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Ajwa Dates Ameliorate Myocardial Toxicities Induced by Clozapine in Rats

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ABSTRACT

Introduction: Clozapine (CLZ) is a highly effective antipsychotic drug for patients suffering from schizophrenia. However its most important side effect is life-threatening myocarditis. Studies have shown that Clozapine-induced myocarditis is accompanied with an increase in cardiac oxidative stress. Since Ajwa dates have strong antioxidant activity, their potential cardio-protective ability against Clozapine induced myocardial toxicity merits investigations. **Aims & Objectives:** To investigate the ameliorating effects of Ajwa dates (Pheonix Dactilyfera) fruit and pit extracts on myocardial toxicities induced by Clozapine in rats. **Place and duration of study:** Post Graduate Medical Institute, Lahore. January, 2017 to March, 2017. **Material & methods:** It was an experimental control group design. 32 female healthy albino rats were randomly assigned to four groups of eight animals each (Table 1).Group 1 the healthy control group, was maintained on standard laboratory diet *ad libitum for* 21 days, all animals in group 2(the diseased control) were injected with CLZ once daily intraperitoneal (IP) injection of 0.1ml dose of 25mg/kg/day for 21 days. Group 3 was fed with CLZ plus Aqueous ajwa date fruit extract (aq.ADFE) 1g/kg/d by gastric gavage. Each rat in Group 4 was given CLZ plus aqueous ajwa date pit extract (aq.ADPE) 1g/kg/d by gastric gavage. All groups were then compared for LDH, MDA, CK-MB, GSH and GSH-Px values on the 22nd day at the end of the study. **Results:** This study found aqueous extract of *Phoenix dactilyfera* fruit and pit to reduce myocardial toxicity induced by Clozapine as shown by an improvement in cardiac biochemical and oxidative stress markers of LDH, CK-MB, GSH, GSH-Px and MDA. **Conclusion:** It was concluded that the myocardial toxicity caused by clozapine in rats can be ameliorated by using aqueous Ajwa dates (*Phoenix dactilyfera)* fruit and pit extracts.

Key words: Ajwa Dates**,** *Pheonix dactylifera*, Myocardial Toxicity, Clozapine

INTRODUCTION

Schizophrenia is a major psychotic disorder that typically begins during adolescence or early adulthood[1]. The disease manifests itself as alterations in thoughts and perceptions i.e. delusions, hallucinations and loosening of associations[2]. According to a study of World Health Organization, the disease was projected as the 10th leading cause of YLDs (years lived with disability) in 2013[3]. The main focus of treatment in schizophrenia includes antipsychotic medicines and psychosocial intervention[4].

The anti-psychotic drugs introduced lately are termed "atypical" or second generation agents (SGAs) that are therapeutically effective in patients showing no response to first generation agents (FGAs)[5]. Common examples include Risperidone, Ziprasidone, Olanzapine and Clozapine. Among these, Clozapine has been termed most effective for treating resistant schizophrenia[6]. Clozapine structurally is a tricyclic compound (dibenzodiazepine). It acts mainly by blocking $5HT_{2A}$ receptors[7]. In 10% to 50% of schizophrenics unresponsive to typical antipsychotic medications, clozapine is an effective alternative[8]. It has been approved by the FDA for treatment of suicidal behaviors in schizophrenics as well as for treatment of severe schizophrenia unresponsive to other medicines[4,9]. It is the most efficacious amongst the atypical antipsychotics in reducing suicidal ideation in schizophrenics[7,10].

Recent literature has highlighted the cardiovascular complications associated with clozapine particularly myocarditis and cardiomyopathy[7,11-13]. Research suggests that myocarditis despite being a mild

disease may result in fatal heart failure[14]. Studies have reported myocarditis as one of the major causes of sudden death among young population[14,16]. Cardiotoxicity induced by clozapine is accompanied by elevated oxidative stress and decreased antioxidant defenses[13].

Natural substances have countless medicinal properties and can cure without any side effects. Cultivated in North Africa and Middle East for at least 5000 years, date is one of the most primitive fruit crops[17]. In the desert region, date is an emblem of life as it can endure drought, high temperatures, and salinity compared to any other fruit[18]. Date fruits are considered to show protective effects against various diseases because of the bioactive non-nutrients generally termed as phytochemicals. These compounds have been gaining increasing importance in the field of medicine due to their cholesterol lowering and anti-oxidant properties and their due role in prevention of diabetes and cardiovascular diseases[19,20]. According to a research, the phytochemicals present in date fruit differ widely in composition and quantity depending upon various factors such as type and variety of date, the stage of ripening, storage and processing etc. Sun drying, for instance is believed to result in loss of carotenoid and anthocyanin content while causing an increase in total polyphenols and phenolic acids[21, 22].

Ajwa is the most expensive variety of date palm and a rare cultivar which is delightfully soft and fruity having fine texture and black colour[23,24]. The Ajwa Date is the fruit of female date palm tree (*Phoenix dactylifera*), belonging to the family Arecaceae and is cultivated only at Al-Madina Al-Nabawiah in Saudi Arabia[25]. The motivation for studying this particular variety came from the sayings of Prophet Muhammad (SAW) about this special date which was planted by his own hands[26].

According to phytochemical analysis, Ajwa dates have higher content of carbohydrates, minerals, proteins, multivitamins, fats and dietary fibers which make Ajwa superior to other dates[23]. These contain alkaloids, steroids, flavonoids, tannins, phenols and polyphenols that act as antioxidants[27,28]. In a study done on three premium quality dates, Rhutab, Khalas and Ajwa, it was found that Ajwa dates had the highest antioxidant activity (free radical scavenging capacity) and significantly higher levels of total phenolic content[29]. Another recent study concluded Ajwa pits being the richest source when compared for total phenols and flavonoid contents with other local varieties[30].

Despite the availability of vast literature on date palm's therapeutic properties, the direct effects of date fruit and pit consumption on cardiovascular system remains under-investigated[31]. No study has yet been done regarding protective role of Date palm fruit and pit against Clozapine induced cardiotoxicity. The present study aims to explore this protective role of a particular cultivar of *Phoenix dactylifera*, the Ajwa dates[20].

Fig-1: Ajwa Dates Fruits and Pits

MATERIAL AND METHODS

Plant Material: Ajwa dates were purchased from a renowned dry fruit shop in Lahore i.e. Sadiq Dry Fruit and verified by PCSIR Laboratories, Lahore.

Drugs: Clozapine provided by Mass Pharmaceuticals, Lahore in powdered active principle.

Chemicals: Malondialdehyde, Thiobarbituric acid, Perchloric acid, reduced Glutathione, Ellman's reagent (5, 5- dithiobis-2-nitrobenzoic acid DTNB solution), Hydrogen peroxide, Chloroform in liquid form, Phosphate buffered saline (PBS), Formalin solution, Eosin/hematoxylin stain, Normal saline (0.9% NaCl) by, Mass Pharmaceuticals.

Instruments: Eppendorf tubes, Serum vials, Glass slides, Multichannel pipettes, Syringes 5ml and 10ml, Sterile glass test tubes (Pyrex), Dissection box, Small plastic containers, Insulin syringes, Filter paper, Electronic balance (for measuring the doses of medicine and plant extracts).

Sample Selection: 32 healthy female Sprague dawley albino rats aged 6-7 weeks weighing 150-200gms purchased from University of Veterinary and Animal Sciences Lahore were used in the experiment.

Biochemical Kits: LDH kit (Analyticon, Germany), CK assay kit (Wieners Lab).

Preparation of Medicine and Extracts: CLZ was dissolved in 0.1M HCl and pH balanced (5.9) in phosphate buffered saline (PBS). It was administered in 0.1ml doses of 25mg/kg/day IP[32] as previous research showed that rats could bear far greater ranges of pH than humans (4.5-8.0)[33].

Aqueous Extract of Ajwa Date Fruits (aq. ADFE): Flesh of fruits was left in distilled water (1:3) for 48 hours at 4°C. The whole solution was ground and centrifuged at 4°C for 20 min at 4000 rpm. The supernatant was collected and stored at -80°C till use[34].

Aqueous Extract of Ajwa Date Pits (aq. ADPE): After separating pits from flesh, the pits were washed with tap water and sun dried. Further drying was done at 50°C for 4 hrs in an oven. Pits were then milled by heavy duty grinder. The milled pit was sieved through 1mm screens, followed by adding 1 litre hot (80°C) water onto 50g pit powder. The mixture was incubated at 30°C for 7h while shaken at 100rpm. The extract was filtered through Whatman no.4 filter papers, lyophilized and kept at -80°C until used[35].

Experimental Setup: The rats were housed in standard polypropylene cages at a controlled room temperature of 25±10°C and relative humidity 60-70% at PGMI, Lahore. They were fed standard laboratory diet ad libitum through the entire study duration i.e. 21 days. Animals were kept on a 12-hour light-dark schedule 6am to 6pm and all experimental testing was conducted during the light phase from 9am to 12pm. Two weeks were given to rats for acclimatization to the new environment before starting the experiment. All efforts were made to minimize animal suffering.

1. Group Formation: Sample was divided into four groups of eight animals each (Table-1). These were given different treatments for 21 days. Group 1 (healthy control) was maintained on standard laboratory diet *ad libitum.* All animals in group 2 (diseased control group) were injected with CLZ once daily intraperitoneal (IP) injection of 0.1 ml dose of 25mg/kg/day. This high dose of CLZ was used for inducing myocarditis based for on an earlier study[36]. Group 3 was administered with CLZ plus aqueous date fruit extract (aq. ADFE) 1g/kg/d by gastric gavage which was equivalent to 7 dates per person/day[35]. Each rat in Group 4 was given CLZ plus aqueous fruit pit extract (aq. ADPE) 1g/kg/d by gastric gavage.

2. Blood Sampling: At the end of the study on the 22nd day the anaesthetized animal was placed in dorsal recumbent position. After marking the lower border of sternum, needle of syringe was introduced just to the left of xiphoid process towards heart at 20–30 degrees. By applying negative pressure at least 3cc of blood was collected from heart in serum vials.

Fig-2: Collection of blood by intra-cardiac puncture

3. Serum Preparation: The blood samples were centrifuged at 3000 rpm for 5 minutes. The serum was then collected with the help of micropipette in eppendorf tubes. The serum was stored in a freezer at -40°Ctill further analysis.

4. Supernatant Preparation: After blood sampling animals were surgically sacrificed. A vertical midline incision was performed to cut sternum, then heart was taken out. The hearts were excised and washed with ice-cold saline blotted with a piece of filter paper and divided into two halves. One half of each heart was homogenized in phosphate buffer (pH 7.4). The homogenates were centrifuged at 3000rpm at 4°C for 30 min. The supernatants of the homogenates were removed and stored in eppendorf tubes at -40°C. The Supernatant was used for biochemical assays.

Fig-3: Rat heart placed on a filter paper

5. Biochemical Parameters:

1) CK-MB activity in serum was measured by using CK assay kit (Weiners Lab).This assay is based on the conversion of creatine phosphate and ADP by CK-MB to creatine and ATP. The ATP and glucose are converted to ADP and glucose 6 phosphate by hexokinase (HK) and G6PD then oxidizes G6P and reduces NAD to NADH. Rate of NADH formation, measured at 340nm, is directly proportional to CK-MB activity[37].

2) LDH (Lactate Dehydrogenase) activity in rats' serum was measured by LDH kit (Analyticon, Germany). It is the kinetic determination of LDH activity.

$$\text{Pyruvate} + \text{NADH} + H^{+} \leftrightarrow \text{Lactate} + NAD^{+}$$

Lactate dehydrogenase catalyzes the conversion from pyruvate to lactate oxidizing NADH into NAD. The rate of NADH decrease is measured photometrically and is directly proportional to the LDH concentration[38].

3) Lipid peroxidation was measured in cardiac homogenate (MDA Quantification).The unknown MDA containing samples were first reacted with Thiobarbituric acid at 95˚C. After brief incubation, the samples and standards were read

spectrophotometrically. MDA content in unknown samples was determined by comparison with predetermined MDA values[39].

4) Determination of GSH level and GSH-Px activity: (Glutathione and Glutathione peroxidase). For intracellular GSH measurement equal volume of perchloric acid was mixed with cardiac homogenate by vortexing, and was then allowed to stand for 5 min. at room temperature. After centrifugation at 3000 rpm, supernatant was collected and was assayed using Ellman's reagent[40]. For GSH-Px activity, enzymatic reaction involving β NADPH, GSH, Glutathione reductase and a standard, was initiated by the addition of Hydrogen peroxide. The change in absorbance was measured spectro-photometrically[41].

Statistical Analysis:

It was done by using SPSS version 20.0. Based on normality and homogeneity, one way ANOVA was applied for test of difference of mean.

RESULTS

A tabular description of the various animal groups and the diet they were given is represented in Table-1.

GROUPS (n=8)
GROUP 1 (CONTROL standard lab. diet)
GROUP 2 (CLZ only)
GROUP 3 (CLZ & aq. ADFE)
GROUP 4 (CLZ & aq. ADPE)

The mean values of each group for the various biochemical parameters are illustrated through bar charts. The overall difference among groups was found significant with p<0.001. After 3 weeks of treatment with clozapine , group 2 had highest levels of isoenzymes LDH and CK-MB levels of 312.6±10.5 and (332.13IU/L) respectively than group 1, 3 and group 4 (p-value <0.001) while group 1 had the lowest. Group 3, and 4 had mean levels close to healthy controls in group 1 (Fig-4). The difference of values LDH, CK-MB between Groups 3 &4 was not statistically significant (p-values of 0.752, 0.914 respectively).

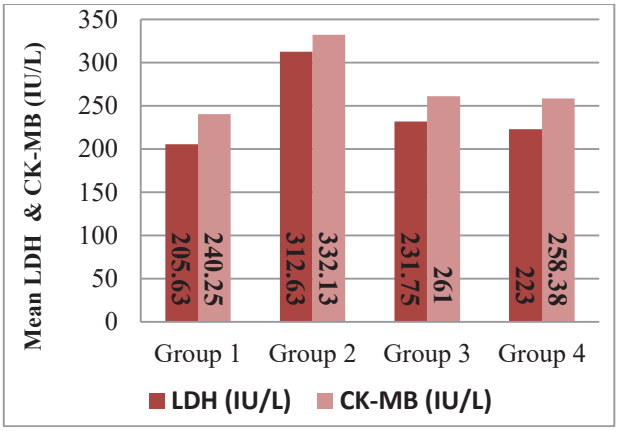

Fig-4: Group-wise comparison of mean LDH & CK-MB values

The mean MDA levels were similarly noted to be the highest for group 2 at 433.88umol/g protein (p-value <0.001) and least for group 1 at 321.13umol/g protein (p-value <0.001).(Fig-5). MDA levels of group 3 and 4 were both closer to normal values of Group 1 and the difference between them was not statistically significant p-value 0.999.

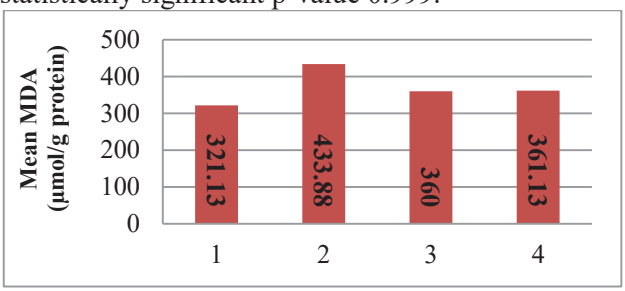

Fig-5: Group-wise comparison of mean MDA values

The GSH value was in reverse order in comparison to previous variables and mean value was lowest for group 2 (P-value <0.001) and highest for group 1(P-value <0.001), and were recorded to be 17.6±1.2 and 34.7±1.7 respectively. The difference between GSH value of group 3 and group 4 was not statistically significant (p-value 0.958). The GSH-Px levels had almost similar results as for GSH (Fig-6).

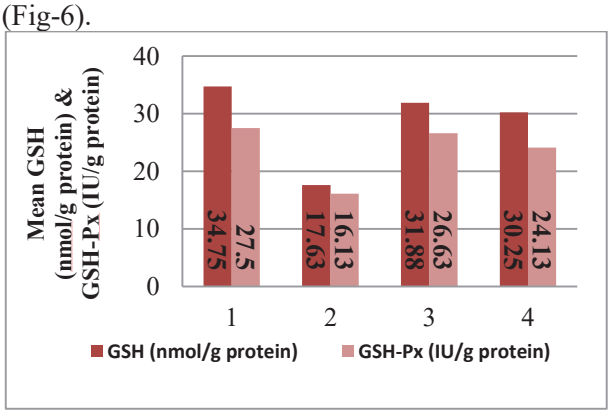

Fig-6: Group-wise comparison of mean GSH & GSH-Px values

DISCUSSION

Clozapine (CLZ) is one of the commonly used SGAs having lesser extra pyramidal side effects[42,43]. However, this potential of CLZ is compromised by its tendency to result in other serious side effects like the life threatening myocarditis[44,45]. Hence the use of CLZ is restricted to patients of treatment-refractory schizophrenia and for patients with suicidal tendencies. Consequently, there is a dire need to find treatments to counter this life threatening myocarditis caused by CLZ so that the use of this very effective antipsychotic drug can be expanded to treat more patients of schizophrenia. Dates are rich in these bioactive non-nutrients[46]. So, the aim of present study is to explore the effects of various extracts of Ajwa date palm fruit and pit, a particular cultivar of dates superior to all varieties[23]. A healthy control group (group 1) was maintained on standard laboratory diet. Group 2 (diseased control) was given CLZ only in 0.1 ml dose of 25mg/kg/day IP (intraperitoneally) for 21 days. 3rd group was given both CLZ and aq. ADFE (1gm/kg/day orally) while Group 4 received CLZ and aq. ADPE (1gm/kg/day orally) for 21 days. No animal loss occured during the study.

1. Serum LDH and CK-MB Levels

The elevated levels of LDH and CK-MB are considered an important indicator of early and late cardiac damage, particularly during clinical follow-up of any drug induced cardiac pathologies[47]. In our study significant differences were found when the groups were tested for serum LDH levels with $p<0.001$. Group 1 (healthy control) had the lowest LDH levels, while those of group 2 (diseased control) rose by 34% as compared to group 1 upon CLZ administration. This showed that biochemical derangements had begun to occur with CLZ. LDH levels of group 2 were the highest among all the groups with $p<0.05$. On the other hand, 3 weeks of administering aq. ADFE and aq. ADPE (group 3 & 4) resulted in significant decline of 25%, and 28% respectively as compared to the diseased control group. A significant decline in CK-MB levels was also noted in groups 3 & 4 of 21% & 22% respectively (Fig-4). Phytochemicals present in date fruit and pit could have proven beneficial in this regard as these contain compounds known to have anti-oxidant properties and play an important role in prevention of cardiovascular diseases[19].

No previous direct research on serum LDH and CK-MB levels could be found to support or refute these results proving the current work to be original in its scope.

Oxidative Stress Parameters:

2. Lipid Peroxidation Product (MDA)

Lipid peroxidation is a well-known mechanism of cell damage and oxidative stress. Lipid peroxides decompose to form malondialdehyde[48]. In our study, the levels of MDA were measured in all rats. Our study showed that CLZ injected rats (group 2) $p<0.001$, had the highest MDA levels with a 26% increase as compared to the healthy control (group1). After 21 days of treatment, aq. ADFE and aq. ADPE (group 3 & 4 respectively) provided less potent antioxidant activity with a decrease in MDA levels to 17%, and 16% respectively (Fig-5).

3. GSH

Glutathione is a fundamental intracellular enzyme that helps in protecting the cells from free radical damage and acts as an antioxidant[49]. In healthy cells, reduced form of glutathione (GSH) is more than 90% of total glutathione pool. When cell is under oxidative stress, GSH levels drop down[50]. In our study GSH levels were observed.

Group 1 (healthy control) had the highest GSH levels while group 2 (in which CLZ was used for inducing cardiotoxicity) had the lowest levels with p-value<0.001. Thus a drop of 97% was recorded in GSH levels due to CLZ generated oxidative stress. GSH levels of group 3 and 4 were closer to healthy control group proving antioxidant activity in Ajwa dates (Fig-6).

4. GSH-Px

Like GSH, the main role of GSH-Px is to protect the cell from oxidative damage. We measured GSH-Px levels in all rats and it also showed heartening results. The healthy control (group 1) had the highest GSH-Px levels. The least GSH-Px levels were observed for group 2 (where myocarditis had been induced by giving CLZ) as reflected by a drop of 70% versus group 1.

The cardiac GSH-Px of rats in Group 3 (aq. ADFE) and group 4 (aq. ADPE) exhibited improvement in of 65% and 49% respectively reflecting perhaps that pit extract had lesser antioxidant activity than the fruit extract (Fig-6).

This showed that aq. ADFE had the highest antioxidant and free radical scavenging ability. These results were in agreement to previous results. Aq. extract of Ajwa date fruit had the highest free radical scavenging ability[51].

CONCLUSION

In conclusion, the myocardial toxicity caused by Clozapine in rat model was improved by consumption of aqueous extracts of Ajwa dates pit and fruit extract. It was demonstrated by an

improvement in cardiac biochemical and oxidative stress marker levels of LDH, CK-MB, GSH, GSH-Px and MDA values in experimental groups 3 and 4, which were administered these Ajwas aqueous extracts.

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