare and understand the amount of glucose release by the hepatocytes of *Sus scrofusa domesticus.* The working standard concentration of glucose taken was 9mg/mL. The absorbance readings for standard graph carried out were 0.197, 0.309, 0.374, 0.417, 0.423 and 0.483.

Hence the glucose so released by the hepatocytes under *ex-vivo* conditions was found to range from 2.43 to 3.393 mg/mL/250mg of the tissue.

It was observed that the glucose released by the hepatocytes on addition of cortisol was greater than that of glucose released by the hepatocytes placed only in normal saline indicating the effect of cortisol on gluconeogenesis pathway. When only sodium pyruvate was added as a substrate it was also observed that there was an increase in the glucose released. When both the substrate *i.e*., sodium pyruvate and the glucocorticoid *i.e*., cortisol was added to cell-culture well a gradual increase in the glucose released by the hepatocytes was observed.

Similar studies were carried out by Friedmann B *et al.,* showed that on addition of glucogenic substrates such as sodium pyruvate the metabolic pathway is directed towards gluconeogenesis pathway thus leading to an increase in glucose production.[40]

Comparison of the Effects of Raw and Boiled Extracts of *Clerodendrum colebrookianum* on the Hepatocytes Induced with Cortisol: The absorbance readings of glucose released by the hepatocytes induced with cortisol were 0.395 ± 0.004, 0.377 ± 0.024, 0.359 ± 0.10 and 0.343 ± 0.007 for the readings carried out at 30, 60, 90 and 120 minutes respectively. The absorbance readings of glucose released by the hepatocytes induced with cortisol to which raw extract was added were 0.374 ± 0.004, 0.389 ± 0.004, 0.376 ± 0.016 and 0.333 ± 0.094 for the readings carried out at 30, 60, 90 and 120 minutes respectively.

The absorbance readings of glucose released by the hepatocytes induced with cortisol to which boiled extract was added were 0.162 ± 0.026, 0.189 ± 0.035, 0.172 ± 0.005 and 0.192 ± 0.020 for the readings carried out at 30, 60, 90 and 120 minutes respectively.

The effect of raw and boiled extract on the hepatocytes induced with cortisol were compared and studied and depicted in the graph as seen in **Fig. 1**. It was observed that the absorbance readings of glucose released by the hepatocytes induced with cortisol and the addition of the raw extract was more or less similar to the absorbance readings of the glucose released by the hepatocytes induced with cortisol.

But there was a noted drastic decrease of absorbance readings of glucose released by the hepatocytes induced with cortisol to which boiled extract was added. Thus from the above readings it can be concluded that the boiled extract was more effective in reducing the activity of cortisol when compared to that of the raw extract.

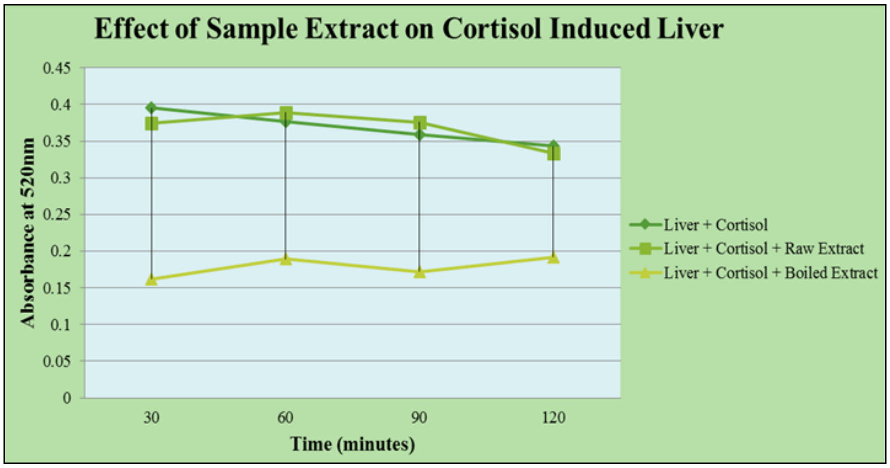

FIG. 1: EFFECT OF AQUEOUS EXTRACT ON CORTISOL INDUCED LIVER. Each experiment was performed in triplicates and the results are presented as mean absorbance ± SD.

Comparison of the Effects of Raw and Boiled Extracts of *Clerodendrum colebrookianum* on Gluconeogenesis: The absorbance readings of glucose released by the hepatocytes induced with cortisol plus sodium pyruvate were 0.314 ± 0.003, 0.335 ± 0.001, 0.356 ± 0.034 and 0.361 ± 0.001 for the readings carried out at 30, 60, 90 and 120 minutes respectively. The absorbance readings of glucose released by the hepatocytes induced with cortisol plus sodium pyruvate to which raw extract was added were 0.383 ± 0.036, 0.346 ± 0.043, 0.341 ± 0.041, 0.378 ± 0.049 for the readings carried out at 30, 60, 90 and 120 minutes respectively. The absorbance readings of glucose released by the hepatocytes induced with cortisol plus sodium pyruvate to which boiled extract was added were 0.136 ± 0.038, 0.124 ± 0.010, 0.137 ± 0.015 and 0.143 ± 0.012 for the readings carried out at 30, 60, 90 and 120 minutes respectively. The effect of raw and boiled extract on the hepatocytes induced with cortisol and addition of sodium pyruvate as the glucogenic substrate were compared and studied and depicted in the graph as seen in **Fig. 2**. It was observed that the absorbance readings glucose released by the hepatocytes induced with cortisol plus sodium pyruvate and the addition of the raw extract was more or less similar to the absorbance readings of the glucose released by the hepatocytes induced with cortisol plus sodium pyruvate. But there was a noted drastic decrease of absorbance readings of glucose released by the hepatocytes induced with cortisol plus sodium pyruvate to which boiled extract was added.

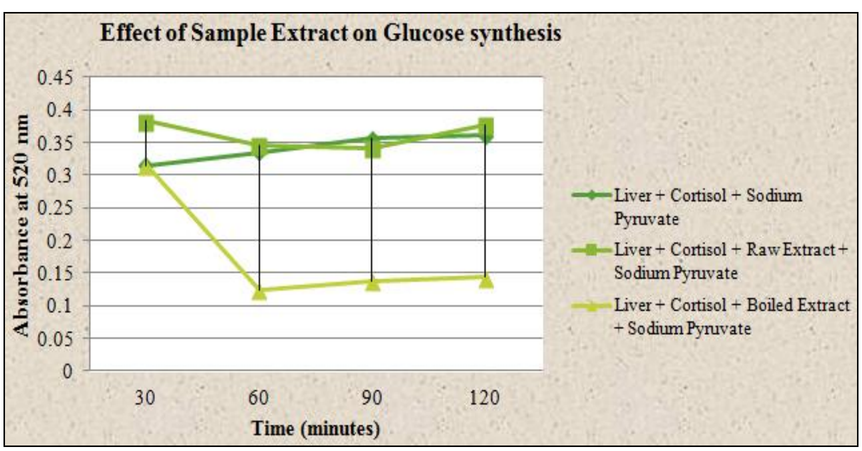

FIG. 2: EFFECT OF AQUEOUS EXTRACT ON GLUCONEOGENESIS. Each experiment was performed in triplicates and the results are presented as mean absorbance ± SD.

From the absorbance readings it can be observed that the boiled extract was very effective in reducing the glucose release thus indicating the reduction in the activity of cortisol which affects the gluconeogenesis pathway. Similar studies carried out by Krebs H. A. *et al.,* showed that the rate of gluconeogenesis from amino acids and other known precursors in slices of mouse liver after depletion of liver glycogen by means of phlorrhizin was high with L-lactate, pyruvate, glycerol, D-glyceraldehyde, dihydroxyacetone, D-fructose, sorbitol, xylitol, a-glycerophosphate, alanine, proline, threonine, serine and propionate.[39]

Study of the Percentage Reduction of Glucose Released by the Boiled Extract: Since the boiled extract was more effective when compared to the raw extract in reducing the activity of cortisol. Hence the percentage release of glucose was calculated and plotted as seen in **Table 2**.

The percentage release of glucose by the hepatocytes induced with cortisol was taken as 100% throughout the duration of experiment carried out and the percentage release of glucose by the hepatocytes induced with cortisol on addition of boiled extract was estimated as seen in **Table 2**. Thus indicating more than 50% of reduction in glucose release.

TABLE 2: EFFECT OF BOILED EXTRACT ON GLUCOSE RELEASE

Time (minutes)	Glucose release% Liver + Cortisol	Glucose release% Liver + Cortisol + Boiled extract	% Reduction of Glucose release
30	100	41.01	58.99
60	100	50.13	49.87
90	100	47.91	52.09
120	100	55.97	44.03

Each experiment was performed in triplicates and the results are presented as mean percentage release of glucose.

The boiled extract also showed an effective reduction on gluconeogenesis when sodium pyruvate is added as the substrate. Hence the reduction of glucose released on addition of sodium pyruvate was calculated and plotted as seen in **Table 3**. The percentage release of glucose by the hepatocytes induced with cortisol and addition of sodium pyruvate as the substrate was taken as 100% throughout the duration of experiment carried and the percentage release of glucose by the hepatocytes induced with cortisol plus sodium pyruvate on addition of boiled extract was estimated as seen in **Table 3.** Thus indicating more than 60% of reduction in glucose released inferring the decrease in activity of cortisol.

TABLE 3: % REDUCTION OF GLUCOSE BY BOILED EXTRACT

Time (minutes)	Glucose release % Liver + Cortisol + Sodium Pyruvate	Glucose release% Liver + Cortisol + Sodium Pyruvate + Boiled extract	% Reduction of Glucose release
30	100	43.31	56.69
60	100	37.01	62.99
90	100	38.48	61.52
120	100	39.61	60.39

Each experiment was performed in duplicates and the results are presented as mean percentage release of glucose.

ACKNOWLEDGEMENT: The authors wish to acknowledge Department of Chemistry (PG Biochemistry) and the management of Mount Carmel College Autonomous, Bengaluru for funding this project and offering their facilities for the analysis.

CONFLICTS OF INTEREST: Nil.

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How to cite this article:

Singh KG, Thangi JJ, Ashwini HA and. D'souza CJM: The reduction in cortisol induced stress markers by the aqueous extract of *Clerodendrum colebrookianum*. Int J Pharm Sci Res 2017; 8(8): 3591-98.doi: 10.13040/IJPSR.0975-8232.8(8).3591-98.