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# BIOACTIVITY GUIDED ISOLATION AND ANTI-CATARACT ACTIVITY OF ALPHA-AMYRIN FROM DICHLOROMETHANE EXTRACT OF CORCHORUS TRILOCULARIS

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## ABSTRACT

***Aim of Study****-The aim of the study was to isolate the active principles and evaluate the anti-cataract activity using goat lens.* ***Material & Methods****- Triterpenoids were isolated from dichloromethane extract using dichloromethane and methanol solvent. α-amyrin was isolated and anti-cataract activity evaluated.* ***Results****- α-amyrin treated lenses showed significantly increased level of $K^+$ (P<0.001) with increasing concentration and the maximum activity was registered at concentration of 500 µg/ml respectively. There is a significant increase in MDA levels in groups treated with glucose. The α-amyrin treated lens had showed significant decrease in lipid peroxidation levels when compared to glucose 55 mM treated lens. The level of MDA was expressed in nmoles of MDA formed/mg protein.* ***Conclusion****- isolation of active compound was followed from dichloromethane and isolated compounds also showed significant activity in In-vivo models. After performing the study, we can summarize that the α-amyrin having potential antioxidant property and can restore the transparency of the crystalline lens by increasing the anti-oxidative enzyme levels. The major cause of the opacity of the lens is structural modification of the lens proteins.*

## KEY WORDS

*Cataract, amyrin, Oxidative stress, Corchorus trilocularis, total protein content*

## INTRODUCTION

Cataracts, which affect more than 50 million people (Taylor and Davies, 1987), are the most common cause of blindness in the world. There have been significant advances in surgical techniques and refinement of intraocular lens implants which have benefited cataract patients (Zigler et al., 2003). It is one of the major causes of visual disability and blindness throughout the world (Spector, 1974).

Oxidative stress, defined as an excess of pro-oxidants relative to antioxidants and a key factor in the gradual loss of lens transparency, is implicated in the initiation of maturity onset cataract which appears late in life and is probably not associated with congenital conditions or other diseases, such as diabetes (Spector, 1995).

A scientific examination of traditional herbal remedies for diabetes may provide precious leads for the expansion of alternative drugs and strategies. Alternatives are clearly needed for better management of diabetes because of high cost and poor availability of current therapies for many rural populations, particularly in rising countries (Li et al., 2004).

Corchorus (Family: Tiliaceae) is a genus of annual herbs. Nearly 40 species are known to occur in nature and distributed in tropics of both the hemisphere. The roots and capsules of Corchorus are antibacterial, demulcent, bitter tonic, laxative, carminative, refrigerant, and febrifuge, diuretic, useful in chronic cystitis, gonorrhea, cadiotonic and in treatment of diabetes.

In our previous study, antidiabetic activities of various extracts were studied in STZ induced diabetic animals. Among all extracts, dichloromethane extract showed potent activity (data not shown here). So, our main aim of study is to isolate the active principles from dichloromethane extract and prove its use in the diabetic complication i.e. cataract formation, so that we are able

to come up with a more effective and potent bioactive extract or phytoconstituents with fewer side effects in comparison with existing synthetic drugs (Khan et al., 2006).

## MATERIAL & METHODS

### Collection and authentication of the plant leaves

The leaves of Corchorus trilocularis were collected from outfield Medicinal garden during the month of July that shows the green color with rough surface. The plant leaves were washed thoroughly in tap water, dried in shade, finely powdered and used for successive extraction methods. Plants were identified by senior botanist.

### Successive Solvent Extraction Methods

The dried leaves of Corchorus trilocularis were powdered and a fixed quantity of powdered drug was defatted by using petroleum ether. Then dried mark was again extracted by using dichloromethane, ethyl acetate, ethanol, butanol and finally with water. All the extracts were dried & % Yield of the Petroleum ether, Dichloromethane, Ethyl acetate, Ethanol, Butanol, & Aqueous extract of Corchorus trilocularis was calculated (Mukherjee, 2002; Kokate, 1996).

### Bioactivity guided isolation from dichloromethane extract of Corchorus trilocularis

Wet packing technique was adopted for packing the column. 20 gm of extract was mixed with 50 gm of silica gel and a very small amount of an appropriate solvent. For the isolation of compounds, dichloromethane and methanol was used as a solvent system. Firstly 100% dichloromethane was passed through column and then increasing quantity of methanol was used in different ratio (90:10, 80:20, 70:30, 60:40 and finally 50:50). Elute was collected at the rate of 20 drops per minute and each fraction was about 25 ml and total 150 fractions were collected. All the collected fractions were monitored simultaneously on a TLC plate using dichloromethane: methanol (83:17) as solvent system. The fractions showing same TLC pattern were pooled together and finally 5 fractions (F1-F5) were obtained. Percentage yield of collected elutes were determined in respect to the total weight of the fraction. All the fractions were evaluated for antidiabetic activity (data not shown here) and fraction 2 showed most promising antidiabetic activity & a single spot in TLC and it was characterized by spectral techniques (Mukherjee, 2002; Kokate, 1996).

### Spectral analysis and Structure elucidation

It was tried to purify compounds, which were obtained by employing Column chromatography and TLC and by re-crystallizing them in different solvents. The compounds were weighted, and their melting point determined. The identification of a molecule was done through the interpretation of the data obtained from spectroscopic analysis i.e. IR, NMR, Mass etc.

### Anti-cataract Activity of α-amyrin

### Collection of Eyeballs

Fresh goat eyeballs of young and healthy goats were collected from the slaughterhouse, immediately after the slaughter. These eyeballs were immediately transferred to the laboratory at 0-4°C. Sliced the Cornea from the front of the eye to gain access to the lens.

### Lens culture

The lenses were incubated in artificial aqueous humor (NaCl 140mM, KCl 5mM, MgCl2 2mM, NaHCO3 0.5mM, Na2HPO4 0.5mM, CaCl2 0.4mM, Glucose 5.5mM) for 72 hours at room temperature at a pH of about 7.8 is maintained. In addition to this 32mg of penicillin and 250mg of streptomycin were added to prevent bacterial contamination. Glucose 55mM served as cataract inducer (Goyal et al., 2012).

The grouping schedules are as follows.

Group I : Normal lens glucose 5.5mM (control)

Group II : Glucose 55mM (induced)

Group III : A. Glucose 55mM+ α-amyrin (100µg/ml) (Treated)

B. Glucose 55mM+ α-amyrin (300µg/ml) (Treated)

C. Glucose 55mM+ α-amyrin (500µg/ml) (Treated)

### Photographic Evaluation

After 72 hours of incubation, lenses were observed for opacity and photographs were taken by placing the lenses on the wire meshes with posterior surface touching the mesh, and the pattern of mesh was observed through the lens as a lens as a measure of lens opacity.

### Preparation of lens homogenate

After incubation, lenses were homogenized in 10 volumes of 0.1M potassium phosphate buffer, pH 7.0. The homogenate was centrifuged at 10,000 rpm for 1 h and the supernatant was used for estimation of biochemical parameters (Goyal et al., 2012).

**Estimation of Biochemical Parameters**

**Estimation of total protein content**

To 0.1 ml of lens homogenate, 4.0ml of alkaline copper solution was added and allowed to stand for 10min. Then, 0.4 ml of phenol reagent was added very rapidly and mixed quickly and incubated in room temperature for 30 mins for colour development. Reading was taken against blank prepared with distilled water at 610 nm in UV-visible spectrophotometer. The protein content was calculated from standard curve prepared with bovin serum albumin and expressed as µg/mg lens tissue (Goyal et al., 2012).

**Estimation of malondialdehyde (MDA)**

Lenses were homogenized in10% (w/v) 0.1 M Tris–HCl buffer (pH 7.5). One milliliter of the homogenate was combined with 2 ml of TCA–TBA–HCl reagent 15% trichloroacetic acid (TCA) and 0.375% thiobarbituric acid (TBA) in 0.25 N HCl and boiled for 15 min. Precipitate was removed after cooling by centrifugation at 1000g for 10 min and absorbance of the sample was read at 535 nm against a blank without tissue homogenate. The values are expressed as nmoles of MDA/ min/ mg lens protein (Goyal et al., 2012).

**Estimation of Different electrolytes**

Sodium and potassium levels were estimated by flame photometry (Goyal et al., 2012).

**Statistical analysis**

The values are expressed in mean ± SEM. The results were analyzed by using one-way analysis of variance (ANOVA) followed by Dunnet's "t" test to determine the statistical significance. $p< 0.05$ was chosen as the level of significance. Statistical analysis was performed using Graph Pad Prism Software 5.0 version.

## RESULTS

**Isolation & Characterization of α-amyrin**

By the bioactivity guided isolation procedure, triterpenoids was isolated from dichloromethane extract. The compound was identified on the basis of various spectral techniques and by comparison to previous literature, isolated compound closely resembles to α-amyrin.

**α-amyrin**

**IR (KBr)-** 3442, 3056, 2860, 2693, 2237, 2159, 1963, 1731, 1643, 1512, 1483, 1419, 1359, 1344, 1279, 1220, 1147, 1049, 946, 842, 657, 537cm$^{-1}$

**1H NMR** (400 MHz, Chloroform)- δ 5.40 (s, 7H), 3.47 (s, 7H), 2.80 (s, 6H), 2.23 (s, 7H), 2.02 (s, 5H), 1.96 (dd, J = 9.5, 1.7 Hz, 27H), 1.85 (d, J = 19.4 Hz, 13H), 1.77 (s, 5H), 1.68 (dd, J = 24.5, 5.8 Hz, 1H), 1.74 – 1.21 (m, 122H), 1.21 – 1.16 (m, 18H), 1.03 – 0.99 (m, 41H).

**$^{13}$C NMR** (100 MHz, Common NMR Solvents) δ 143.02 (s), 125.43 (s), 78.57 (s), 57.72 (s), 54.69(s), 46.52 (s), 42.14 (s), 40.20 (s), 39.67 (s), 38.71 (s), 38.39 (s), 38.04 (d, J = 8.4 Hz), 37.47 (s), 34.06 (s), 32.86 (s), 32.39 (s), 29.16 (s), 27.68 (d, J = 8.7 Hz), 25.88 (s), 24.13 (s), 23.89- 23.67 (m), 23.36 (s), 19.61 (s), 18.79 (s), 18.02 (d, J = 16.2 Hz), 17.05 (s).

**Mass Spectra-** In α-amyrin, IR absorption bands were appeared at 3442 cm$^{-1}$ indicating the presence of hydroxyl group, 3056 cm$^{-1}$ ( C-H str. in $CH_3$ ), 2860 cm$^{-1}$ ( C-H str. in $CH_2$ ), 1731 cm$^{-1}$ ( C=O str. ), 1483 cm$^{-1}$ ( C-H def. in $CH_3$ ), 1359 cm$^{-1}$ ( C-H deformation in gem dimethyl ), 842 cm$^{-1}$ ( =C-H out plane bending ). The $^1$H-NMR spectrum shows that H-2 proton appeared at δ 3.47 as a multiplet and H-13 olefinic proton shows a singlet at δ 5.40. Also, eight methyl protons appeared as singlet as well as multiplet at δ1.13, δ 1.02, δ 1.01, δ 0.96, δ 0.94, 0.88 and δ 0.88 which were quite similar with α-amyrene as mentioned by Saha et al. The $^{13}$C-NMR has shown recognizable signals at 143.02 and 125.43ppm, which corresponds to double bond at C-12 and C-13. The δ value at 78.56 ppm is due to C-2 β- hydroxyl group. The peaks also showed that the isolated compound had eight methyl group, ten -$CH_2$ group and four –CH groups. The results were compared with the available literature and confirmed the presence of α-amyrin.

**Anti-cataract Activity of α-amyrin**

Glucose (55mM) treated lenses showed significantly higher Na+ and lower K+ activity ($P<0.001$) compared with normal lenses. α-amyrin treated lenses showed significantly increased level of $K^+$ ($P<0.001$) with increasing concentration and the maximum activity was registered at concentration of 500 µg/ml respectively.

In Table No. 1, α-amyrin treated lenses showed significantly decreased level of $Na^+$ ($P<0.001$) with increasing concentration and the maximum activity was registered at concentration of 500 µg/ml respectively.

**Table No. 1: Effect of α-amyrin on $Na^+$ & $K^+$ activity in lens homogenate after 72 hrs of incubation**

| Description | $Na^+$(meq/gm) | $K^+$(meq/gm) |
|---|---|---|
| Normal Goat lens | 153±10.1 | 14.1±1.4 |
| Goat lens + Glucose55mM | 220.4±12.3*** | 6.4±0.3*** |
| Goat lens + Glucose55mM + α-amyrin in conc. of | | |
| a) 100µg/ml | | |
| b) 300 µg/ml | 170.8±11.2** | 8.6±0.94* |
| c) 500 µg/ml | 165.5±12.3** | 10.8±0.9** |
| | 158.5±19.4*** | 12.7±1.8*** |

**Where- *p<0.05, **p<0.01, ***p<0.001 compared with diabetic control vs treated groups**

**Photographic Evaluation**

All the lens of group I developed dense opacities. The opacity was progressively increased towards centre with complete opacification at the end of 72 hrs. While α-amyrin treated at 100, 300 and 500 µg /ml retarded the development of opacity. The grades of opacity was +++, ++, +.

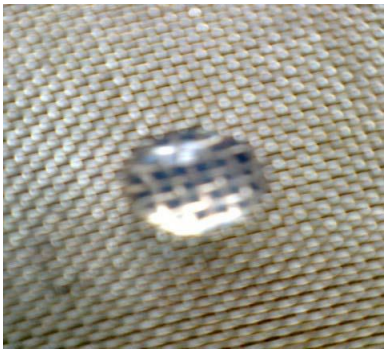

Figure No.1: Normal lens before 72 h of incubation.

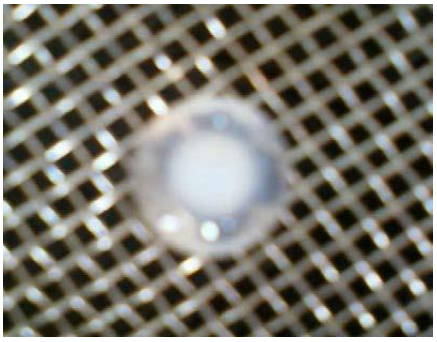

Figure No. 2: Lens after 72 h of incubation with glucose 55 mM.

**Figure No. 3: Lens after 72 h of incubation with glucose 55 mM+ α-amyrin 500 µg /ml.**

**Effect of α-amyrin on total protein and MDA level**

Group II (disease control) showed significant decrease (P<0.001) in protein content as compared to group I. On the treatment of α-amyrin, the total protein content was highly significantly increased in treated groups.

There is a significant increase in MDA levels in groups treated with glucose. The α-amyrin treated lens had

showed significant decrease in lipid peroxidation levels when compared to glucose 55 mM treated lens. The level of MDA was expressed in n moles of MDA formed/mg protein.

**Table No. 2: Effect of α-amyrin on total protein and MDA level in lens homogenate after 72 hours of incubation.**

| Description | Total Protein (mg) | MDA (nmoles/mg) |
|---|---|---|
| Normal Goat lens | 180.4±5.4 | 32.8±2.4 |
| Goat lens + Glucose 55mM | 155.2±5.4** | 59.8±3.1 |
| Goat lens + Glucose 55mM + α-amyrin in conc. of | | |
| a) 100µg/ml | 162.4±0.4* | 54.5±0.45* |
| b) 300 µg/ml | 165.8±0.65** | 49.3±0.12** |
| c) 500 µg/ml | 180.8±1.3*** | 42.2±0.64*** |

**Where- *p<0.05, **p<0.01, ***p<0.001 compared with diabetic control vs treated groups**

## DISCUSSION

Prolonged exposure to free radicals is a pivotal cause of tissue stress and injury. The free radical permanent damage to tissue structures results from a permanent alteration in the molecular pattern of carbohydrates, lipids, proteins and even nucleic acid bases. In diabetes, the level of free radicals was reported to increase in alloxan and streptozocin treated rats an elevated level of free radicals was detected in several tissues including the kidneys (Shabeer et al., 2009).

The research was envisaged for isolation of active principles from dichloromethane extract of Corchorus trilocularis and to find out or isolate the most possible active compounds from the active extracts showing the best activity. The isolated compound has been evaluated in diabetic complications like cataract activity.

Bioactivity guided isolation was performed to separate Phytoconstituents from bioactive dichloromethane extract of Corchorus trilocularis by applying variety of mobile phases.

One compound was isolated in significant amount and their characterization was done using different spectral techniques ($^1$H-NMR, Mass, IR and UV analyses) based on the following deliberations, to reveal their identity as triterpenes.

Based on the Mass, IR, NMR analysis, the isolated compound was identified as α-amyrin acid, which was compared to the standard.

Literature review also revealed that individually amyrin, β-sitosterol and stigmasterol showed no hypoglycemic activity but the presence of both is necessary to produce synergism effect between the two (Alam et al., 2012; Nazreen et al., 2011).

Several studies on different experimental models and on human cataract have now suggested that the level of various antioxidants in lens has important role in cataract development. But, a number of unresolved issues require further investigation.

In our study we found that, on administration of isolated compounds has significantly increased protein content in lens. The probable mechanism of anti-cataract activity may be via alteration of protein content in eye.

The lens is equipped with a number of detoxifying enzymes, which is another means of self-protection. Detoxification occurs through a multitude of antioxidants, oxidation defense enzymes and by other means such as through NAD+/H dependent reductases, oxidases and dehydrogenases. Antioxidant enzymes such as catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPX) have the ability to reduce reactive oxygen species. Together with catalase, an important lenticular antioxidant enzyme, contain a heam protein with one molecule of NADPH, which is tightly bound to each of the four subunits of catalase in mammals are important enzymatic antioxidant defense systems in the lens.

Antioxidant enzymes are able to catalytically remove free radicals and other reactive species. A wide array of enzymatic antioxidant defenses exists, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) (Halliwell & Gutteridge, 1999). Expression of some of these enzymes is controlled by redox-sensitive transcription factors, allowing the

antioxidant system to respond to fluctuations in production of oxidizing species caused by photooxidative processes, especially during sustained exposure (Rahman, 2000).

In our study we found that, on administration of isolated compounds has significantly decreased the MDA level. MDA is a powerful oxidant radical and can damage the lens. Since triterpenoids and flavonoids act as a antioxidant derivatives, so by inhibiting the formation of these radicals they prevent further damage by oxidation. Na+ K+ ATPase is important in maintaining the ionic equilibrium in the lens, and its impairment causes accumulation of $Na^+$ and loss of $K^+$ with hydration and swelling of the lens fibers leading to cataractogenesis (Wilbur, 1949). This alteration in the $Na^+$ $K^+$ ratio alters the protein content of the lens, leading to a decrease in water soluble proteins content and increase in insoluble proteins. This causes lens opacification (Chylack and Kinashita, 1969). In the present study, cataract lens treated with isolated compound elevated the activity of total proteins and $K^+$ ions to the level of normal level whereas reduced concentrations of $Na^+$ ions. This clearly evidenced that isolated compounds seem to prevent the alteration of $Na^+$ and $K^+$ imbalance, which may be due to a direct effect on lens membrane Na+ K+ - ATPase or indirect through their free radical scavenging activity.

## CONCLUSION

The isolation of active compound was followed from dichloromethane and isolated compounds also showed significant activity in In-vivo models. After performing the study, we can summarize that the α-amyrin having potential antioxidant property and can restore the transparency of the crystalline lens by increasing the anti-oxidative enzyme levels. The major cause of the opacity of the lens is structural modification of the lens proteins.

The present findings are significant for the development of alternative, inexpensive and perhaps safer strategies for the treatment of diseases.

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