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RESEARCH ARTICLE

The Effects of Topical Adenosine Agonists (Limonene) on Induced Ocular Hypertension in Rabbits

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Abstract

Glaucoma is a multi factorial disease characterized by progressive loss of retinal ganglionic neurons, if glaucoma is not treated can progress to blindness. Controlling of intraocular pressure is the only approved medical therapy to decrease glaucoma progression. Limonene is a selective adenosine A2A receptors agonist, activation of this receptor can alter outflow resistance at the Schlemm's canal cell level, and there by decrease IOP. Because vasodilatory effects of adenosine are primarily mediated by adenosine A1& A2A receptors. A fourth (40) rabbits (albino) included in this study &divided into 8 groups, travaprost 0.004% group A (n=8) used as positive control, isotonic buffer group B (n=8), limonene group C, D&E (1%, 2% and 4%) respectively (8) rabbits in each group. The present study was conducted to evaluate the intraocular pressures lowering effect of adenosine agonist (Limonene) and to explore their effect on adenylyl cyclase activity by measuring the level of cAMP changes in the aqueous humor in rabbits with induced ocular hypertension, and to explore the possible side effects of the tested agent on the eyes after instillation. The adenosine system is one of the potential target systems as new therapeutic approaches in glaucoma as concluded by the lowering IOP effect of limonene.

Keywords: *Intraocular pressure, Adenosine agonists, Limonene and travaprost.*

Introduction

Glaucoma is a group of eye diseases (optic neuropathies) recognized by progressive retinal ganglion cells (RGCs) degeneration, resulting in cupping, a characteristic appearance of the optic disc, gradual damage to the optic nerve and visual loss that generally starts with a subtle loss of side vision (peripheral vision).If glaucoma is not diagnosed and treated, it can progress to loss of central vision and blindness [1]. According to The World health Organization, glaucoma accounted for 2 percent of visual impairment and 8 percent of global blindness in 2010 [2]. The pathophysiology of glaucoma is not completely understood, but the AH is secreted by the ciliary body and drainage via two independent pathways (the trabecular meshwork and the uveoscleral outflow pathway), the normal physiological balance between the secretion and the drainage of the aqueous humor is affected by this condition [3].

IOP is defined as the pressure of the fluid inside the eye. It is an important characteristic in the evaluation of patients at risk from glaucoma. As a part of the definition of glaucoma elevation of ocular pressure is a major risk factor for the progression of visual field loss and it consider the only parameter that can be alter by pharmacologically intervention [4]. Adenosine is a ubiquitous local modulator that regulates diverse physiological and pathological functions by stimulating it's membrane receptors. Studies have been identified four AR subtypes: A1, A2A, A2B, and A3. All ARs belong to the G protein-coupled receptor (GPCR) family, A2A AR activate adenylyl cyclase and increase cAMP levels via the stimulatory Gs proteins [5].

Adenosine is a potent endogenous vasodilator in most vascular beds, including that of the retina.

Its vasodilatory effects are primarily mediated by $A_1$and A_{2A} AR receptors [6]. A2A AR on endothelial and smooth muscle cells is responsible for adenosine-induced vasodilation [7]. Limonene is a major aromatic compound in essential oils extracted from citrus rind obtained from oranges, grapefruits, and lemons. Limonene have been used popularly in aroma therapeutic practice due to it is potent calming and sedative effects that alleviate nervous disorders, heart problems, colic, asthma, and depression. In addition, limonene has anticancer activity [8]. Limonene is a natural cyclic terpene, It acts as a selective A2A receptor agonist [9]. Since A2A receptors are coupled to a Gs G protein, so the activation of this receptor lead to activate adenylyl cyclase and increased cytosolic cAMP level via the stimulatory Gs proteins (5). A2A AR activation can alter outflow resistance at the SC-cell level, and thereby decrease IOP [10].Also the activation of this receptor increases guanylate cyclase activity and the subsequent increased cyclic guanosine 3; 5′-monophosphate (cGMP) levels relax vascular smooth muscle causes vasodilation [11]. Limonene is colorless liquid with an odor of lemon, it is readily absorbed from gastrointestinal tract, skin, and respiratory tract [12].The half-life of limonene has been estimated to be 12- 24 hours [13]. Limonene is considered to have fairly low toxicity. It does not pose a mutagenic, carcinogenic, or nephrotoxic risk to humans [14]. Limonene oxidative product are known to cause eye and airway irritation by trigeminal stimulation [15].Figure (1).

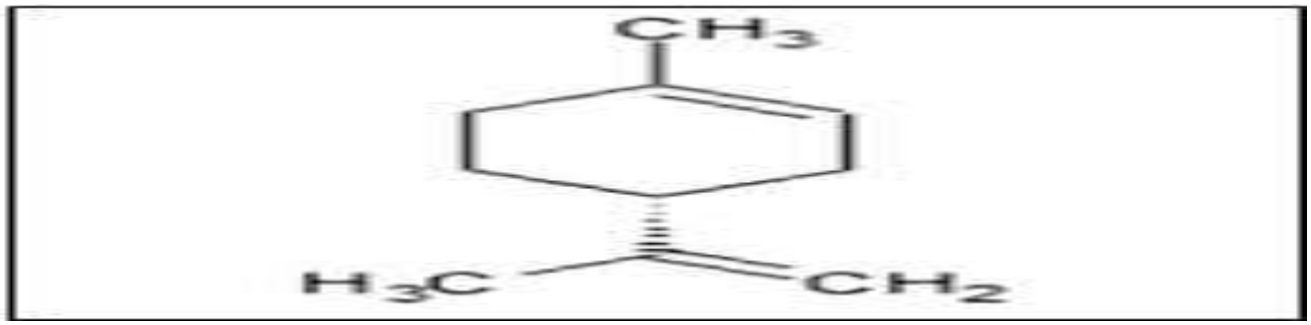


Figure 1: chemical structure of limonene [16]

Materials and Methods

A fourty (40) adult male rabbits (albino) weighing (1.5- 2 kg) were included in this study, and were divided into 5 groups. Preparation of ophthalmic solutions of the tested drugs done by mixing the desired volume (0.1, 0.2, 0.4 ml) with appropriate volume of phosphate buffer solution with subsequent addition of sufficient amount of NaCl to make solution isotonic and to stabilize the buffer. Stirring these mixtures well to accelerate the dissolution of the undissolved particles and mixing well till we have a clear solution.

Then the benzalkonium chloride solution (to concentration of 0.01%) and ethanol (to concentration of 1%) were added and mixed well prior to addition of phosphate buffer solution to achieve the final volume to get (1%, 2%, 4% v/v) of limonene solution. The eye drop is isotonic, equivalent to tonicity of 0.6% w/v sodium chloride solution, so it is better to be tolerated by the eye. In general, the eye can tolerate solution usually equivalent to a range of (0.45% to 1.8% w/v) sodium chloride [17].

Limonene solution is light sensitive so it kept in amber color container [18].In this study, sterilization by filtration method is used in the preparation of a sterile solution by passage the final solution through a syringe affixed with a microbial filter to be ready for instant instillation [19].

Induction Technique of Ocular Hypertension in Rabbits

The procedure of induction was done under sterile condition starting with the anesthesia. The animals anesthetization was performed by using ketamine 50 mg/kg intramuscularly plus diazepam 2 mg/kg intramuscularly, the anesthesia onset which provided by ketamine initiated after six minutes and the peak effect after ten minutes which persist about forty minutes, ketamine with diazepam combination produce surgical anesthesia in rabbit that continue for up to 30 minutes [20].

The right and the left eyes was prepared by instillation of 4% povidine iodine and then washed with distilled water. Tetracaine HCL o.5% eye drops was instilled and then (27G X

13 mm) needle on micro syringe was inserted into the anterior chamber of the eye at 2 o´clock on the limbus. Then aqueous humor was withdrawn without moving the needle, then a single dose of an equal volume of 2% (w/v) HPMC was injected into the anterior chamber by another micro syringe).Finally, the needle was then removed without significant loss of aqueous humor [21].

In this study the volume of hydroxypropyl methylcellulose that injected into the eye was 0.4ml. The rate of a failure in the induction of ocular hypertension was approximately 43% and in these cases the induction had been repeated on another intact rabbits. Chloramphenicol eye drops used as antibiotic prophylaxis; it was administered twice daily on the day before the induction also on the day of the induction and continued later.

Animals Groups

Group (A) Travaprost 0.004% Group: (8 Rabbits)

Both eyes of this group have been induced for ocular hypertensive, the right eyes instilled with Travoprost 0.004% drop (1-2 drops) once daily which considered as a positive control group, while the left eyes instilled with DW (1-2 drops) once daily which considered as a negative control group.

Group (B) Isotonic Buffer Group: (8 Normotensive Rabbits)

This group was instilled with isotonic buffer solution (1-2 drops) once daily in the right eye and DW (1-2 drops) once daily in the left eye to show if there is any effect of the vehicle (isotonic solution) on the eye.

Limonene Group (24 Rabbits)

Divided in to (3) subgroups (8rabbits) in each. The right eyes of this group have been induced for ocular hypertensive and instilled with (1-2drops) of Limonene drop once daily for 7 days.And measuring IOP after (1hour) of each application.

Group C-(8 rabbits): instilled with 1% Limonene drop

Group D- (8 rabbits): instilled with 2% Limonene drop.

Group E- (8 rabbits): instilled with 4% Limonene drop.

Experimental Techniques (Parameters)

Intraocular Pressure (IOP) Measurement

The IOP measurement was done on the eye of the rabbits with the assistance of Schiotz tonometer. After local anesthetization of the cornea with 1-2 drops of (Tetracaine HCL o.5%) ophthalmic solution, the animal was hold laterally and fixed then tonometer is placed on the cornea and a scale reading was taken from the tonometer. Then scale reading taking from the tonometer is transformed to the corresponding mmHg of tension by referring to a standard chart that is supplied with the device. Obtained results would be represented in millimeter of mercury.

A control or zero time value of IOP was taken 15 minutes (min) before the application of tested drug. One to two drop of freshly prepared tested drug was instilled in the middle of inferior conjunctival sac followed by lid closure. After that, IOP was measured after (60 minute) of topical application of tested drug. The instillation of tested agent as one drop for 7 days once daily [22].

Pupil Diameter

Pupil diameter measurement was accomplished by using the pupil gauge.The results obtained would be represented in millimeter unit [23].

Light Reflex

The pupillary response or the light reflex of both eyes was examined by applying flash light suddenly to the eye to detect if the reflex to the flashlight present or not [24].

Corneal Sensation Reflex

Both eyes examined by using piece of cotton wool it applied from the side and award of its approach to detect if the corneal reflex present or not [23].

Conjunctival Redness

The conjunctival redness could be examined for both eyes by inspection of conjunctiva [25].

Lacrimation

Lacrimation could be detected by inspection of conjunctiva of both eyes [25].

Aqueous Humor Collection& Measurement of cAMP in the AH Samples

This step would be done for the measurement of the cAMP level of the tested drugs. Aqueous humor was collected carefully from the anterior chamber of rabbit's eye using 27-gauge needle without causing any injury to the iris or lens throughout the procedure [26].It was performed after administration of ketamine 50 mg/kg intramuscularly plus diazepam 2 mg/kg intramuscularly.

After collection, samples were immediately stored at temperature (-20°C) until the performance of the biochemical analysis from AH. Limonene was instilled into the eye. cAMP in the aqueous humor was measured 60 min after drug instillation, and it was determined with the cAMP-Screen TM System kit (Elabscience, USA) according to the manufacturer's instructions.

Statistical Design and Analysis

The results were presented by means of means ± standard error of mean (SEM). One way analysis of variance (ANOVA) followed by Turkey test comparison t-test (2-tailed) was utilized to compare between groups. The differences between the means are studies as significant at the 0.05 confidence level. The concentrations that decrease 50% of the IOP this value was analyzed by linear regression equation and logarithmic equation.

The statistical analysis was done by using Windows SSPS 16.0 (SPSS Inc. Chicago, IL), the level of significance was set at P<0.05 as significant.

Results

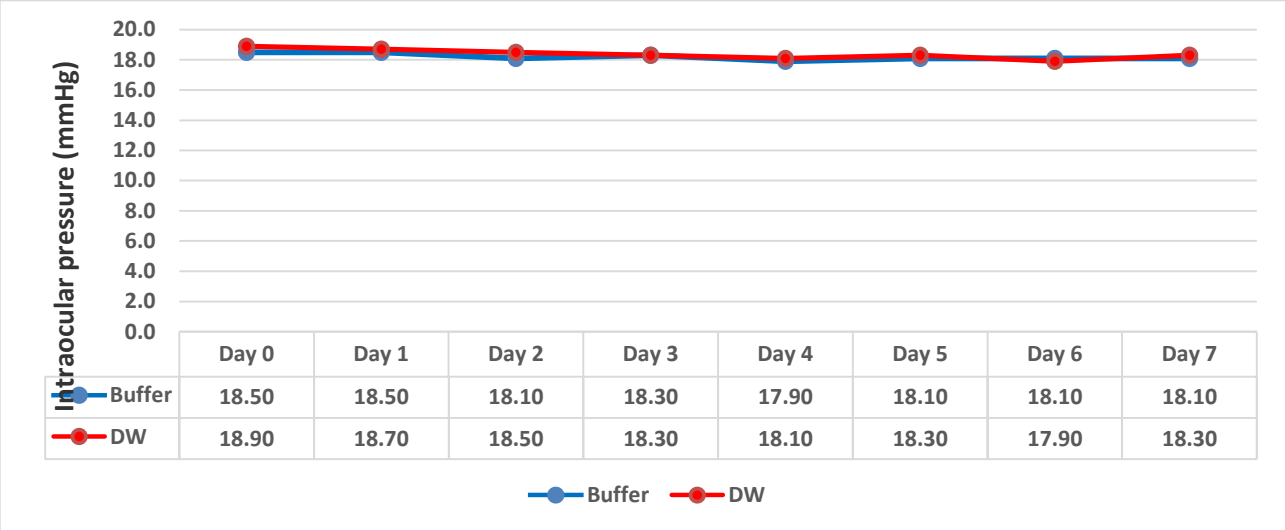
Response of Mean Intra Ocular Pressure& Other Parameters

Effect of Isotonic Buffer Solution on Normotensive Rabbits Eyes

There was no effect of isotonic buffer solution on the mean intraocular pressure (IOP) of rabbits right eyes (P value > 0.05) during the time course of the experiment (7 days), and there was no significant difference between the IOP means of the right and left eyes on the individual days (P> 0.05), figure (1-2).there was no effect on the other parameters.

Effect of Distilled Water on Normotensive Rabbits Eyes

There was no effect of DW on the mean intraocular pressure (IOP) of rabbits left eyes (P value > 0.05) during the time course of the experiment (7 days), and there was no significant difference between the IOP means of the right and left eyes on the individual days (P> 0.05), Figure (2).There was no effect on the other parameters.



	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Buffer	18.50	18.50	18.10	18.30	17.90	18.10	18.10	18.10
DW	18.90	18.70	18.50	18.30	18.10	18.30	17.90	18.30

Figure 2: Comparison of intraocular pressure of normotensive group between buffer (right eye) and distilled water (left eye) P value > 0.05 in all days (Day 0 is the day before starting drops application. Days 1-7 are the ongoing days of treatment)

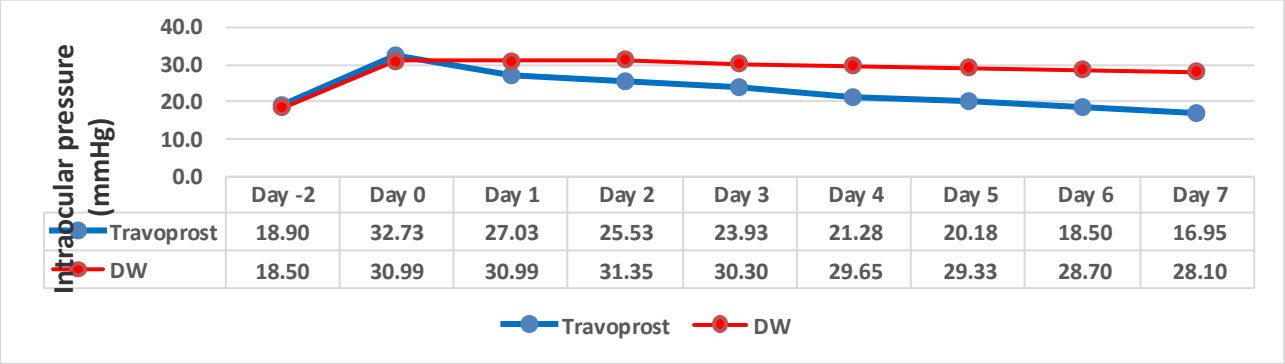
Effect of Travoprost (0.004%) Drop

Pre induction of ocular hypertension, the IOP mean for right and left eyes was (18.90 ±0.1mmHg) & (18.50 ±0.74mmHg) respectively with no significance difference between them (p<0.05).At post induction of ocular hypertension, the mean IOP for right eyes was (32.73 ±2.07 mmHg).

After one hour of travoprost (0.004%) application the mean IOP decreased by (5.7mmHg) that was significant effect compared to distelled water.

The reduction effect on IOP of travoprost started from the first day of treatment, where it was significant decrease (P value < 0.05) at this day, and it was highly significant decrease (p<0.001) from the second day of treatment till the last 7th day of treatment compared to distelled water, figure (3).The total percent of IOP reduction after 7 day treatment with travoprost (0.004%) constitute (48.21%) of the based elevated IOP.

Three of the eight rabbits developed conjunctival redness after instillation of travoprost eye drop that would constitute approximately 8.9% per day of application as conjunctival redness side effect. There was no effect on the other parameters.



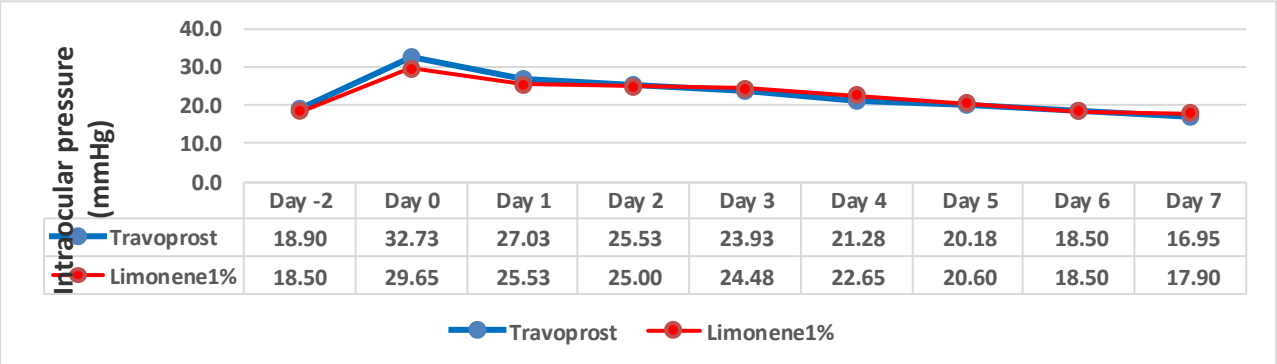
	Day -2	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Travoprost	18.90	32.73	27.03	25.53	23.93	21.28	20.18	18.50	16.95
DW	18.50	30.99	30.99	31.35	30.30	29.65	29.33	28.70	28.10

Figure 3: Comparison of intraocular pressure between positive control group Travoprost 0.004% (right eye) and negative control distilled water (left eye) P value < 0.05 in day 1, P value < 0.001 in days 2-7 (Day -2 is the day before induced-elevation of IOP. Day 0 is the day 48 hours after the induction and where the treatment is started. Days 1-7 are the ongoing days of treatment)

Effect of Limonene (1%, 2%, 4%) Ophthalmic Drop

Comparing the Mean IOP of Group C Limonene (1%) and Travoprost 0.004% and distelled Water

Pre induction of ocular hypertension to the right eyes, the IOP mean was (18.50 ±0.74 mmHg). Post induction of ocular hypertension, the mean IOP was (29.65±1.20 mmHg) which was significant (P<0.05) when compared to travoprost. After one hour of tested drug instillation the mean IOP decrease by (4.12 mmHg) which was non-significant (P<0.05) compared to travoprost, at days four and seven of limonene (1%) instillation the mean IOP reduced by (7mmHg)&(11.75 mmHg) but travoprost 0.004% reduce IOP by(11.45mmHg)&(15.78mmHg) that was significant effect (p< 0.05) than limonene (1%), figure(4).The total percent of IOP reduction after 7 day treatment with limonene (1%) constitute (39.62%) of the based elevated IOP. There was highly significant difference in IOP reduction that found in all days (P value < 0.001) of limonene (1%) instillation when compared with distilled water.



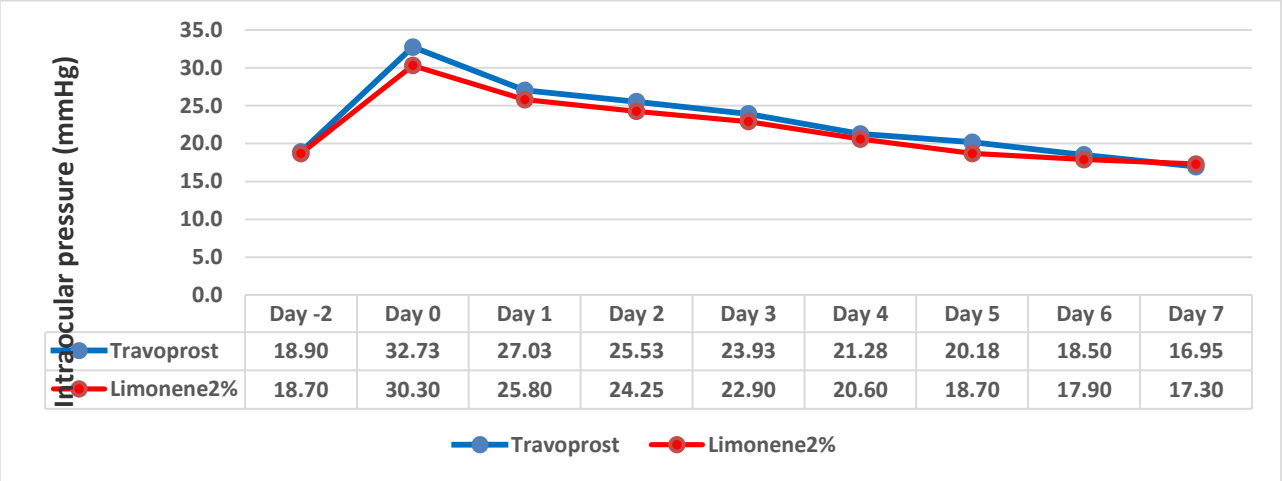
	Day -2	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Travoprost	18.90	32.73	27.03	25.53	23.93	21.28	20.18	18.50	16.95
Limonene1%	18.50	29.65	25.53	25.00	24.48	22.65	20.60	18.50	17.90

Figure 4: Comparison of intraocular pressure between positive control group (Travoprost 0.004%) and limonene 1% group P value < 0.05 in day 0, 4, and 7 only (Day -2 is the day before induced-elevation of IOP. Day 0 is the day 48 hours after the induction and where the treatment is started. Days 1-7 are the ongoing days of treatment)

Comparing the Mean IOP of Group D Limonene (2%) and Travoprost 0.004% and Distilled Water

Pre induction of ocular hypertension to the right eyes, the IOP mean was (18.70 ±0.56 mmHg). Post induction of ocular hypertension, the mean

IOP was (30.30±1.38 mmHg) which was significant (P<0.05) when compared to travoprost. After one hour of tested drug instillation the mean IOP decrease by (4.5 mmHg), which found to be non significant (P> 0.05) when compared with travoprost (0.004 %).There was no significant difference when compared with (travoprost 0.004%) (p>0.05) in all days, except in day 5th there was significant difference between them (P< 0.05) where the limonene (2%) had a maximum reduction effect on this day where it was (11.6mmHg) of the based elevated IOP. At day seven of limonene (2%) instillation the mean IOP reduced by (13mmHg) but travoprost 0.004% reduce IOP by (15.78mmHg), that was non-significant (p> 0.05) when compared to limonene (2%) at this day, figure (5).The total percent of IOP reduction after 7 day treatment with limonene (2%) constitute (42.90%) of the based elevated IOP. There was highly significant difference in IOP reduction that found in all days (P value < 0.001) of limonene (2%) instillation when compared with distilled water.

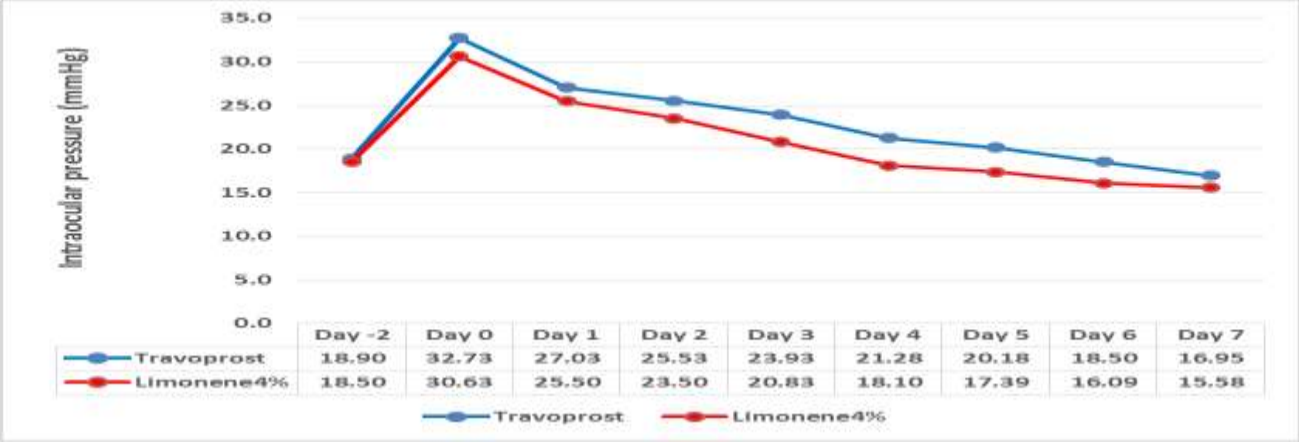


	Day -2	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Travoprost	18.90	32.73	27.03	25.53	23.93	21.28	20.18	18.50	16.95
Limonene2%	18.70	30.30	25.80	24.25	22.90	20.60	18.70	17.90	17.30

Figure 5: Comparison of intraocular pressure between positive control group (Travoprost 0.004%) and limonene 2% group P value < 0.05 in days 0 and 5

Comparing Means IOP of (group E) Limonene (4%) with Travoprost 0.004% and Distilled Water

Pre induction of ocular hypertension to the right eyes, the IOP mean was (18.50 ±0.74 mmHg). Post induction of ocular hypertension, the mean IOP was (30.63±1.34 mmHg) which was significant (P<0.05) when compared to travoprost. After one hour of limonene (4%) instillation the mean IOP decrease by (5.13mmHg) which found to be non significant (P> 0.05) when compared with travoprost. From the day 3th to the day 6th, a maximum reduction in the mean IOP by limonene (4%) when compared with travoprost (0.004%) that was found to be highly significant effect (p< 0.001).At day seven of limonene (4%) instillation the mean IOP reduced by (15.05mmHg) but travoprost (0.004%) reduce IOP by (15.78mmHg) that was significant effect (P< 0.05) by the limonene (4%) when compared to travoprost at this day, Figure (6).The total percent of IOP reduction after 7 day treatment with limonene (4%) constitute (49.13%) of the based elevated IOP. There was highly significant difference in IOP reduction that found in all days (P < 0.001) of limonene (4%) instillation when compared with distilled water.



	Day -2	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Travoprost	18.90	32.73	27.03	25.53	23.93	21.28	20.18	18.50	16.95
Limonene4%	18.50	30.63	25.50	23.50	20.83	18.10	17.39	16.09	15.58

Figure 6: Comparison of intraocular pressure between positive control group (Travoprost 0.004%) and limonene 4% group P value < 0.05 in days 0 and 7, P value < 0.001 in days 3-6

Other Parameters: Pupil Diameter

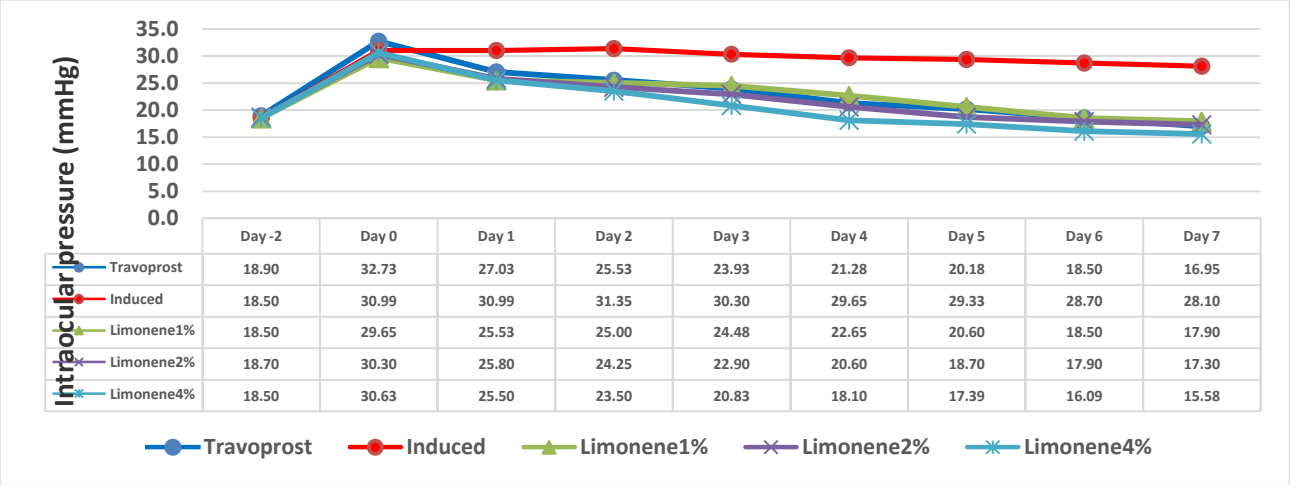
Instillation of limonene (1%, 2%, 4%) eye drops in the induced-ocular hypertensive

right eyes did not cause any change in pupil diameter of the eyes (during the trial period. However, the induction technique significantly changed (reduced) the pupil diameter where it was (8.25 ±0.46 mm, 8±0.75 mm, 7.25±0.7 mm) respectively before the induction and became (7.38 ±0.91mm, 7.25±1.28 mm, 6.38±1.06 mm) respectively after the induction ($P \leq 0.05$).

Conjunctival Redness

One of the eight rabbits developed conjunctival redness in the induced-ocular hypertensive right eyes after instillation of limonene (1%) for four of the seven days of drug application. That would constitute approximately 7.1% per day of application. Four of the eight rabbits developed conjunctival redness in the induced-ocular hypertensive right eyes after instillation of limonene (2%) that would constitute approximately 14.2% per day of application. Two of the eight rabbits developed conjunctival redness in the induced-ocular hypertensive right eyes after instillation of limonene (4%) for three of the seven days of drug application.

That would constitute approximately 10.7% per day of application as conjunctival redness side effect, and it might be due to the invasive nature of the induction technique. There was no effect on the other parameters (Light reflex, corneal reflex, Lacrimation).



	Day -2	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Travoprost	18.90	32.73	27.03	25.53	23.93	21.28	20.18	18.50	16.95
Induced	18.50	30.99	30.99	31.35	30.30	29.65	29.33	28.70	28.10
Limonene1%	18.50	29.65	25.53	25.00	24.48	22.65	20.60	18.50	17.90
Limonene2%	18.70	30.30	25.80	24.25	22.90	20.60	18.70	17.90	17.30
Limonene4%	18.50	30.63	25.50	23.50	20.83	18.10	17.39	16.09	15.58

Figure 7: Comparison of intraocular pressure in limonene groups with positive and negative control groups

Dose Response Curve of Limonene

Table 1: Data of limonene concentration

Tested drug	Concentration L/100 ml	Concentration L/L	Log Concentration L/L	Effect IOP mmHg
Limonene 1%	1	0.1	-1	17.9
Limonene 2%	2	0.2	-0.699	17.3
Limonene 4%	4	0.4	-0.398	15.58

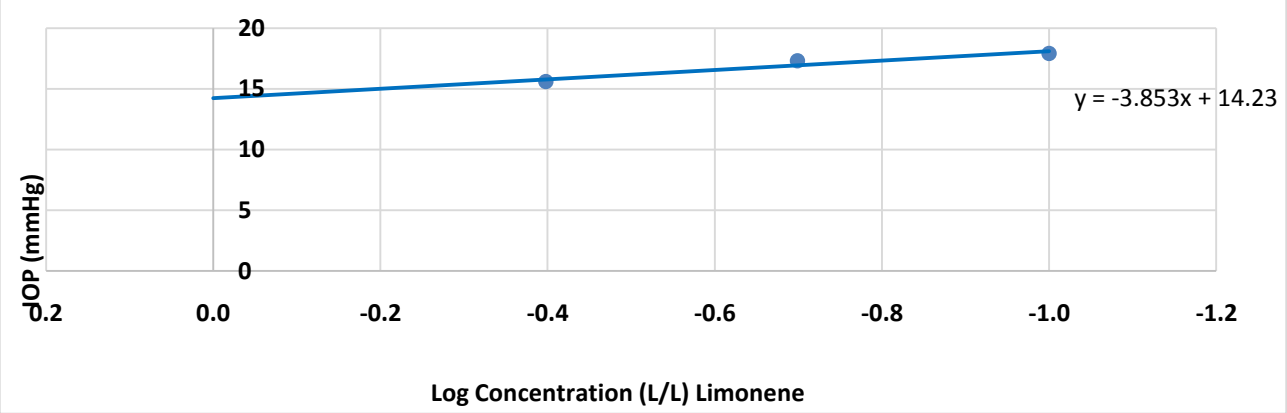


Figure 8: Dose response curve of Limonene
This figure showed that 0.00009 L/L of limonene required to produce 50% decrease in IOP, y = -3.8534x+ 14.233
When y= 50, x= 0.00009 L/L required to produce 50% decrease in IOP ($9*10^{-5}$)

Cyclic Adenosine Monophosphate (cAMP) Levels in Rabbit's Aqueous Humor

- **Comparing Travoprost 0.004% Treated Group with Healthy Group**

There was no elevation of the cAMP levels (259.88+3.77 pmole/ml), (p>0.05)

- **Comparing Travoprost 0.004% Treated Group with Limonene (1%, 2%, 4%)**

There was significant elevation of the cAMP levels in limonene (1%) treated group (268.44+3.37pmole/ml) during the period of the treatment (p>0.001).

There was highly significant elevation of the cAMP levels in limonene (2%) treated group (295.16+7.9pmole/ml) during the period of the treatment (p<0.001).

There was highly significant elevation of the cAMP levels in limonene (4%) treated group (323.5+14.24pmole/ml) during the period of the treatment (p<0.001).

Table 2: Comparison between cAMP levels in control positive with other study groups by unpaired test

cAMP (pmol/ml)	Control +ve	Control -ve	Limonene 1%	Limonene 2%	Limonene 4%
Mean± SD	261.67+5.04	259.88+3.77	268.44 +3.37	295.16 +7.9	323.5 +14.24
P value		**>0.05***	**0.008***	**<0.001***	**<0.001***
			0.008**	**<0.001****	**<0.001****
			<0.001***		

* P value of comparison between control +ve and other study groups by unpaired test, ** p value of comparison between control -ve and other study groups by unpaired test, *** p value of comparison among six groups (apart from control) by ANOVA

Discussion

A cumulative data indicates the importance role of adenosine receptor agonists in treatment of glaucoma by modulate aqueous humor formation and outflow facility, reduction of IOP and retinal neuroprotection. The IOP reduction effect achieved byA2A AR activation that can alter outflow resistance and thereby lower IOP. Also A2A agonists have been shown to reduce vascular resistance and increase retina and optic nerve head blood flow. Using these data, limonene will be a good candidate to be study in the animal models of glaucoma [27]. The present study clearly demonstrated that limonene (1%) was able to reduce mean IOP in hypertensive models after one hour of instillation. Peak mean IOP reduction achieved in day 7 of hypertensive eyes, which constitute (39.62%) of the based elevated IOP.

The hypotensive effect of limonene (1%) was highly significant when compared with DW and significant comparable to that of travoprost (0.004%) (p<0.05) in ocular hypertensive eyes, were the last one act more effectively. Limonene (2%) and (4%) was able to reduce mean IOP in hypertensive models after one hour of instillation. Peak mean IOP reduction achieved in day 7 of hypertensive eyes that constitute (42.90%) & (49.13%) respectively of the based elevated IOP, which was found to be highly significant comparing with DW (P< 0.001), limonene (2%) has a significant hypotensive effect when compared with travoprost (p> 0.05) were the positive control act more effectively, while limonene (4%) also has a significant effect when compared with travoprost (0.004%) (p<0.05) but it act more efficiently than the positive control. The IOP- lowering effect of limonene demonstrated in the present study could be due to A2A AR activation can alter outflow resistance at the SC-cell level and there by decrease IOP (10). Also A2A AR activation causes vasodilation due to increases guanylate cyclase activity and the subsequent increased cGMP levels relax vascular smooth muscle [11]. Some studies conformed that A2A receptor activation causes vasodilation, because they found that targeting the A2A AR could potentially treating pulmonary arterial hypertension (PAH).

Because A2A AR activation induces pulmonary vasodilation [28].The results of this study are compatible with previous study that test the effects of A2A AR activation on the IOP where topical application of (2-O-Ado and 2-CNAdo) cause IOP reduction in the eye of the rabbits, and this ocular hypotensive responses might be mainly due to activation of the A2A AR. They significantly increased the outflow facility and increased cAMP in the AH at 60 min after their applications and this clarify their mechanism of ocular hypotension [29].

In this study, limonene had no significant effect on pupil diameter (P > 0.05), a significant redness had been reported in the hypertensive eyes group after instillation of limonene (1%, 2%, 4%) the percent was (7.1%, 14.2%, 10.7%) respectively. This hyperemia could be resulted from A2A AR activation of the cojuctival blood vessels which is vasodilatory in nature. In the present study, limonene (1%) cause significant elevation of the cAMP levels in treated group after seven days of treatment (p>0.001) compared to travoprost.

While the data shows a highly significant elevation of the cAMP levels in limonene (2%) & limonene (4%) treated group after seven days of treatment (p<0.001) compared to travoprost, suggesting a direct binding and a selective affinity of limonene to A2A AR subtype this clarify the mechanism of ocular hypotension induced by this agent, this agreed with (9) about limonene as agonistic ligand for adenosine A2A receptors directly binds and exhibits a selective affinity to this receptor. The elevation in the cAMP levels was caused by the action of the tested agents that preferably coupled to the Gs family and stimulate adenylate cyclase to increase the production of cAMP [30]. Since the vasodilatory effects are primarily mediated by A2A AR (6), So it`s activation can alter outflow resistance at the SC-cell level, and thereby decrease IOP [10]. The results of this study are compatible with previous study has been shown that dibutyryl cAMP administered directly into the anterior chamber of the rabbit eye increased outflow facility, indicating that cAMP modulates outflow facility[31]. All tested drugs were found to be relatively safe in their administered doses [32].

Conclusions

The topical instillation of Limonene has a significant intra-ocular lowering effect as compared to negative control group (DW group) and positive control groups (Travoprost group) and had a significant effect on adenylyl cyclase activity and cAMP level. The most effective agent on IOP reduction was Limonene (4%) it was more effective than the positive control. Finally, the adenosine system is one of the potential target systems as new therapeutic approaches in glaucoma.

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