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ISOLATION, CHARACTERIZATION AND PHARMACOLOGICAL EVALUATION OF THE ANTICATRACTOGENIC ACTIVITY OF THE ESTERIFIED LUTEIN FROM MARIGOLD FLOWERS

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ABSTRACT

Tagetes erecta, the mexican marigold also called Aztec marigold is a species of genus tagetes. Tagetes erecta is known for its high therapeutic values. These plantsare rich in alkaloids, terpenes, flavonoids, phenolic compounds etc. The dried and cleaned marigold flower petals were taken and lutein was extracted from hexane through conventional extraction by soxlet exractor. The esterified lutein was subjected to analytical procedures like TLC, UV-Visible spectroscopy and IR Spectroscopy. The biological activities like Anti catarctigenic activity was evaluated in Dexamethasone and glucose induced cataract.

KEYWORDS: Tagetes erecta; esterfied lutein, Dexamethasone, Glucose, Cataract.

INTRODUCTION

Marigold flower is one of the richest sources of natural carotenoids. The major carotenoid in marigold is lutein, which has been reported to be beneficial in several aspects to human health such as supporting eyes and skin, and reducing the failure of the eyesight due to age-related macular degeneration (AMD), coronary heart disease and cancer.[1] Therefore, lutein has gained much interest due to its potential in nutraceutical and pharmaceutical applications. In marigold flowers, lutein generally exists in the form of lutein fatty acid esters. Conventional method for marigold lutein fatty acid esters extraction is achieved by solvent extraction (generally using hexane). Alternatively, the environment friendly and non-toxic extraction solvent such as supercritical carbon dioxide (SC-CO2) can also be used so as to provide milder extraction conditions.[2] Since only in its free form that lutein can be taken up

by human body[3-4], marigold extract or marigold oleoresin must therefore be saponified with an alkali solution, i.e. KOH solution, to obtain free lutein.[5] Unfortunately, the saponified lutein mixture contains many impurities such as soap, oil, unreacted lutein fatty acid esters. Thus, a purification process is generally required to obtain purified lutein for human applications. Crystallization is a common process for purifying free lutein, however it results in rather low yield and purity. Although high purity could be achieved by re-crystallization, the process requires several steps, making it rather complicated, and thus lowering the overall yield.[6]

Lutein is a yellow plant pigment that belongs to the carotenoid family, namely to xanthophylls. It occurs in many kinds of fruits and vegetables, especially in leafy vegetables, but also in the yolk and eye tissues Lutein acts as an effective antioxidant, namely in the protection of eyes, because it neutralises free radicals formed by the action of ultraviolet radiation on eye retina. Humans are not able to synthesise lutein, so they can acquire it solely by the consumption of fruits, vegetables, and/or food supplements. Plant materials contain all-trans-isomer of lutein; nevertheless, cis-isomers of lutein aregenerated, apart from other agents, also by the actions of light and temperature, and other factors were also detected during extraction and sample analysis. In plants, lutein is present either in the form of free lutein in leafy vegetables such as spinach, cabbage, and broccoli, or in the form of esters with fatty acids in the following fruits and vegetables: mango, orange, papaya, red or green pepper, yellow corn etc. The content of lutein in natural sources depends on their kind, variety, level of maturity, part of fruit, and also on the way of processing by heat, preservation, or storage.[7]

Today, cataracts affect more than 22 million Americans age 40 and older. And as the U.S. population ages, more than 30 million Americans are expected to have cataracts by the year 2020, PBA says.[8]

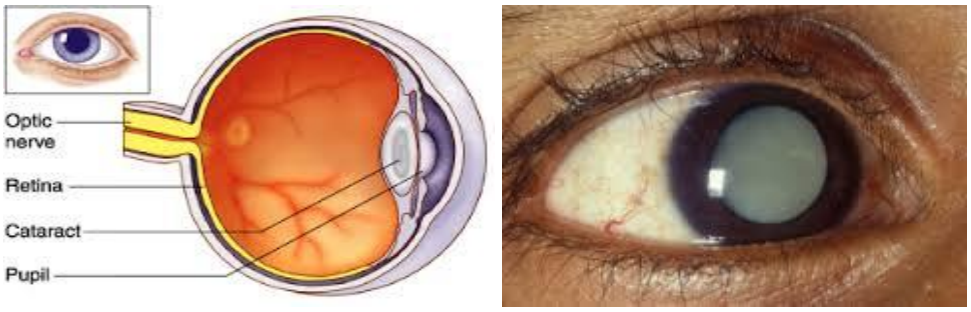


Fig:1: Normal and cataract eye.

Classification

Cataract may be partial or complete, stationary or progressive, hard or soft. The main type of age related cataracts are nuclear sclerosis, cortical and posterior subcapsular.

Nuclear cataract

Corticalcataract

Posteriorsubcapsularcataract

Animmaturecataract

Congential cataract

Role of Lutein in Prevention of Hypoxia-Induced Cell Damage in the Eye Free radicals, as defined by the presence of one or more unpaired electrons in an atom or groups of atoms, come in many different forms. The most common type of free radical in biological system is the reactive oxygen species (ROS). ROS such as singlet oxygen species are generated in the retina because of oxygen consumption as well as high energy photon light conversion into electrochemical signaling ROS generated in the retina are believed to be the byproducts of extramitochondrial oxidative phosphorylation in the rod outer segment. The accumulation of oxygen radicals and lipid peroxidation, resulting from increased retinal oxygen utilization, has been postulated as a mechanism for photoreceptor apoptosis. Oxidative damage occurring in the rod outer segment causes the release of extra-mitochondrial components like cytochrome c, which initiates apoptosis via caspase 9 activation. One of the major protective roles lutein has in the retina is to serve as an oxygen free radical scavenger during oxidative stress conditions. The ability of lutein to provide effective removal of free radicals, such as singlet oxygen particles, is primarily governed by the chemical structure of two hydroxyl groups acting as strong sinks for reactive oxygen species.

MATERIALS AND METHODS

Plant source: The Marigold flowers (Tagetus erecta) were brought from Rythu bazaar, Behind RTC bus stand. They were aunthenticated by Pharmacognosy department. The flowers that cleaned and the petals were removed from the flowers and the petals were dried under sunlight such that water content would be removed from them.

Extraction Process: The dried marigold flowers were packed and taken into the soxhelet extractor, the extraction process was performed by using HEXANE as the solvent The conditions to be maintained are: Temperature - 40◌c and the extraction process was continued upto 3 continous days. After 3days of extraction the extract obtained from the

extraction process was subjected to distillation in order to recover the solvent. The obtained distilled hexane was collected and stored for future use. The obtained leutin ester was stored in EPHEDRON TUBES and kept in room temperature.

Characterization of lutein

- **Uv – visible spectroscopy**

Preparation of the test sample: The unknown amount of sample was taken and it was dissolved in hexane.

Procedure: Initially the equipment UV – VISIBLE SPECTROPHOTOMETER (LAB INDIA UV-3000^{+}) was runned by placing the solvent hexane in both reference and test cuvettes and the system was made auto zero by arranging the wave length range 420 - 460nm. Now, the test sample (esterified leutin) was taken into the test cuvette and the equipment was runned by placing the wavelength range 420 - 460nm.

IR Spectroscopy

We used BRUKER ALPHA F.T.IR instrument and identified different functional groups by observing their respective wave numbers (cm^{-1}).

In vitro evaluation of anti-cataract activity

In this study, goat lens was used as they were easily available. Fresh goat lens were collected from slaughter house.

Lens culture

Fresh goat eyeballs were obtained from slaughter house was immediately transported to the laboratory at 0-40 c. The lens were removed by extra capsular extraction and incubated in artificial aqueous humour (Nacl:140mM, Hcl:5mM, Mgcl2: 2mM, naHCO3:0.5mM, NaH(PO4)2:0.5mM,CaCl2:0.4 and glucose :5.5mM) at room temperature and PH 7.8 for 72 hours. Cefixime 500mg were added to the culture media to prevent bacterial contamination.[22]

Dexamethasone induced Cataract on isolated goat lens

Induction of In vitro Cataract

Dexamethasone 10mg was used to induce cataract. Dexamethasone induced posterior subcapsular cataract by oxidative stress, osmotic change, hydration and conformational change of proteins. A total of 16 lenses were used for the study. These lenses were incubated

in artificial aqueous humour with Dexamethasone 10mg/kg served as toxic control for 72 hours.

Study group

A total 16 lenses were divided into following groups.(n= 4 in each group).

Group I: Aqueous humor (Normal control).

Group II: Aqueous humor + Dexamethasone 10mg (Toxic/model control).

Group III: Aqueous humor + Dexamethasone 10mg + Lutein 50µg/ml.

Group IV: Aqueous humor + Dexamethasone 10mg +Lutein 100µg/ml.

Glucose Induced Cataract

Preparation of Lens Culture

The lenses were removed by extra capsular extraction and incubated in artificial aqueous humor (NaCl: 140 mM, KCl: 5 mM, MgCl : 2 mM, NaHCO: 0.5 mM, NaH(PO): 0.5 mM, CaCl: 0.4 mM, andGlucose: 5.5 mM)at room temperature andpH7.8 for 72 hours.PenicillinG32 mg% and Streptomycin 250 mg% were added to the culture media to prevent bacterial contamination. Glucose at the concentration of 55mMwas used to induce cataract.

Experimental Design

Group I: Lens + Glucose 5.5mM(Normal control)

Group II: Lens + Glucose 55mM(Negative control)

Group III: Lens + Glucose 55mM+ (50ug/ml lutein)

Group IV: Lens + Glucose 55 mM + (100ug/ml Lutein)

Preparation of Lens Homogenate

After 72 hours of incubation, homogenate of lenses was prepared in tris buffer (0.23 M, pH 7.8) containing 0.25 × 10-3 M EDTA and homogenate was adjusted to 10% w/v which was centrifuged at 10,000 G at 4ºC for 1hour and the supernatant was used for the estimation of biochemical parameters.

Evaluation parameters

- Photographic evaluation.
- Weight of the lens.
- Estimation of sodium ions.

- Estimation of potassium ions.
- Estimation of proteins colorimetry.
- Colorimetric assay of catalase.
- Estimation of Malanaldehyde
- Assay of Superoxide dismutase (SOD)
- Determination of Aldose Reductase (AR)Activity.

Photographic Evaluation

After 72 hours of incubation, lenses were placed on a wired mesh with posterior surface touching the mesh and the pattern of mesh (number of squares clearly visible through the lens) was observed through the lens as a measure of opacity.

The degree of opacity was graded as follows:

'0' - Absence of opacity

'1' - Slight degree of opacity

'2' - Presence of diffuse opacity

'3' - Presence of extensive thick opacity.

RESULTS

- **UV – VISIBLE SPECTROSCOPY**

The absoption maximum (max) of esterified leutin was found to be 442nm.

Table: 1: FTIR Functional groups.

Wave Number (cm^{-1})	Functional group
3000-2950	Alkanes (stretch)
2900-2800	Aldehydes
3000-2850	Alkanes
1740-1720	Aldehydes
1725-1705	Ketones
1725-1700	Carboxylic acid
1680-1630	Amides
1730-1750	Ester
1680-1600	Alkene
1550-1350	Nitro
1350-1000	Amine
1350-1140	Sulphones, Sulphonyl chloride, Sulfates, Sulphonamides
1400-1000	Flourides
1300-1000	Alcohol, Ether, Ester, Carboxylic acids
785-540	Chloride

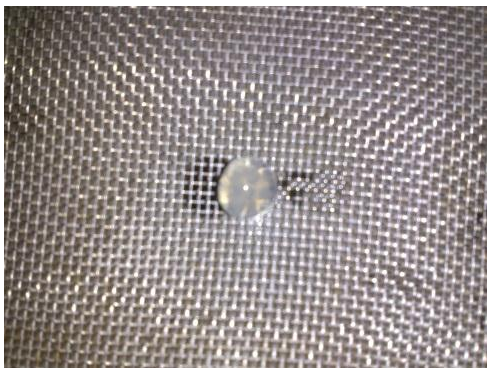
Anti Cataractogenic activity

Photographic Evaluation

Incubation of lenses with Dexamethasone 10mg showed opacification starting after 8 hours at the periphery, on the posterior surface of the lens. This progressively increased towards the centre, with complete opacification at the end of 72 hours as compared to lenses incubated in normal aqueous humour where transparency maintained and squares were clearly visible. Incubation of lenses with Lutein at (50 µg/ml, 100 µg/ml) concentrations seems to retard the progression of lens opacification.

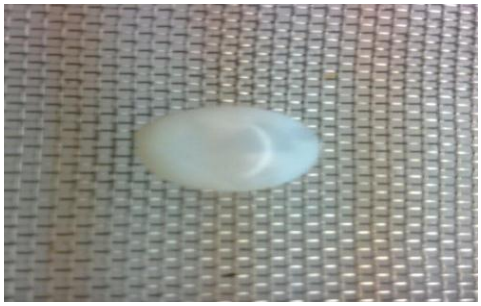
Dexamethasone induced cataract model

Group I (Normal) **Group II (Model control)**

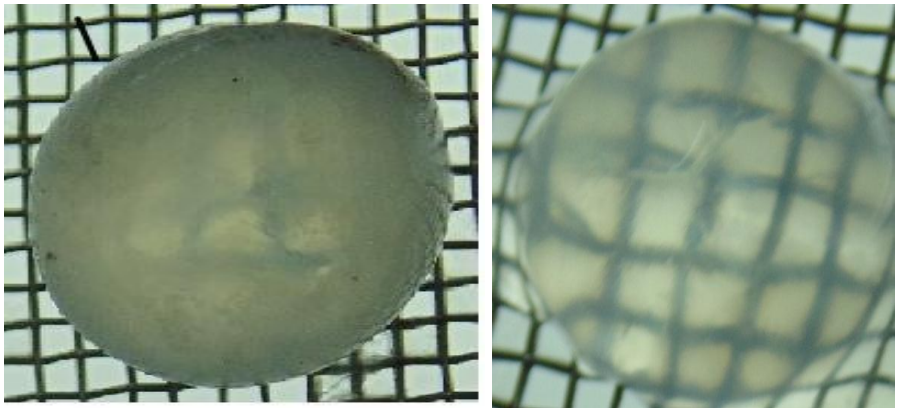


Group III (MEAS 50µg/ml) **Group III(MEAS 100µg/ml)**

Glucose induced cataract model



Group I (Normal) **Group I (Toxic)**



Group I (50ug/ml lutein) **Group I (100ug/ml lutein)**

Fig 9: Various lens.

Table 2: Grades of Lens.

Study Groups	Grade
Group I(Normal control)	0
Group II(Model control)	3
Group III(Test I)	1
Group IV(Test II)	1

Table 3: Effect lutein on weight of the lens in Dexamethasone induced Cataract.

Groups	Wt. of lens before drug treatment(gm)	Wt. of lens after drug treatment(gm)
Group I	0.41	0.41
Group II	0.45	0.73
Group III	0.47	0.65
Group IV	0.37	0.55

Table 4: Effect of Lutein on Sodium leaves in Dexamethasone induced Cataract.

Groups	Sodium levels µg/ml
Group I	105.5±+2.10
Group II	227.3±3.30
Group III	165.8±1.93
Group IV	116±1.29

Table 5: Effect of Lutein on Potassium levels in Dexamethasone induced Cataract.

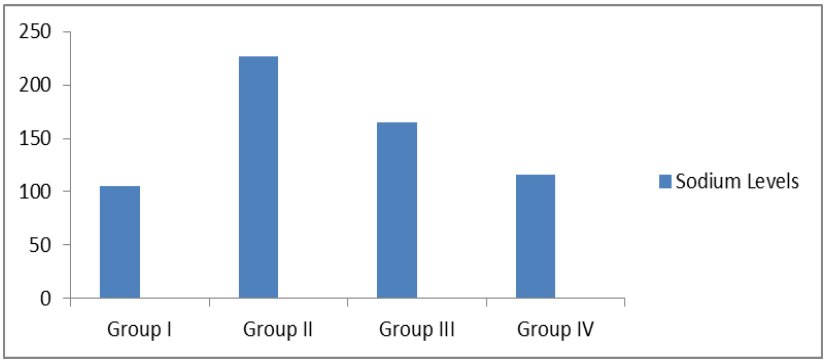
Groups	Potassium levels µg/ml
Group I	10.8±0.44
Group II	6.17±0.11
Group III	8.95±0.12
Group IV	10.18±0.15

Table 6: Effect of Lutein on Total protein content in Dexamethasone induced Cataract.

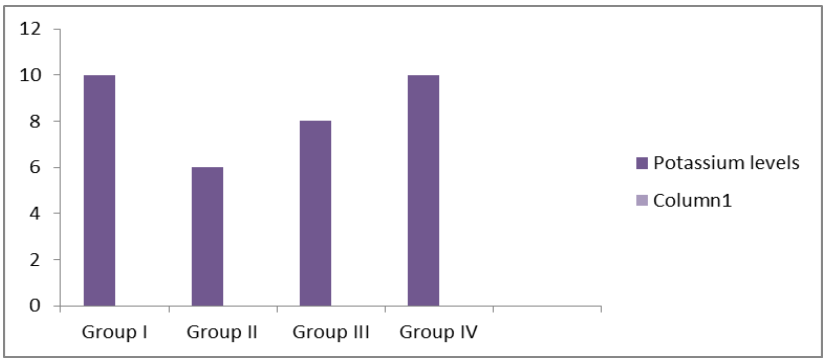
Groups	TPC level gm/dl
Group I	3.25±0.01
Group II	1.87±0.03
Group III	2.52±0.04
Group IV	2.86±0.04

Table 7: Lutein on Catalase levels in Dexamethasone induced Cataract.

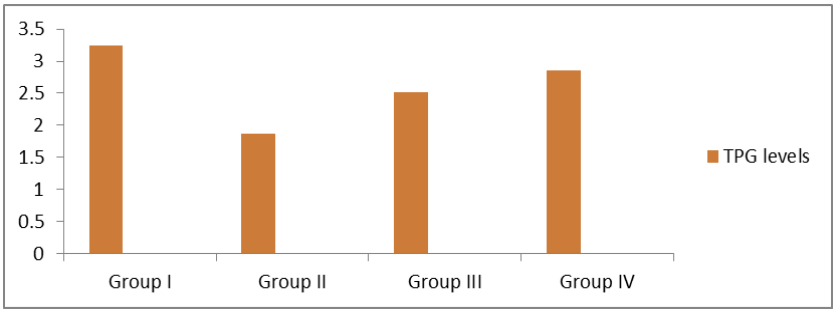
Groups	Catalase levels µm of H2O2/min
Group I	228.3±0.85
Group II	143±0.91
Group III	267.5±1.04
Group IV	304±1.86



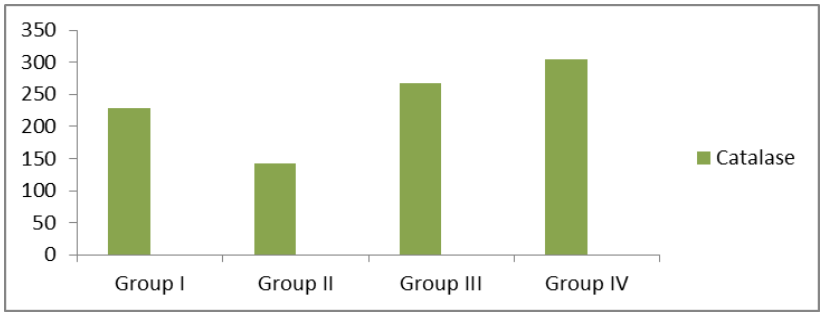
Graph 1: Effect of Lutein on Sodium leaves in Dexamethasone induced Cataract.



Graph 2: Effect of Lutein on Potassium leaves in Dexamethasone induced Cataract.



Graph 3: Effect of Lutein on TPG leaves in Dexamethasone induced Cataract.

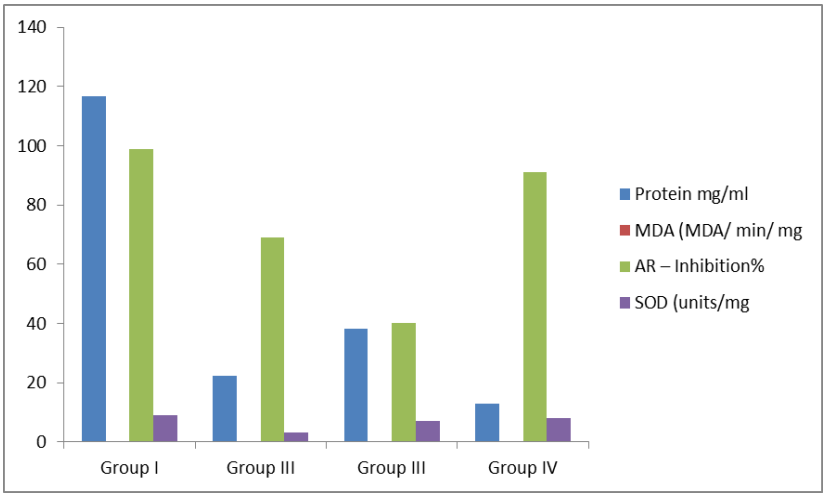


Graph 4: Effect of Lutein on Catalase leaves in Dexamethasone induced Cataract.

Glucose induced cataract model

Table 8: Effect of lutein on different parameters on glucose induced cataract.

Group	Protein mg/ml	MDA (MDA/ min/ mg lens protein)	AR – Inhibition Activity (%)	SOD (units/mg tissue
Group I	1 16.6 ± 0.316	0.0003 ± 0.00158	98.98 ± 0.0509	9.21 ± 0.01
Group II	2 2.2 ± 0.158	0.0026 ±0.000354	69.22 ± 0.0316	3.11 ± 0.0223
Group III	3 8.8 ± 0.412	0.0021 ± 0.0001	40 ± 0.0509	7.81 ± 0.10024
Group IV	12.8 ± 0.224	0.0005 ± 0.000316	91.40 ± 0.0509	8.50 ± 0.10024



Graph 5: Effect of lutein on different parameters on glucose induced cataract.

DISCUSSION

Lutein is a colour pigment is isolated from marigold flowers and it identified by analytical methods like UV Spectroscopy and IR.

Cataract is a major cause of blindness all over the world. It is an age related phenomenon, over and above oxidative stress also plays its role. Surgical treatment has remained the only remedy till now. Hence, if a drug is sought which can either reverse or prevent lenticular opacity, it will be a great advance in the treatment of this disorder. A number of drugs have been shown to interfere with the process of cataract formation like aldose reductase

inhibitors, restatin, sulindac, aspirin, etc. Cataract is one of the universal processes of ageing and is consequence of cumulative effect of various insults to the lens. The oxidation of lens proteins by free radicals and reactive oxygen species play an important role in the process leading to lens opacification. This oxidative crisis is one of the reasons for generation of cataract.

In vitro model for inducing cataract using Dexamethasone 10mg provides an effective model on isolated lenses of goat. Incubation of goat lenses in the media containing Dexamethasone (10mg) concentration induce cataract has shown to cause considerable drop in Na+/K+-ATPase activity, with progression of opacity. The impairment of Na+/K+-ATPase causes accumulation of Na+ and loss of K+ with hydration and swelling of the lens fibers leading to cataractogenesis. This alteration in the Na+, K+ ratio change the protein content of the lens, leading to a decrease in total proteins causing lens opacification. In this study showed higher total proteins ($P < 0.05$ at all concentration) and K+ ions ($P<0.05$ at all concentration) whereas lower concentrations of Na+ ions ($P<0.05$ at all concentration) with Lutein treated groups. The imbalance of Na+ and K+ is prevent due to an action of Lutein which corrects imbalances in the polyol pathway by decreasing aldose reductase activity, sorbitol concentrations. Catalase is an important part of the innate enzymatic defense system of the lens which is responsible for the detoxification of H2O2. Decrease in the activities of this enzyme in tissue has been linked with the build up of highly reactive free radicals leading to injurious effect such as loss of integrity and the function of the cell membranes. The catalase keeps the level of free radicals below toxic levels. In cataractous lenses its concentration is decreased. Hence, with the use of antioxidants cataract formation can be prevented. In this study the level of Catalase was found to be less in to experimentally induced cataract lenses as compared to normal control group ($P<0.05$). The lenses treated with Lutein showed significant rise in enzyme level suggesting maintenance of antioxidant enzyme integrity.

As the role of oxidative stress in cataract development had been established, and thus the importance of antioxidants in prevention of cataract has, also been accepted in human ophthalmology. Three molecular mechanisms may be involved in the development of diabetic cataract: nonenzymatic glycation of eye lens proteins, oxidative stress, and activated polyol pathway in glucose disposition. All of these changes accelerate generation of reactive oxygen species (ROS) and increases in oxidative chemical modification of proteins in the lens of diabetic patients. In the present study, opacity in the lens (cataract) occurred due to the

incubation of lens in the media containing high concentration of glucose (55mM) which was because of the formation of free radicals like superoxide, H O, MDA inside the cataractous lens (that led to the increase in the oxidative stress). Lutein treated lens are decreased levels of prptein and MDA and increased levels of SOD and catalase observed These free radicals are inhibited by enzymatic antioxidants such as SOD and CAT.

CONCLUSION

Research is an never ending process where the new things will the discovered based on the available proofs and from past work. In our current study we have worked on the esterified lutein extracted from the marigold flower petals.

The Present investigation suggests that Lutein effectively prevent the cataractogenic condition which was indicated by increase in the total protein content, potassium level and decrease in the sodium and calcium level. However, antioxidant property of lutein leaves was confirmed by increase in lens, Catalase. In conclusion all the above finding lends credence to lutein in the treatment of cataract.

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