EFFECT OF VARIOUS FRACTIONS OF CAMEL MILK ON DIABETIC AND SUPEROXIDE DISMUTASE PROFILE

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ABSTRACT
Diabetes is steeply turning into epidemic especially in third world countries. Efforts are being pooled to counter this present day menace. In this connection, existing diabetic treatment is expensive and at time with lots of side effects. Along with new regime of medicines, scientists are looking for complementary medicines. Camel milk has been shown in literature for its anti diabetic potential and a promising candidate as a complementary tool for the prevention and treatment of diabetes. In present study, camel milk was extracted with Alcohol, Chloroform and Ether at low temperature. Standard methods of extraction at low temperature have been adopted. Streptozotocin induced diabetes was produced in experimental mice with three consecutive intraperitoneal injections. Along with solvent fractions, left over residues have been collected and analyzed for anti diabetic activities. It was revealed that Residues of Extracted Camel Milk (RECM) have maximum hypoglycemic and reduction of Hb1ac activity. Alcohol ranked second in this line. Chloroform and Ether fractions also have anti diabetic activity but at lesser extent. RECM by stimulation or regeneration released more insulin in the blood of diabetic mice. RECM also raised Superoxide Dismutase in streptozotocin treated mice. Alcohol, chloroform and ether extracts also raised superoxide Dismutase. Elevation of Superoxide dismutase and suppression of glucose profile in response to various fractions of camel milk shows that it is a plausible agent to lessen the long term complications of diabetes and have potential to become modern day neutraceutical.

KEYWORDS: Camel milk, RECM, Streptozotocin, Insulin, SOD.
INTRODUCTION

Long term micro- and macrovascular complications poses a burning challenge to the scientists in tackling the prevention and treatment of diabetes mellitus a menace all around the world in mankind history. Diabetes mellitus is the most common chronic endocrine disorder. A growing body of evidence emerged which clearly indicates that hyperglycemia leads to increased oxidative stress which in turn may be held responsible for the complications of diabetes Mellitus.\(^1\) Evidence about oxidative damage has been gathered in arterial samples obtained from experimental diabetic animal models and from human diabetic patients.\(^2\) It has been observed that oxidation of glucose can generate oxidative free radicals and excess reactive oxygen species such as superoxides a dangerous stuff for our body. These molecules can instigate lipid peroxidation, leading to the production of excessive oxidative free radicals in diabetic patients.\(^3\) Molecules especially proteins in the arterial wall can also be glycated, which usually is associated with oxidation in result of hyperglycemia. The advanced glycation end-products emerged during the metabolism of proteins, lipids and nucleic acids.\(^4\) The end product molecules thus formed may lead to increased production of oxygen free radicals and therefore, can play a role in the development of microvascular disease and atherosclerosis in diabetic patients.\(^4\) Numerous studies have also suggested that patients with diabetes appear to have decreased antioxidant defense capability, measured as lower levels of specific antioxidants, such as vitamins (vitamin C, E), and micro elements (Cu, Zn, Mg), or reduced activities of antioxidant enzymes, such as catalase, superoxide dismutase (SOD) or glutathione peroxidase.\(^5, 6\)

The diabetes altered activities of these enzymes and reduced level of GSH have been observed which affect the ability to defend against oxidative stress.\(^7\) Sustained reductions in hyperglycemia decrease the risk of developing microvascular complications.\(^8\) However, the powerful inhibitory actions of drugs on high blood glucose levels cause side effects such as flatulence, diarrhea, serious hepatic injury and renal failure.\(^9\) As an alternative approach, natural food with antihyperglycemic activities has been increasingly used by diabetic patients and health care professionals.\(^10\) Therefore to suppress the oxidant radicals, provide and boost antioxidant profile and thereby delay the diabetic complications, different adjuvent therapies have been developed.\(^11, 12\)

Despite the impressive advances in health sciences and medical care with regard to diabetes, a trend has been increasing to develop alternative therapies. Complementary to the prescribed
Traditional plant remedies or herbal formulations are being used since ancient times[15] and are still widely used even in developed countries to treat hypoglycemic and hyperglycemic conditions.[16] It is of interest that many ethno-botanical surveys on medicinal plants used by the local population have been performed in different parts of the world and there is a considerable number of plants described as antidiabetic.[17] Several natural products such as Momordica charantia, Azadirachta indica, Gymnema sylvestre, Pterocarpus marsupium, Coccinia indica, Trigonella foenum graecum, Allium sativum and Ocimum sanctum, etc. are being used for the management of diabetes and to overcome its complications. These plants are found to be effective and their low cost and minimal side effects have increased the interest of scientists to develop plant based drugs for managing diabetes as the interest grow scientists also looked for animal natural products.[18, 19]

Research interest and activities in the areas of animal derived medicine have increased tremendously in the last decade.[20] Animals and their products utilized as medicines by the inhabitants surrounding the Ranthambhore National Park, India. The use of animals for medicinal purposes has been a tradition in various cultures. It is increasingly becoming a trend to research new animal derived medicines due to cost effective and less or no side effects.[21]

Camel milk (CM) is known as an essential nutritional source. Its vitamin content is known to help immune-deficient patients as well as those recovering from diseases. it also shows protective effects against heavy metal toxicity and viral and bacterial infections. It is thought that CM may also be beneficial for asthma, anemia, jaundice, and spleen problems.[22] Recent studies have shown that CM has antihypertensive, anti-cancerous, hepatoprotective, and hypocholesterolemic effects[23] and corrective effect of milk of camel on some cancer biomarkers in blood of rats intoxicated with aflatoxin B. [24] The hypocholesterolemic effect have been found of Gariss and Gariss containing bifidobacteria in rats fed on a cholesterol-enriched diet.[25]

The low prevalence of diabetes in the Raica community was attributed to the regular consumption of CM. It has been suggested by Dr. R.P. Agrawal that 500 ml of raw, fresh camel milk daily improves the lives of diabetics due to an insulin-like protein that is absorbed rapidly and does not coagulate. However, Agrawal also states that insulin remains the most efficient treatment for diabetes, unless it is not an option. While research appears promising,
additional scientific studies are needed to prove the effectiveness of camel milk for the treatment of diabetes.\[26]\) Recently camel milk has been deeply studied for its special properties because of higher hepatoprotective, insulin like, antibacterial and antiviral activities.\[23]\) Camel milk is considered to have anti-cancer, hypoallergenic and anti-diabetic properties.\[23,26]\) Other components such as lactoferrin, immunoglobulins, lysozyme and vitamin C are in good quantity in camel milk, were reported to play a crucial role in the determination of these properties.\[27]\) Camel milk possesses insulin like activities, which decreases the requirement of exogenous insulin in Type 1 diabetic patients.\[28]\) Presence of half-cystine, lactoferrin or unknown insulin like factor in camel milk may be contributory factor in its anti diabetic activities. In addition, presence of antioxidant trace elements (Cu, Zn and Mg and vitamins (Vitamin C, E) may be a cause for the enhancement of its antioxidant activity to revert the complications of Diabetes Mellitus.\[29]\) Though every single one of the antioxidant enzymes (Superoxide Dismutase (SOD), Catalase and Glutathione Peroxidase) may play a crucial role in Diabetes Mellitus, yet the role of Superoxide Dismutase (SOD) is vital, proverbial and particular in scouring the complications of Diabetes Mellitus.\[30]\) SOD helps the body to use zinc, copper, and manganese appropriately. Cu/Zn SOD protects the cells cytoplasm, and Mn SOD (mitochondria SOD) protects mitochondria of the cell from free radical damage.\[31]\)

Although a few studies reported that the camel milk has some degree of effect against oxidative stress induced in diabetic animal models but they didn’t explore the specific reason behind this potential activity. Underlying molecular mechanisms to prevent the oxidative stress in such experimental animal models remaining unexplored. In this regards, hypothesis of the present work would lead to understand the underlying mechanism of the antioxidants in camel milk in the prevention of the complication of diabetes mellitus Moreover, present study may be helpful to elucidate the exact biomolecule which may prove a potential anti diabetic agent.

**MATERIAL AND METHODS**

**Collection of Camel Milk**

Camel milk was collected from camel centre Aljouf from C.Hamra bread dromedaries in clean 500ml bottles and transported to the laboratory while on ice and stored at $4^\circ$C. The milk obtained so was the lyophilized and kept frozen at $-80^\circ$C for further experimentation.
Extraction of Milk
Extraction of camel milk was carried out by adopting and modifying the method illustrated by R. Aschaffenburg, 2009. [32] Extraction with Alcohol 250 ml Ethyl Alcohol was added to the lyophilized came milk and stirred overnight. This Alcoholic mixture was centrifuged. supernatant was decanted as alcoholic extract and residues were collected and air dried for successive Ether and Chloroform extraction. Alcoholic extract was concentrated at low temperature on rotary evaporator. Concentrated material marked as Alcoholic extract was kept for further experiments.

Extraction with chloroform & Ether
250ml chloroform was added to the left over residues of Alcoholic extraction and stirred overnight. The mixture was transferred to the separating funnel and let it stand for 1 hour. Clearly two phases were emerged. Chloroform portion separated, transferred and air dried in petri dishe. This was marked as Chloroform extract. The residues were also air dried and immersed in 250 ml Ether and let it stirrer overnight. After stirring mixture was poured in the separating funnel. After 1 hour Ether phase was separated and air dried and marked as Ether Extract. The left over residues was air dried and marked as Residues of Extracted Camel Milk (RECM) for further experiments.

Animals
The study protocol, and all of the animal handling procedures was followed according to the guidelines of the Ethic committee of College of Medicine, Aljouf University was adopted with some modifications.

Two to three months old 50 male Swiss albino mice weighing 30-40gm were generously donated by Animal House of College of Pharmacy, Aljouf University. Animals were housed in polyplastic cages in air conditioned room at standard temperature and humidity (70° F &50-60% relative humidity). The cages were well ventilated and suitable for continuous airflow and exposure to 12hrs light/dark cycle. Standard food and water were supplied to the animals ad libitum. After 15 days of acclamitization for experimental design, animals were randomly divided into three groups. Each group is comprised of10 animals. First group was named as Group A left untreated and supplied normal diet, water. Group B animals were treated with streptozotocin to produce experimental diabetes. The third group called Group C received streptozotocin injections to induct diabetes but also treated with various extracts/residues of camel milk.
Induction of diabetes
Method of Duraisamy Gomathi [33] was adopted with some modifications. Diabetes in mice was produced by three days successive intra peritoneal injection of 45mg/kg body weight streptozotocin STZ) in 0.1M cold citrate pH 4.5. The level of blood glucose was checked by drawing blood from tail of the mice with Accua check Performa (Roche, Germany) prior to the STZ injection and after three days of last injection. Glucose level above 300mg/dl was considered as induced diabetes.

Biochemical Estimations
The animals were fasted for 6 hours and blood was obtained from the tip of the tail of both experimental and control mice. The Blood Glucose was measured at days, 0, 15 and 30 of the present study. On the culmination of the study day i.e. day 30 all the animals were anesthetized and blood was collected by cardiac puncture. Blood was processed for further studies.

The plasma insulin was measured with a mouse ELISA kit for insulin (Morinaga, Yokohama, Japan) as adopted by yong et al.[34] Plasma glucose has been estimated by Glucose oxidase method.[35] Hb1ac was determined by using the Mouse hemoglobin (Hb1ac) measuring kit by suppliers named Beckman Coulter SYNCHRON Systems as described by Dudley et al.[36] Estimation of Superoxide dismutase was carried out by commercial Kit obtained from Biovision, USA. The method has been followed according to the supplier’s instructions.[37]

RESULTS AND DISCUSSION
Body Weight
Body weight of the animals has been checked at the time of start and after completion of the experimental work. It was noted that Streptozotocin injection produced diabetes and consequently suppressed 33% body weight of animals Figure 1. When treated with the residual material of extracted camel milk (RECM), 27% recovery in body weight was observed. Alcoholic, ether and Chloroform extracts recover, 11%, 3.8% and 2% body weight respectively as shown in Figure 1.
Figure 1: body weight of streptozotocin induced diabetic mice before and after the treatment with different fractions of camel milk

Values are Mean± Standard Deviation, n= 10

Abbreviations: RECM (residues of extracted camel milk), D Diabetes, Al (Alcohol), Ch (chloroform), E (ether), ext (extract)

Control vs diabetic: p<.001, RECM vs Diabetes: p<.001 Diabetes vs D+Al ext: p<.05
Diabetic vs D+Ch ext: p>.05
Diabetes vs D+E ext: p>.05

Glucose level

Streptozotocin treatment raised the blood glucose level 156% of the normal in the experimental mice Table 1. When mice was treated with left over residues (RECM), 40% glucose was found to be reduced in the diabetic mice Table 1. Alcohol, Ether and Chloroform extracts of camel milk suppressed the blood glucose level in the streptozotocin induced diabetic mice 9.2%, 6.8% and 5.8% respectively as evident in Table 2,3,4.

Insulin Levels

As shown in figure 2 in response to Streptozotocin treatment, 47% decrease in insulin levels was observed in diabetic mice. Treatment with residues of milk extraction (RECM) enhanced 42% insulin level in diabetic mice from day 0 to day 30 Figure 2. This response was much less when the animals were treated with the Alcohol, Ether and Chloroform extract of camel milk i.e increase in insulin levels was noted 11.6%, 7.6% and 3.8% respectively as revealed in Figure 2.
Figure 2: Serum insulin level of streptozotocin induced diabetic mice before and after the treatment with different fractions of camel milk

Values are Mean± Standard Deviation, n= 10
Abbreviations: RECM (residues of extracted camel milk), D Diabetes, Al (Alcohol) , Ch (chloroform), E (ether), ext (extract)
Control vs diabetic: p< .001, RECM vs Diabetes: p< .001, Diabetes vs D+Al ext: p<.05 , Diabetes vs D+Ch ext:  p>.05,
Diabetic vs D+E ext:  p>.05

Hba1c
As evident from Table 1, streptozotocin injection caused 113% increase in Hba1c level. Camel milk Residual treatment for 4 weeks brought down 29% Hba1c level in diabetic mice. Treatment with Alcohol, Ether and Chloroform extracts of camel milk suppressed the elevated Hba1c level by 17%, 11% and 11.2% respectively as shown in Table 1,2,3,4.

Superoxide Dismutase
As shown in figure 3, 57.9% SOD levels were dropped in Streptozotocin induced diabetic mice. When diabetic mice were treated with left over residues of extracted milk 85 % recovery in SOD levels was noted. While recovery of SOD levels in Diabetic mice in response to the treatment with Alcohol, Ether and Chloroform extracts was observed as 52.5%, 46%, 39.3% respectively as evident in Figure 3.
Figure 3: Serum SOD level in streptozotocin induced diabetic mice before and after the treatment with different fractions of camel milk

Values are Mean± Standard Deviation, n= 10

Abbreviations: RECM (residues of extracted camel milk), D Diabetes), Al (Alcohol) , Ch (chloroform), E (ether), ext (extract)

Control vs diabetic: p< .001, RECM vs Diabetes: p< .001, Diabetes vs D+Al ext: p<.05, Diabetic vs D+Ch ext: p>.05. Diabetes vs D+E ext: p>.05

In present study Animals used in experiment was treated with streptozotocin. As can be seen in result section Figure 1, there was a considerable weight loss in response to streptozotocin induced diabetes. Weight loss in response to streptozotocin may be attributed to the damage of beta cells causing deficiency of Insulin, in addition to tissue destruction. Since insulin is an anabolic hormone, its deficiency might have caused a considerable weight loss. The nitrosourea moiety of streptozotocin is responsible for beta cell toxicity. While deoxyglucose moiety of streptozotocin facilitates transport across the membrane. Involvement of free radicals generation and resulting alterations of endogenous scavengers of these reactive species have been reported in STZ diabetogenicity. Further, STZ causing alkylation or breakage of DNA strands and a consequent increase in the activity of poly-ADP-ribose synthetase, an enzyme depleting NAD in beta cells finally leading to energy deprivation and death of beta cells is reported. It can be speculated that weight loss in experimental diabetic mice may be attributed to the reduction in insulin level and tissue destruction. Equally it can be said that weight gain in response to RECM and Alcohol, Chloroform and Ether fractions of camel milk may be credited to increase insulin level and its anabolic activates.
Table 1: Effect of RECM on glycemic control in untreated and streptozotocin treated diabetic mice

<table>
<thead>
<tr>
<th>Glycemic profile</th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic RECM. extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose(mg/dl)</td>
<td>114±3.2</td>
<td>292±4.4</td>
<td>176±6.2</td>
</tr>
<tr>
<td>Hb1ac(%)</td>
<td>4.6</td>
<td>9.8</td>
<td>6.9</td>
</tr>
</tbody>
</table>

Control vs diabetic:  p< .001, RECM vs Diabetes:  p< .001, Diabetes vs D+Al ext:  p<.05,
Diabetic vs D+Ch ext: p> .05 Diabetes vs D+E ext: p> .05

After streptozotocin induced diabetes, and treatment of the same with RECM and different solvent fractions, the animals were sacrificed and blood was collected for analysis of Glucose. As it is evident in Table 1, streptozotocin elevates 156% glucose level as compared to control group. As it is mentioned earlier this effect may be exerted due to cytotoxicity of streptozotocin to the pancreatic beta cells and free radicals generation in STZ treated diabetogenecity. [39] The beta cell destruction may lead to hypoinsulinemia unable to counter the blood glucose level in the mice employed in present study. As can be seen on table 1 RECM considerably reduced the glucose level that was 40% of that of diabetic mice.

Table: 2 Effect of D+Al ext on glycemic control in untreated and streptozotocin treated diabetic mice

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic alcohol. extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose(mg/dl)</td>
<td>114±3.2</td>
<td>292±4.4</td>
<td>265±5.4</td>
</tr>
<tr>
<td>Hb1ac(%)</td>
<td>4.6</td>
<td>9.8</td>
<td>8.7</td>
</tr>
</tbody>
</table>

Control vs diabetic:  p< .001, RECM vs Diabetes:  p< .001, Diabetes vs D+Al ext:  p<.05,
Diabetic vs D+Ch ext: p> .05 Diabetes vs D+E ext: p> .05

The reduction in response to Alcohol, Chloroform and Ether was much less than was found with the RECM treatment as shown in Table 1,2,3,4. To our knowledge, in present work, the extraction of camel milk to explore the anti diabetic fractions is unique and no similar study has been come across when literature was surveyed. However, whole camel milk has been reported as anti diabetic by number of studies. [40] It may be implied that residues after extraction of milk with different solvents may have insulin or insulin like molecules which caused lowering of glucose level.

Table 3: Effect of D+Al ext on glycemic control in untreated and streptozotocin treated diabetic mice

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic chloro. extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose(mg/dl)</td>
<td>114±3.2</td>
<td>292±4.4</td>
<td>275±6.2</td>
</tr>
<tr>
<td>Hb1ac(%)</td>
<td>4.6</td>
<td>9.8</td>
<td>8.7</td>
</tr>
</tbody>
</table>

Control vs diabetic:  p< .001, RECM vs Diabetes:  p< .001, Diabetes vs D+Al ext:  p<.05,
Diabetic vs D+Ch ext: p> .05 Diabetes vs D+E ext: p> .05
It may also be conform that the camel milk residues may have stimulated remaining beta cells which release more insulin to reduce the blood glucose level in the diabetic mice. Interestingly, some antidiabetic activity has also been found in Alcohol, Chloroform and Ether extracts of camel milk as revealed in Table 2,3,4. Similar camel milk action has been predicted by Saachin et al. [41] Among Alcohol, Chloroform and Ether, alcoholic fraction has more insulinogenic activity and ether fraction contains minimum as revealed Table 4. This may be implied to the possible presence of short molecular peptides in Alcohol fraction of the camel milk.

Hyperglycemia leads to non enzymatic glycosylation of hemoglobin. It means that during diabetes, the excess glucose present in the blood reacts with hemoglobin to form HbA1c. In present study, it is quite likely that hypglycemia in dibetic mice as shown in Table 1, may have caused 113% increase in the level of Hb1ac Table 1. Residues of extracted camel milk (RECM) significantly cut down this extremely raised Hb1ac level. It was observed that in RECM treated mice 29% Hb1ac was reduced as evident in Table 1. Similar results have been reported by Abd El- Azia et al. [42]

Table 4: Effect of D+e ext on glycemic control in untreated and streptozotocin treated diabetic mice

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Control</th>
<th>Diabetic</th>
<th>Diabetic Ether. extract</th>
</tr>
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<tr>
<td>Glucose(mg/dl)</td>
<td>114±3.2</td>
<td>292±4.4</td>
<td>272±4.5</td>
</tr>
<tr>
<td>Hb1ac(%)</td>
<td>4.6</td>
<td>9.8</td>
<td>8.1</td>
</tr>
</tbody>
</table>

Control vs diabetic: p<.001, RECM vs Diabetes: p<.001, Diabetes vs D+Al ext: p<.05 , Diabetic vs D+Ch ext: p>.05Diabetes vs D+E ext: p>.05

However, in above referred research they used whole milk and in present study different fractions/residues of camel milk have been used. A significant increase (17%) in Hb1ac in response to Alcoholic extract has also been noted in Table 2. There is some Hb1ac increasing activity which has been observed in response to Chloroform and Ether extracts Table 2,3,4. It is to be found out that this activity actually belongs to chloroform and Ether, if it is so chemical nature of these molecules have to be determined. Streptozotocin is known to destroy the beta cells which produce insulin. Destruction of beta cells may cause lower level of insulin. In present study, streptozotocin severely suppressed the insulin in diabetic mice. In diabetic mice 47% insulin was decreased as compare to control Figure 2. As compare to control. The RECM treatment increase the insulin level by 42% as compared to diabetic
mice. Figure 2. This increase may be via stimulation of beta cells or regeneration of beta cells. In either case insulin will be released more and cause increased blood level of insuline in treated diabetic mice. There is another possibility that camel milk may contains insulin which may have added in the level of insulin. Similar results with camel milk have been reported elsewhere.[43, 44]

Alcoholic extraction has also been found to be contained a considerable insulin enhancing activity (11.6%) as shown in Figure 2. Chloroform and Ether extract were also contained the activity in favor of insulin enhancing levels i.e. 7.6% & 3.8% respectively as shown in Figure 2. However, at present we do not know the nature of active molecules in these fractions.

Oxidative stress is thought to be a leading cause of long term diabetes complications. As adaptive response antioxidant defense is stimulated. However, when antioxidant defense becomes weak, the balance between oxidants and antioxidants is severely hampered. Overwhelming increase in free radicals incurred damage causing life threatening complications of diabetes. SOD is one of the prominent antioxidant defense enzyme, which found to be lower. (57.9%) in diabetic mice as compare to control mice as shown in Figure 3.

(57.9%) in diabetic mice as compare to control mice as shown in Figure 3. This shows that hyperglycemia is instrumental for the lower level of SOD in this case. RECM treated mice significantly recover SOD level (85%). Similar results have been reported by Laila et al.[45]

Alcohol, chloroform and ether fractions recovered SOD levels in diabetic mice 52.5%, 46%, 39.3% respectively (Figure 3). This elevation of SOD in RECM treated diabetic mice may be attributed to high content of antioxidant viatamins and minerals in camel milk.[46, 47]

**CONCLUSION**

Previously, camel milk has been shown to contain anti diabetic activity. In present study various fractions of camel milk after extraction with different solvents have been obtained and their anti diabetic activity has been determined. Left over residues named RECM showed maximum anti diabetic activity. RECM not only raised Insulin level but also reduced Hb1ac and raised Super oxide dismutase in diabetic mice. All the solvents used in this study were found active but lower than the activity shown by RECM. From the results it can be safely
mentioned that camel milk can be used as a whole or concentrated active molecules through fractionation technique which have every potential to be used as neutraceutical in diabetic patients.

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