

Potential of Allylmercaptocaptopril as an Anti Cataract Agent against Galactosemic Cataract in Rats

An in Vitro and in Vivo Studies

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ABSTRACT

Aim: Allylmercaptocaptopril (AMC) is a covalently bonded product of allicin and captopril has been evaluated for its anticataract activity against selenite induced cataract in experimental animals. We wanted to evaluate its anticataract potential in galactosemic cataract to elucidate biochemical mechanism to appraise its activity.

Method: We examined the protective effect of AMC in both in vitro and in vivo models of galactose-induced cataract in rats and compared the effect with captopril. We evaluated the effect of both captopril and AMC on onset and maturation of cataract in galactosemic cataract.

Result: AMC reduced the rat lens polyol level, the marker of osmotic stress induced by galactose when compared with galactose treated and captopril treated lens. Glucose-6-phosphate dehydrogenase, succinate dehydrogenase, lactate dehydrogenase activity and reduced glutathione level were decreased in the galactose treated group compared with normal lenses. AMC treatment significantly restored these biochemical levels compared with the galactose treated group. The second, in vivo phase of the study revealed that AMC treatment significantly delayed the onset and maturation of cataract in galactose treated rats compared to captopril treatment.

Conclusion: These results support the view that AMC counteracts the effects of galactose in inducing cataract. The anticataract effects of AMC may be related to its intrinsic ability to protect and restore the activities of lens enzymes and the bioavailability of glutathione respectively.

Key words: Cataract, captopril, galactose, osmotic stress, polyol, allylmercaptocaptopril.

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Ratlarda Galaktazomik Katarakta Karşı Alilmerkaptokaptopril'in Anti Katarakt Ajan Olma Potansiyeli

Amaç: Alilmerkaptokaptopril(AMC), deney hayvanlarında, selenit ile indüklenmiş katarakta karşı anti katarakt aktivasyona sahip olduğu gösterilen Kaptopril ve Allisinin kovalent bağlanması ürünüdür. Bu çalışmada AMC 'nin galaktazomik katarakt da antikatarakt potansiyelinin biyokimyasal mekanizmasını aydınlatmak amaçlandı.

Metod: AMC 'nin koruyucu etkisi in vivo ve invitro olarak ratlarda galaktoz ile indüklenmiş katarakt da değerlendirildi ve kaptoprilin etkisi ile karşılaştırıldı. AMC ve Kaptoprilin galaktozemik katarakt da kataraktın başlangıcı ve olgunlaşması üzerine olan etkisini incelendik.

Bulgular: AMC, galaktoz ve kaptopril ile tedavi edilen lenslerle karşılaştırıldığında rat lenslerinde ki galaktoz ile indüklenen osmotik stresin belirteci olan polivol düzeyini azalttı. Galaktoz verilen grupta normal lensler ile karşılaştırıldığında Glucose-6-phosphate dehidrogenaz, suksinat dehidrogenaz, laktat dehidrogenaz aktivitesi ve indirgenmiş glutatyon düzeyleri azaldı. İkinci olarak, çalışmanın in vivo aşamasında AMC tedavisi, kaptopril tedavisi ile karşılaştırıldığında galaktoz verilen sıçanlarda kataraktın başlangıcı ve olgunlaşması önemli derecede geciktirdi.

Sonuç: Bu sonuçlar galaktozin indüklediği kataraktı AMC nin dengelediği görüşünü desteklemektedir. AMC nin anti katarakt etkisi lens enzimlerinin etkisini koruma ve yerine getirme gücü ve glutatyon kaynağı olmasından dolayıdır.

Anahtar kelimeler: Katarakt, kaptopril, galaktoz, ozmotik stres, poliyok, allylmerkaptokaptopril

INTRODUCTION

Cataract, opacity of the eye lens, is the leading cause of blindness worldwide. Nearly 19 million people are blind due to cataract in the world (1). The age-adjusted prevalence of cataract in India is three times that of the United States (2). Apart from aging, various risk factors such as nutritional deficiencies or inadequacies, diabetes, sunlight, environmental factors, smoking, and lack of consumption of antioxidants are known to increase the risk of cataract (3,4). Studies indicate that hyperglycemia and the duration of diabetes increases the risk of development of cataract (3). Though the etiology of cataract is not fully understood, oxidative damage to the constituents of the eye lens is considered to be a major mechanism in the development of cataract (5,6). Oxidative stress associated with diabetes may play an important role in the initiation and progression of diabetic cataract. The toxic effects of reactive oxygen species (ROS) or free radicals are neutralized in the lens by antioxidant systems, both enzymatic and non enzymatic (5,7). A number of observational studies suggest that intake of foods containing micronutrients having antioxidant potential may be protective against cataract (8). Angiotensin-converting enzyme inhibitors are commonly used in the therapy of hypertension and chronic heart failure (9). Captopril, a well known sulfhydryl angiotensin-converting enzyme (ACE) inhibitor is a synthetic 3-mercapto-2-methylpropionyl derivative of L-proline which has been used extensively for more than 2 decades (10). It acts as a competitive inhibitor of peptidyl dipeptidase, which catalyzes the conversion of angiotensin I to angiotensin II. Thus, it is a potent systemic hypotensive drug that functions by decreasing the

peripheral vascular resistance in essential hypertension (11,12). It also stimulates the kallikrein-kinin system. Besides these effects, captopril may decrease intraocular pressure (13,14). Captopril is easily absorbed through the mucosa, and its bioavailability is about 65% (15,16). It has been suggested that absorbed captopril is carried as disulfides with the endogenous sulfhydryl-containing compounds and it is also a scavenger of oxygen free radicals (17-19). Its ability of quenching toxic oxygen free radicals could be an additional therapeutically useful property in the prevention of diquat and streptozocin induced cataract (20,21). In our previous study, we have extensively evaluated the anticataract effect of captopril in selenite induced cataract in rat pups with respect to their potential to restore the level of reduced glutathione, inhibition of lipid peroxidation and maintain the antioxidant enzymes level in ocular lens (22). The anticataract effects of garlic (*Allium sativum*) have been recorded since very early times. In addition, experimental studies have demonstrated the ability of garlic to prevent the development of diabetic cataract (23).

The present study was sought to investigate the advantages of both molecules, captopril and allicin, which operate by different mechanisms. The covalently bonded reactive product of captopril and allicin leads to allylmercaptocaptopril (AMC), a nonsymmetric disulfide. Its efficacy as an anticataract agent remains to be addressed. In the present study, we investigated the effect of AMC on galactose induced cataract and also studied the biochemical mechanisms involved behind its protective effect.

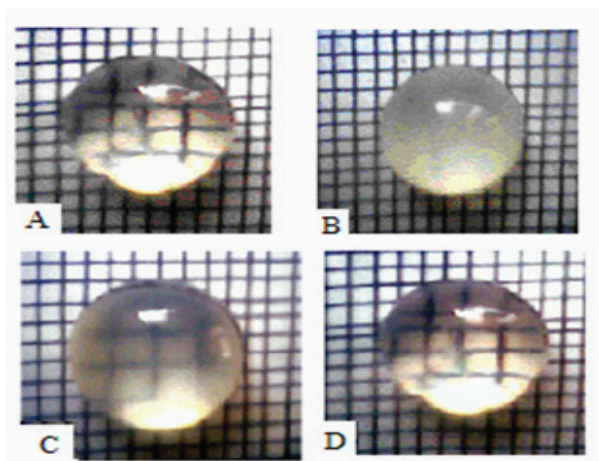


Figure 1. (A) Normal lens after 72 h of incubation (Transparency maintained, more squares clearly visible). (B) Complete cataractogenesis after 72 h of incubation in galactose 30mM (Complete loss of transparency, no squares visible through lens). (C) After 72 hours of incubation in galactose 30mM + captopril 5 mM, lens appears slightly hazy (less no. of squares slightly clearly visible). (D) After 72 hours of incubation in galactose 30mM + AMC 5 mM, lens appears slightly hazy (more no. of squares clearly visible).

MATERIALS AND METHODS

Chemicals

Captopril was kindly provided by Wockhardt Ltd (Aurangabad, Maharashtra, India) approximate purity was 98%. Dulbecco's modified Eagles medium (DMEM) was obtained from Hi Media Laboratories (Mumbai, India). Fetal calf serum and chemicals required for enzymes assay were purchased from Sigma Chemical Company, St. Louis, MO. 24 wells Falcon plastic culture plate was acquired from Genei, Bangalore, India. All other chemicals and AR grade solvents were procured from SRL, Mumbai, India. Allylmercaptocaptopril was synthesized according to our previously reported method (24).

Animals

Wistar male rats (60-80 g) were procured from the animal house stock of the Department of Pharmacology, SRM College of Pharmacy, Kattankulathur, India. The animals were housed under normal conditions (22°C) in separate groups and handled in accordance with the guidelines as per the "Institutional Animal Ethical

Committee" and CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) rules.

Pharmacological studies

In vitro phase of the study

Eyes were enucleated from the young Wistar rats and lenses were dissected from the eyes using a posterior approach. Each isolated lens was incubated in 2 ml of Dulbecco's modified Eagle's medium (DMEM) in a 24 wells Falcon plastic culture plate at 37°C in an incubator at 95% air and 5% CO₂. The medium was supplemented with 10% fetal calf serum and 0.9 g/l sodium bicarbonate. Streptomycin, 100 µg/ml and penicillin, 100 IU/ml were also added to prevent bacterial contamination. After 2 h of incubation, opaque lenses which were damaged during dissection were discarded and transparent lenses were taken for the subsequent experimental studies.

Transparent cultured lenses were randomly divided into Group I: normal, Group II: galactose only (control), Group III: galactose plus captopril and Group IV: galactose plus AMC, each group comprising six lenses. Normal lenses were incubated in DMEM alone, whereas control lenses were incubated in DMEM supplemented with 30 mM galactose. Medium in the treated groups was additionally supplemented with 5 mM captopril and AMC along with galactose respectively. All lenses in the different study groups were incubated for 72 hours in the above mentioned experimental conditions followed by examination for presence of any opacity and photo-documentation. Fresh medium were replaced after every 24 hours. Post-incubation, lenses from different groups were washed, fresh weight recorded and processed for biochemical estimation. Separate set of experiment was carried out for the measurement of each biochemical parameter.

Biochemical Measurements

Lens Polyol estimation

Lens polyol was estimated by the method of West and Rapoport (25). Briefly, the lenses were homogenized in 0.6 N perchloric acid and centrifuged at 5000xg for 20 min. The supernatant so obtained was neutralized with 2 N KOH and again centrifuged. The supernatant was reacted with 0.25 ml of periodic acid (0.03 M) for 10 min followed by the addition of freshly prepared 0.25 ml of

Table 1. Levels of Polyol, G-6-PDH, SDH, LDH and GSH in Group I, Group II, Group III and Group IV lenses

Parameter	Group I	Group II	Group III	Group IV
Polyol (µg/mg lens)	0.01±0.00*	1.40±0.21	1.11±0.01 ^{ns}	0.012±0.01*
G6PDH (U/mg/min)	0.684±0.17*	0.378±0.02	0.546±0.02 [#]	0.612±0.01*
Sdh (µg formazan/mg/hr)	1.984±0.015*	1.332±0.059	1.378± 0.052 ^{ns}	1.791±0.01*
LDH (U/mg/min)	1.864±0.059*	1.126±0.037	1.439± 0.026*	1.532±0.01*
GSH (µmol/g)	1.29±0.31*	0.34±0.02	1.04± 0.25*	1.12±0.01*

All values are expressed as mean±SD. Group I: Normal, Group II: treated with 30 mM galactose only. Group III: lenses treated with 30 mM galactose and 5 mM captopril, Group IV: lenses treated with 30 mM galactose and 5 mM AMC. Statistically significant difference (#P<0.05, *P<0.01, nsP<0.05) when compared with group II values.

stannous chloride (0.125 M) and 2.5 ml of chromotropic acid (0.2%). The reaction mixture was heated on a boiling water bath for 30 min. The absorbance of the purple-colored complex was measured at 570 nm in a UV-visible spectrophotometer (LAMBDA 1050, PerkinElmer). A parallel standard was also prepared using dulcitol.

Glucose 6 phosphate dehydrogenase activity (G-6-PDH)

The activity of G-6-PDH was analyzed by the method of Lohr and Waller (26). The lens was homogenized in 0.9%

physiological saline with 6.6×10⁻⁴ M EDTA at 0-40C and then centrifuged for 20 minutes at 15000 rpm at 00C. 2.4 ml of 0.05 M Triethanolamine buffer pH 7.5 (with 0.2% EDTA sodium salt), 0.5 ml of the above supernatant and 0.05 ml of 3×10⁻² M NADP (in 1% NaHCO₃) were mixed and allowed to stand for 5 minutes at 250C. Then 0.05 ml of 4×10⁻² M glucose-6-phosphate solution was added. The optical density of the test mixture was read at 366nm.

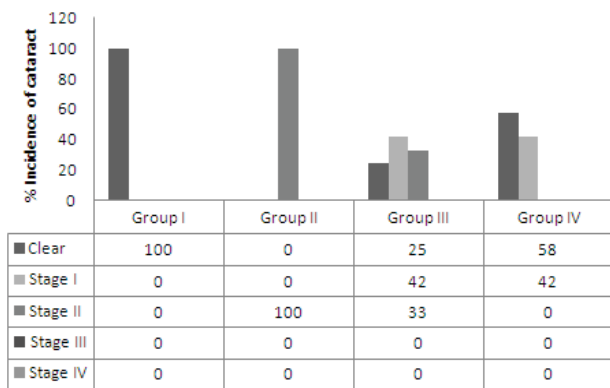


Figure 2. Effect of captopril and AMC on the onset of galactose-induced cataract in rats. Cataract development in rats was observed on day 12 of the experiment by slit lamp biomicroscopy one h after dialation of pupils. The number of lenses that developed opacity against total number of lenses was considered for calculating the percentage of incidence of cataract in each group.

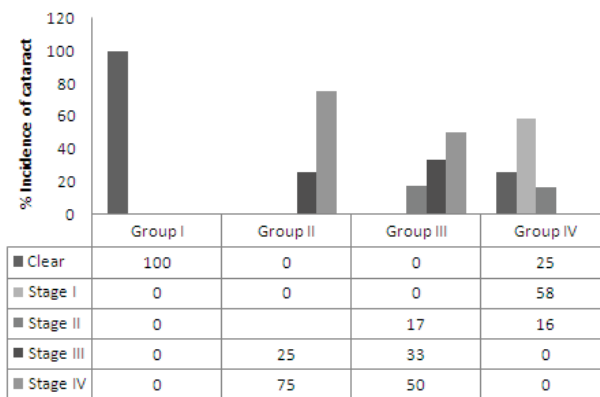


Figure 3. Effect of captopril and AMC on the maturation of galactose-induced cataract in rats. Cataract development in rats was observed on day 24 of the experiment by slit lamp biomicroscopy one h after dialation of pupils. The number of lenses that developed opacity against total number of lenses was considered for calculating the percentage of incidence of cataract in each group.

Succinate dehydrogenase activity (SDH)

The activity of SDH was determined by the method of Beatly et al (27). The assay mixture contained 1ml of 0.1 M sodium succinate, 1ml 0.2 M phosphate buffer pH 7.5, 1ml of INT (0.1% Tetrazolium Salt-INT) solution and 0.4ml of lens aqueous homogenate. After thorough mixing, all the tubes were incubated at 37°C for 1 hour, and the reaction was stopped by adding 0.1ml of 30% TCA (trichloroacetic acid) in assay mixture. Ethyl acetate, 7 ml was added to each tube and this was centrifuged for 4 minutes after thorough mixing to extract the color. The optical density was read at 420 nm.

Lactate dehydrogenase activity (LDH)

The activity of LDH was determined by the modified method of Bergmeyer et al (28). The aqueous lens supernatant after 3000 rpm centrifugation for 20 minutes, was mixed with 2.85 ml phosphate pyruvate solution (0.05M phosphate buffer pH 7.5; 3.1×10^{-4} M Na pyruvate) and 0.05ml reduced nicotinamide adenine dinucleotide (8×10^{-3} M NADH-1 mg/1.5ml of phosphate pyruvate solution). The optical density difference of the reaction mixture was read immediately after mixing at 1-minute intervals for 5 minutes at 366 nm.

Reduced glutathione (GSH)

GSH was determined by the modified Sedlak and Lindsay method (29) using Ellman's reagent. The 2.5ml of 0.02 M EDTA lens homogenate was mixed with 2.0ml of distilled water and 0.5ml of 50% TCA. The mixture was shaken intermittently for 15 minutes and then centrifuged for 15 minutes at 3000xg. The supernatant of this centrifugation was mixed with double volume of 0.4 M Tris buffer pH 8.9 (in 0.02 M EDTA) and 0.1ml of 0.01 M DTNB (5-5' dithiobis 2- nitrobenzoic acid). The optical density was read at 412 nm within 5 minutes of the addition of DTNB. Reduced GSH was used as a standard.

In vivo phase of the study

Male Wistar rats (21 days old) having an average body weight of 64 ± 2.64 g were randomized in four groups containing twelve animals in each group. Each group was fed a different diet as follows; Group I: Animals in this group were fed a normal stock diet based on the AIN-93 formula (30); Group II: Animals were fed 30% galactose in the above diet; Group III: Animals in this group received the II-group diet along with captopril and Group IV: Animals in this group received the II-group

diet along with AMC. 1 % solution of both the drugs was applied topically two drops two times daily in both the eyes until the end of study. The fresh solution of both the drugs were prepared daily in 0.9 % sodium chloride by taking water for injection as a vehicle. Animals were housed in individual cages in a temperature and humidity controlled room having a 12 h light/dark cycle. All of the animals had free access to water. Animals were fed for 24 days. Eyes were examined every other day using a slit lamp biomicroscope (TOPCON, Japan) after dilating the pupil with topical ophthalmic solution containing tropicamide with phenylephrine (Maxdil Plus, Hi-Care Pharma, Chennai, India). Initiation and progression of lenticular opacity were graded according to Sippel's classification (31) as follows: stage 0: normal; stage I: faint peripheral opacities; stage II: irregular peripheral opacities with slight involvement of the center of the lens; stage III: pronounced opacity readily visible as white spots; and stage IV: mature cataract.

Statistical Analysis

All data were expressed in mean \pm standard deviation. The groups were compared by one-way ANOVA using post-hoc Dunnett's test with significance level set at $P < 0.05$.

RESULTS

Lens Morphology in vitro

All lenses incubated in DMEM alone remained transparent. However, after 72 hours of incubation with galactose, all control lenses developed dense opacities. Incorporation of captopril and AMC (5 mM) in the culture medium prevented the development of opacity (Figure 1).

Effect on lens Polyol, G-6-PDH, SDH, LDH and GSH level in vitro

To investigate the possible mechanisms of differential effects of captopril and AMC on galactose-induced cataract, we investigated various biochemical parameters related to the oxidative stress/antioxidant system, the polyol pathway, enzymes involved in the energy metabolism pathway such as G-6-PDH, Sdh and LDH. The results of Polyol, G-6-PDH, Sdh, LDH and GSH level in the lenses of four groups treated with and without galactose (30 mM) along with captopril and AMC were depicted in table 1. The lens polyol level, as an index of osmotic stress, was significantly elevated in galactose treated

lenses ($p < 0.01$), indicating that there was a substantial osmotic stress was generated in the lenses in response to the galactose treatment. The observed increase in polyol was significantly attenuated by AMC treatment ($p < 0.01$), but it was insignificant in captopril treated group ($p < 0.05$). Galactose supplement resulted in marked reduction in mean G-6-PDH, Sdh, LDH and GSH level in lenses ($p < 0.01$) as compared to normal group. AMC treatment led to significant increase in lens G-6-PDH activity ($p < 0.05$) as well as Sdh, LDH and GSH level ($p < 0.01$), but in captopril treated lenses, SDH activity was unaffected ($p < 0.05$) but rest of the three were significantly changed when compared with control group.

Effect on Galactose Cataract Formation in vivo

Results of slit lamp examination indicate that all of the animals fed on the galactose diet developed stage II cataract after two weeks (Figure 2). After four weeks 75% of the lenses developed mature cataract (stage IV) and 25% of the lenses were in stage III (Figure 3). Topical application of captopril delayed the onset of galactose induced cataract since only 33% of the lenses in this group demonstrated stage II cataract while 42% were in stage I cataract and 25% of the lenses had not developed any lenticular opacity at the end of two weeks (Figure 2). Furthermore, maturation of cataract was also slowed by the captopril treatment, as only 50% of the lenses in this group demonstrated stage IV cataract and 33% of the lenses in this group demonstrated stage III cataract and 17% were still in stage II after 24 days (Figure 3). But the effect was more pronounced in AMC treated group since none of the lenses in this group demonstrated stage II cataract while 42% were in stage I cataract and 58% of the lenses had not developed any lenticular opacity at the end of two weeks (Figure 2). Furthermore, maturation of cataract was also slowed by the AMC treatment, as only 16% of the lenses in this group demonstrated stage II cataract and 58% of the lenses in this group demonstrated stage I cataract and 25% still had clear lenses and none of the lenses were in stage IV after 24 days (Figure 3).

DISCUSSION

Oxidative stress has been suggested as a common underlying mechanism of cataractogenesis, and augmentation of the antioxidant defenses of the lens has been shown to prevent or delay cataract (32). Different agents with

diverse chemical structures have shown antioxidant properties in different systems, and their beneficial effects have been demonstrated in various pathologic conditions including cataract. In our previous study, we have extensively evaluated the antioxidant role of captopril in the attenuation of selenite induced cataract in experimental animals (22).

Studies are ongoing to explore the potential of antioxidant agents against cataractogenesis in various experimental models of cataract. Among these models, the galactose induced cataract is commonly used, as the model is reasonable to assume that factors initiating galactose cataracts in young rats are similar to those involved in the human galactose cataract model (33).

Furthermore, the galactose produces a large amount of its reduced form, galactitol, inside the lens that leads to osmotic stress. Accumulation of high concentration of polyols in the lens leads to an increase in the intracellular ionic strength resulting in excessive hydration, eventually loss of membrane integrity and leakage of free amino acids, glutathione and myo-inositol (34) etc.

In the present study, chemical analysis of galactose treated lenses clearly demonstrated a significant increase in the level of galactitol due to osmotic stress and depletion of reduced glutathione as compared to normal lenses and this effect was significantly altered by AMC treatment but not in captopril treated group. It might be due to the ability of AMC to block the enzyme system which is responsible for the formation of polyols.

Further, the accumulation of dulcitol disturbs the osmotic pressure, which in turn leads to a change in metabolic activities. There is considerable loss of SDH activity in the lens during the initiation and progression of galactose-induced lens opacity (35). Sdh is an essential enzyme of citric acid cycle and aerobic energy flow. But the citric acid cycle is not very active in the ocular lens metabolism. Although 3 to 5% of glucose is catabolized within the citric acid cycle, this is of importance only in the lens epithelium (36). The decreased Sdh level reverts to normal by AMC treatment indicates its role in the maintenance of cellular respiration which is important for carbohydrate metabolism.

In vivo studies suggested that as the concentration of galactose penetrating the lens from the aqueous humour increases, the rate of reduction of galactose increases and galactitol accumulates. The continuous produc-tion

of galactitol which leaks out of the lens very slowly and which is not further metabolised leads to the existence of its high levels within the fibres and especially in epithelial cells (37,38). As LDH and G-6-PDH are loosely linked to the tissue, they may be targets for extensive oxidative insults. It is obvious that the decreased activities of G-6-PDH and LDH in the galactose exposed lenses disturb the energy metabolism in the lens. Since the metabolism of glucose-6-phosphate via hexose monophosphate shunt pathway depends mainly on the activity of G-6-PDH, it is suggested that the inhibitory effect of galactose on the activity of G-6-PDH may disturb the hexose monophosphate pathway (39). Iwig et al (40) reported that LDH activity depends on the growth rate of cultivated lens cells. The differentiation of lens epithelial cells into fibre cells is connected with a decrease in LDH activity (41). The significant decrease in the activity of LDH during the initiation of lens opacification may be due to the involvement of the epithelial cells in the lens opacification brought about by galactose.

One of the main functions of G-6-PDH is the maintenance of lens protein sulfhydryl groups in the reduced state. Another possible mechanism for inactivation or loss of activity of the enzyme may be the degradation of protein and depletion of reduced glutathione. The oxidized glutathione is reduced by glutathione reductase in the presence of NADPH produced by HMP shunt (42,43). The present study is in agreement with these findings, since the restoration of GSH level by the treatment of both captopril and AMC protects G-6-PDH activity. Moreover, restoration of LDH and SDH activity, protection against osmotic stress and maintenance of lens clarity without doubt establish the protective action of AMC against galactose induced cataract. It is clear from the above discussion that AMC is acted through both the mechanisms i.e. it protects the enzymes which are responsible for energy metabolism and second by its antioxidant activity. Conversely, Captopril can prevent cataractogenesis only due to its antioxidant activity. In vivo experimental findings revealed that AMC treatment significantly delays the onset and progression of galactose induced cataract in rats as compared to captopril treatment.

The present results support the view that AMC protect against galactose-induced cataract and could be used as an anticataractogenic agent. The protective effect may be related to its intrinsic ability to sustain activities of free-radical-scavenging enzymes and to the bio-

availability of GSH, which protects lens epithelial cells against osmotic stress by decreasing polyol formation. In vivo study confirms its potential to delay the onset and maturation of cataract and its superiority over captopril.

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