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PHYTOCHEMICAL ANTIOXIDANT AND REDUCING EFFECT OF INTRA OCULAR PRESSURE OF *Lentinus squarrosulus* IN RABBITS

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ABSTRACT

The evaluation of preliminary phytochemicals, antioxidants, and the reducing effect of intra-ocular pressure in *Lentinus squarrosulus* aqueous mushroom extract in rabbits. The phytochemical (qualitative and quantitative), antioxidant screening (ferric reducing antioxidant power (FRAP), total antioxidant capacity, and 1, 1-diphenyl-2-picrylhydrazyl (DPPH)) were investigated using an established protocol. The study involved 6 groups (n=5) of New Zealand male rabbits. The whole groups excluding the normal control were induced with 1% Prednisolone acetate suspension for three (3) days to acquire the baseline intraocular pressure (IOP) >5-10 mm Hg, subsequently treated with 100, 200, and 400 mg/kg *L. squarrosulus*. The administration was carried out in the right eye (OD) for 7 days and the IOP measurements were taken. The phytochemical screening showed the presence of alkaloids, phenol, saponins, and terpenoids, but phenol and alkaloids were abundant (40 and 19 mg/kg). *L. squarrosulus* extract had a scavenging effect against oxidative stress at 50 %, 93.7 %, and 25.26 % of the extract compared with ascorbic acid. The result of *L. squarrosulus* extract at 200 and 400 mg/kg elicited a significant decrease in the intraocular pressure compared with untreated control ($p<0.05$). In conclusion, *L. squarrosulus* intraocular pressure was scientifically validated its folklore claim.

KEYWORDS: Phytochemical, Antioxidant, Intraocular Pressure, *Pleurotus tuber-regium*

Ocular hypertension is one of the risk factors responsible for the development of glaucoma, which is the second-leading cause of irreversible blindness in Nigeria and the world at large (Kass *et al.*; 1980). About 60 million people have been affected by this disease worldwide, and approximately 8.4 million of the population are blind. It has been predicted that this figure will increase to 80 million, with 11.2 million going blind by the year 2021 (Friedman and Weinreb; 2004; Quigley and Broman; 2006; Mohammed *et al.*; 2009). Glaucoma is characterized by: increased IOP, enlarged cupping because of excavation of the disc, and disorientation of the neuro-retinal rim. There is also the asymmetry of the cup/disc ratio (difference in the C/D ratio in both eyes), visual field defect, and gradual loss of vision (Kanski; 2007). Ocular hypertension is defined as an elevation in intraocular pressure that exceeds the normal limit of 21mmHg with no accompanied ocular tissue (e.g. optic nerve damage); visual field defect and vision loss (Liang *et al.*; 2007). The elevation in IOP could be due to excessive secretion of aqueous humor or resistance to its outflow which could occur as a result of an obstruction in the drainage pathway. It is noteworthy that, about two third of persons with ocular hypertension not properly managed could develop glaucoma, hence an elevated IOP, especially in younger persons should be of great public concern. This is because the situation could predispose them to the early development of glaucoma and eventual blindness over some time (Aihara *et al.*; 2003).

Lentinus squarrosulus is an edible mushroom that belongs to the fungi family known as "Polyporaceae", genus *Lentinus*, and class Agaricomycetes. (Roy and Krishnappa, 2019). It is broadly distributed across sub-Saharan Africa and many parts of Asia (Adenipekun *et al.*; 2012). In Nigeria, it is called '*ero ata achichianya'* in Igbo and '*olu-awo'* or '*erirokiro'* in Yoruba, (Abdullah and Lau; 2016; Adebiyi and Yakubu; 2016; Akpaja *et al.*; 2005; Adeoye-isijola *et al.*; 2018). Traditionally, it has been in use for a long time due to its medicinal and health properties such as decreasing high blood pressure (anti-hypertensive) because of its high potassium contents and lower high blood sugar level in diabetic management (Omar *et al.*; 2015; Tran *et al.*; 2014; Adeyi *et al.*; 2021). It is useful in the healing and prevention of ulcers (Srichaikul; 2020). It is useful in the treatment of cancer (anti-cancer) because it attacks cancerous cells and prevents their growth (Prateep *et al.*; 2017). It is useful in the treatment of microbial infection because it inhibits the growth of

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harmful microorganisms such as *Salmonella typhi, Staphyloccocus aureus, Trichoderma rubum, and Apertrillus fumigatus* (Borokini *et al.*; 2012). It is useful as an antioxidant in the prevention of oxidative stress and consequent damage (Omar *et al.*; 2015).

MATERIALS AND METHODS

Plant Collection and Identification

Lentinus squarrosulus (shiitake mushroom) was cultivated at the African Centre for Mushroom Research and Innovation (ACMRIT) in the Department of Plant Biology and Biotechnology, University of Benin, Benin City for 6 months (April-Sept). At maturity, they were harvested by the Mycologist. A herbarium voucher number was issued (UBV-X264).

Plant Preparation and Extraction

Lentinus squarrosulus mushroom was chopped into pieces, air dried for three (3) weeks, ground into powder using a British milling machine, and refrigerated in an air-tight container for further use. The ground samples were macerated using cold method extraction processes. This entails soaking the weighed ground sample (1200 g) into a jar diluted with (2500 mL) distilled water for 72 hours with constant steering and shaking. The filtrate was freeze-dried to achieve a semi-solid extract of the concentrate (Jonathan *et al.*; 2012).

Phytochemical Screening

This process involved the qualitative and quantitative analysis of the phytochemical properties of *L. squarrosulus* (Harborne; 1998; Borokini*et al.*; 2012)

Quantitative Phytochemical Analysis

This was done with the methods described by Sofowora (1993), Trease and Evans (2002), Muraleedharannair *et al.* (2012); Daniel *et al.* (2011). The mushroom extract was screened to identify the qualitative and quantitative phytochemical ingredients found (alkaloids, flavonoids, phenolic compounds, tannins, terpenoids, saponins, and glycosides).

Antioxidant Assay

This study investigated the antioxidant property with the methods described by Buchner *et al.* (2014); Büttner *et al.* (2015); Cai *et al.* (2015)

Total Antioxidant Capacity

The extract (*L. squarrosulus*) at 1 mg/ml was added to 3 ml of Molybdate reagent solution in the tubes and incubated for 95 °C at 90 mins. The tubes were left for 20-30 mins to get cool at 37^{o}C and the absorbance of the mixture was measured at 695 nm wavelength. Ascorbic acid (vitamin C) was used as the standard (Buchner *et al.*; 2014).

Diphenyl-2-picrylhydrazyl (DPPH)

The free radical scavenging property of the mushroom extract against 1, 1–diphenyl–2–picrylhydrazyl (DPPH) radical was resolute by a slightly adapted method by Büttner *et al.* (2015). The assay is established on the bases of the antioxidant compounds to decrease DPPH by donating a pair of hydrogen atoms resulting in the color alteration from deep violet to golden yellow. The change in color from deep violet to light yellow was measured spectrophotometrically at 517 nm. Briefly, 0.5 mL of 0.3 mM DPPH solution in methanol was added to 2 mL of several volumes (0.2 - 1.0 mg/mL) of the extract. The reaction tubes were shaken and incubated for 15 mins at 37^{o}C in the dark and the absorbance was read at 517 nm wavelength. All tests were experimented with in triplicate. Ascorbic acid was used as the standard with equal volume as the prepared test samples. A blank containing 0.5 mL of 0.3 mM DPPH and 2 mL methanol was prepared and treated as the test samples.

Ferric Reducing Antioxidant Power (FRAP)

The Ferric Reducing Antioxidant Power (FRAP) assay was experimented with using an adapted method by Cai *et al.* (2015). The assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of 2, 4, 6-tripyridyls-triazine (TPTZ), forming an intense blue Fe^{2+}- TPTZ complex with an absorption maximum at 593 nm. To 1.5 mL of freshly prepared FRAP solution (25 mL of 300 mM acetate buffer pH 3.6, 2.5 mL of 10 mM 2,4,6-tripyridyls- triazine (TPTZ) in 40 mM HCl, and 2.5 mL of 20 mM ferric chloride ($FeCl_3.6H_2O$) solution was added to 1mL of the extracts (1 mg/ml) and standard at concentrations of 100-600µM. The response mixtures were incubated at 37^{o}C for 30 min and the increase in absorbance at 593 nm was measured. FeSO4 was used for the calibration curve and ascorbic acid served as the positive control. FRAP values expressed as (mg Fe (II)/g) of the extracts were then extrapolated from the standard curve Zeng *et al.* (2012).

Experimental Animals

The experiment was carried out using thirty (30) healthy adult New Zealand rabbits and ninety-five (95) healthy Wister rats, purchased from Aduwawa market in Benin-city, Edo state, Nigeria. The animals were kept in iron cages in the Department of Biochemistry, University of Benin. They were acclimatized for 14days with free access to Bendel pelleted grower mesh feed, and water *ad libidum*. They were uncovered for 12 hours light and

darkness. Their care was in line with the standard Ethical Right Guidelines for experimental animals. Life Sciences Ethical Committee certified the use of animals for this study with the ethical number LS19346.

Experimental Design

This study entailed thirty (30) healthy adult New Zealand rabbits selectively divided into six groups (n=5); normal control, 0.5 % timolol (standard drug), 1% Prednisolone acetate suspension (negative control), and the treatment groups at graded doses of *L. squarrosulus* (100, 200 and 400 mg/kg) respectively (Suvarna and Sangave; 2019). Before the study, the external eye tissues of the animals were screened with a penlight and hand magnifier while the internal eye examination was done with a direct ophthalmoscope for possible complications. The baseline intraocular pressure was obtained using a Perkins hand-held tonometer every morning before the study for 3 days to determine the average reading (Lim *et al.*; 2005). All the animals except those in the normal control group were induced with ocular hypertension using topical corticosteroid (1% Prednisolone acetate) suspension. The application was done daily in the right eye (OD) only for 7 days and the IOP measurements were taken until the IOP reading increased by >5-10 mm Hg above the baseline readings (Quigley and Broman; 2006).

Statistical Analysis and Data Presentation

The statistical analysis was performed using the statistical package for Graph-pad prism version 7. The results obtained for the quantitative phytochemical screening, *in-vitro* antioxidant, and intra-ocular pressure were expressed as Mean ± SEM. One-way analysis of variance (ANOVA) test was used to determine the significant differences between the treatment groups. Also, IC_{50} was calculated to establish the free radical scavenging and antioxidant potential of the plant extract. Statistical significance was declared as ($p< 0.05$).

RESULTS

The phytochemical properties screened include; the presence of alkaloids, flavonoids, phenolic compounds, tannins, terpenoids, saponins, and glycosides, as well as quantitatively determining their various concentrations (Table 1).

L. squarrosulus aqueous extract elicited the scavenging capacity capable to reduced or inhibiting oxidative stress, which could be responsible for the occurrence of glaucoma and related eye disorders. It elicited a radical scavenging effect with a significant decrease in percentage inhibition of DPPH, Total antioxidant capacity, and Ferric reducing antioxidant capacity. Antioxidants can donate hydrogen atom that neutralizes free radicals and changes the absorption process calorimetrically (Figure 1 to 7).

Table 1: Preliminary qualitative and quantitative phytochemical properties of *Lentinus squarrosulus* (LS)

Phytochemicals	Qualitative	Quantitative
Constituents	*Lentinus squarrosulus*	*Lentinus squarrosulus*
Alkaloid (%)	+	19
Flavonoids (%)	-	0.0
Phenols (mg/100 g)	+	40
Tannins (mg/100 g)	-	0.00
Saponins (%)	+	9.5
Glycosides (%)	-	0.0
Terpenoids (%)	+	14

Keys: - undetected, + detected

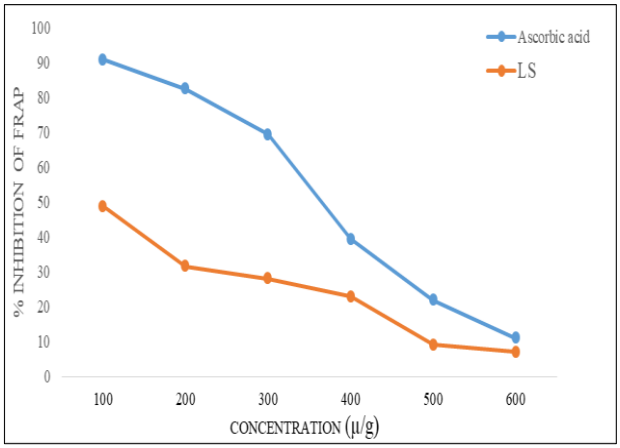

Figure 1: Effect of *Lentinus squarrosulus* extract on ferric reducing antioxidant power

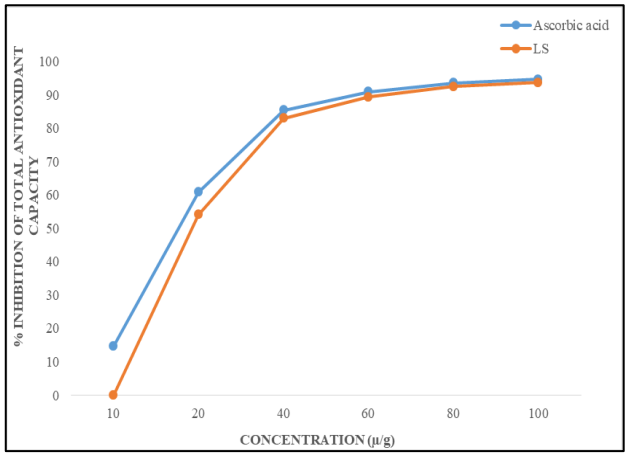

Figure 2: Effect of *Lentinus squarrosulus* extract on total antioxidant capacity

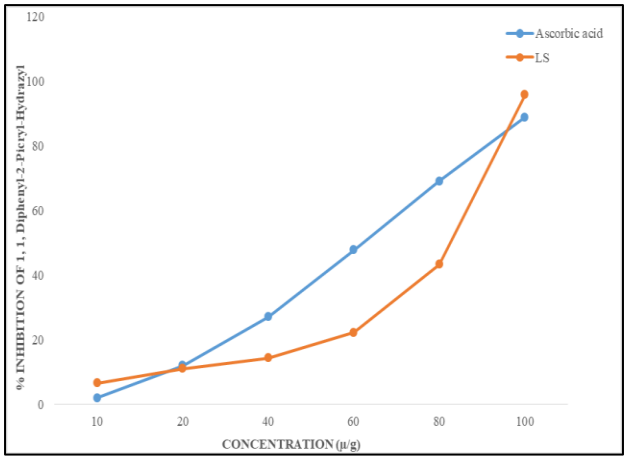

Figure 3: Effect of *Lentinus squarrosulus* extract on 1, 1, Diphenyl-2-Picryl-Hydrazyl antioxidant assay

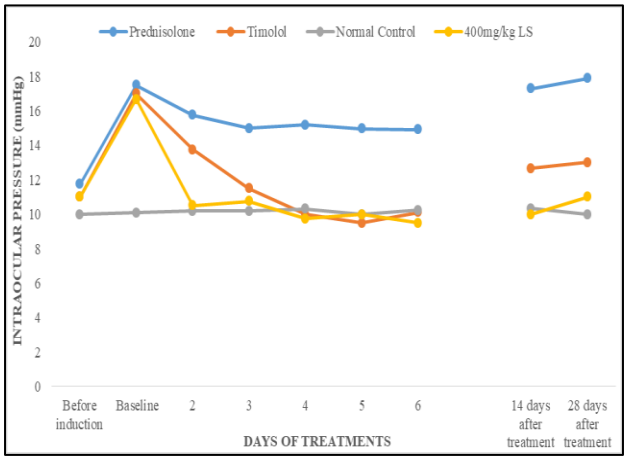

Figure 4: Effect of *Lentinus squrrosulus* in ocular pressure at highest doses of treatment

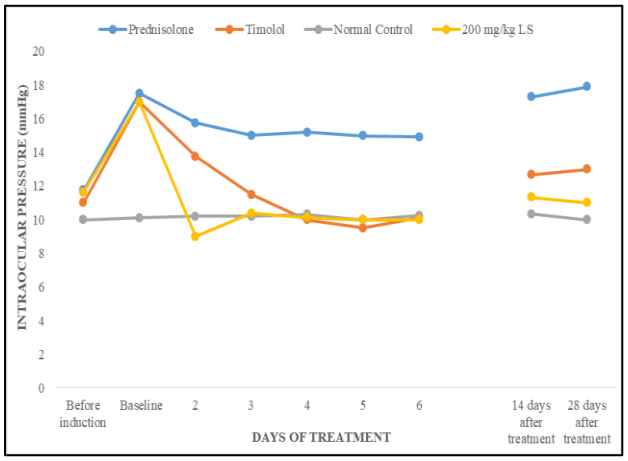

Figure 5: Effect of *Lentinus squrrosulus* in ocular pressure at media doses of treatment

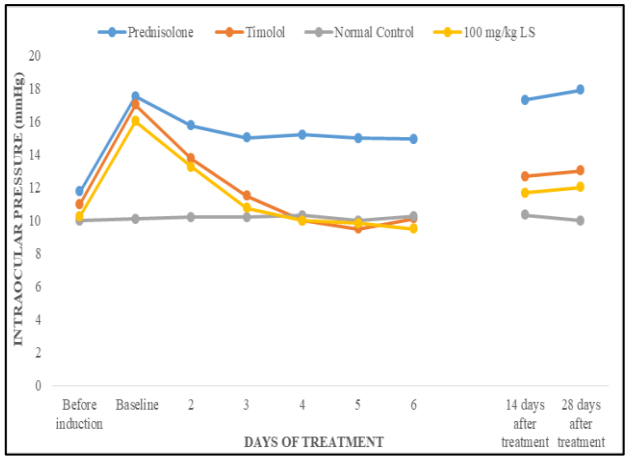

Figure 6: Effect of *Lentinus squrrosulus* in ocular pressure lowest doses of treatment

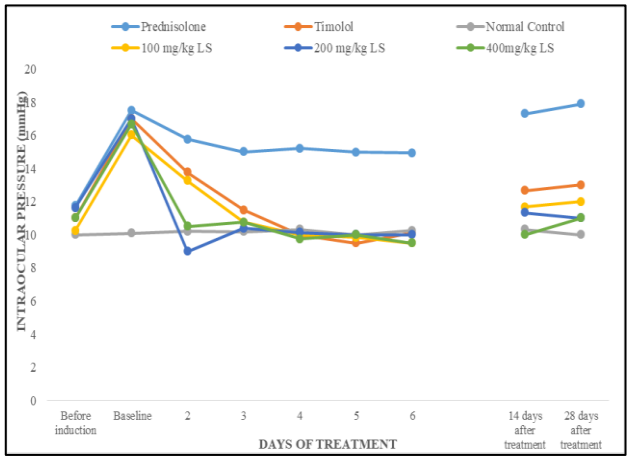

Figure 7: Effect of *Lentinus squrrosulus* graded doses on intraocular pressure (IOP)

DISCUSSION

Phytochemical screening of *L. squarrosulus* mushroom extract showed the presence of flavonoids, phenols, tannins, and saponin were found as shown in Table 1. This present study is similar to the work of Rajurkar and Kunda (2012); Agati *et al.* (2012), whose study is centered on the phytochemicals and metal content evaluated in *Adiantum capillus – veneris* extract. These compounds are active against potentially significant diseases (Daniel *et al.*; 2011). Apart from their potential biological activity, the phytoconstituents such as saponins, flavonoids, terpenoids, phenols, and tannins, were known to be a potent anti-malaria, immunoprotection, anti-diarrhea, anti-ulcer, nociceptive, anti-hypertensive, anti-diabetes, anti-lipidemia, and anti-depressant. Phenolic compounds potentiated the regenerations of blood cells which is an effective anti-anemic agent (Higawara *et al.*; 2014). The number of phytochemicals found in *L. squarrosulus*, was determined using standard procedures, and the result exhibited a

different quantity of phytochemical constituents. Tannin content is the highest in *L. squarrosulus* extract at 28.00 mg/100 g, followed by phenol compounds in *L. squarrosulus* at 20.00 mg/100g in the extract. This study concurred with the preliminary phytochemicals screening conducted by Kumudhavalli and Jaykar (2012) that evaluate petroleum ether, chloroform, acetone, ethanol, and aqueous extracts of fern *Hemionitis arifolia*. The evaluated mushroom extract revealed the major phytoconstituents such as phenol, alkaloids, flavonoids, tannins, saponins, and terpenes compounds. This report is in line with the research work done by Muraleedharannair *et al.* (2012), which examined the Phyto-constituents of *Adiantum caudataum, Adiantum latifolium, Adiantum lunulatum, Christella dentate,* and *Christella parasitic* extracts.

The oxidation of ferric reducing antioxidant power reduces the presence of hydrogen-donating antioxidant capacity, thereby impelling antioxidant concentration, and the duration of reaction taking during antioxidant activity. The evaluation of total antioxidant capacity indicated higher antioxidant content of *L. squarrosulus* aqueous extracts at different concentrations (10, 20, 40, 60, 80, and 100 µg/ml). This present study agreed with the work of Yildiz *et al.* (2014) on some physiochemical characteristics, bioactive content, and antioxidant characteristics of non-sprayed Barberry (*Berberis vulgaris* L.) fruits from Turkey. It was observed that for any particular level of antioxidant activity, the higher the concentrations (above 40 µg/ml) of the extract the better the scavenging activity when compared with ascorbic acid. This phenomenon was observed when measuring the antioxidant activity of the 50% ferric reducing antioxidant scavenging effect in the form of IC_{50} (µg/ml). The free radical scavenging effect was measured as percentage inhibition in the case of stable ferric reducing antioxidant properties at 50.0, 50.0, and 55.0%, of the mushroom extract when compared with the 95.0% ascorbic acid result. The report concurred with Tupe *et al.* (2013) on the evaluation of Antioxidant potentials and Total Phenolic contents of Selected Indian Herbs powder extracts. The standard drug (vitamin C) elicited high percentage inhibition at low concentration when compared with the mushroom extract. Since ascorbic acid is water-soluble, it serves as a potent antioxidant, biological and/or biochemical applications to curtain oxidative stress (Onuegbu *et al.*; 2020).

Free radical scavenging effect evaluated as percentage inhibition of *L. squarrosulus* aqueous extract at diverse concentrations to scavenge DPPH radicals at 100 % when compared with 98 % ascorbic acid at the same concentration. Hence, the inhibitory percentage increase is adequate with an increase in the concentration of *L. squarrosulus* extract. This, therefore, concurred with the possibility of the mushroom extract being capable of donating an electron to a lipid radical by converting the ascorbate radical to terminate the lipid peroxidation chain reaction. *L. squarrosulus* extract elicited a reduction in DPPH radical in line with hydrazine when reacted with hydrogen donors.

The results from 100, 200, and 400 mg/kg of *L. squarrosulus* elicited a significant decrease in intraocular pressure levels. At 200 and 400 mg/kg of the mushroom extract had a better significant decrease in the mean IOP level compared with 0.5 % timolol and 1% Prednisolone acetate suspension. More so, the result obtained from *L. squarrosulus* aqueous extract exhibited a prolonged half-life and possibly triggered ocular-hypotensive activity when compared with timolol. The action of carbapol polymer, triggered the drainage of aqueous humor competence to cause an increase in intraocular pressure by exciting the trabecular meshwork (Wang *et al.*; 2010). Hence, a decrease in intraocular pressure amounts to the endogenous release of Neostigmine, causing a rise in the level of acetylcholine, by an inhibitory action on Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE). This exhibited the action of some exogenous or endogenous mediators which was capable of altering the frontal or anterior chamber angle, thereby instigating the primary inflammatory reaction with late stringy changes. This inflammatory feedback action ensued in the early phase with reduction or total extinction after some weeks (Sawaguchi *et al.*; 2005.). Fibrous disintegration and linkage barrier also take place majorly in the frontal chamber angle later than 4 weeks. Thus, the trabecular meshwork with normal lymph morphology, showed an extended and deformed eye caused by prednisolone administration, leading to collagen hyperplasia and elastic fibers. There the detachment of the endothelial cells from the trabecular meshwork is comparable to that of the macrophages (Realini; 2011). During the induction of intraocular pressure with prednisolone suspension, the phagocytized carbomer elements were elated via vacuoles in the Schlemm's canal of the endothelial chambers. The large vacuoles progressively decreased, leading to extreme carbomer particles amassed in the carbapol polymer, affecting the discharge of aqueous humor capability via the distorted trabecular meshwork. This present study evaluates the IOP lowering effect of *L. squarrosulus* in rabbits when compared with untreated IOP (Quigley and Broman; 2006; Masoud *et al.*; 2013; Nemesure *et al.*; 2003).

At graded doses of (100, 200, and 400 mg/kg) *L. squarrosulus* resulted in a significant decrease in normal

intraocular pressure levels. Specifically at 200 and 400 mg/kg of the mushroom extract had a pronounced significant reduction in the mean IOP level when compared with timolol control and untreated control. This agreed with the report of Liang *et al.* (2007). The result obtained from *L. squarrosulus* mushroom extract exhibited a delayed onset of action and possibly triggered ocular-hypotensive activity when compared with timolol. The possible IOP lowering effect of *L. squarrosulus* could be attributed to the action of anticholinesterase action by reducing the excessive secretion of acetylcholine. This is in line with a previous study by Ji *et al.* (2005) worked on the effect of elevated IOP on mouse ganglion cells in mice. Proofs have been suggested that gluco-corticoids are implicated in the metabolism of carbohydrates, fats, and protein, displaying their main role in the physiological directive of the pathway associated with the outflow capacity and intraocular pressure. Though glucocorticoid receptors located in the trabecular meshwork pathway enhance aqueous outflow, biochemical, and resultant physical procedures, its competence in instigating reductive action. The mechanisms of action may be consequential to the modulatory action of macromolecular breakdown, or adrenergic/prostaglandin interfaces encompassing the outflow system. More so, prednisolone-induced fibrous proliferation destroys the anterior chamber (Kymes *et al.*; 2006; Kierstan; 2021). The eye is encompassed by several muscarinic receptors and their subtypes implicated in the ocular surface, lens, retina, ciliary body, and sclera.

CONCLUSION

In conclusion, *Pleurotus tuber-regium* extract is a potent therapeutic regime for managing intraocular pressure in rabbits. Scientific validation carried out affirms the ethnomedicinal claim that it is effective in the management of ocular hypertension.

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