SPECTROSCOPIC AND CHROMATOGRAPHIC CHARACTERISATION OF MUSHROOM EXTRACT (Pleurotus tuberregium) IN COMPARISON WITH SELECTED ANTI-GLAUCOMA MEDICATIONS

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ABSTRACT
Pleurotus tuberregium, the king tuber mushroom, is an edible gilled fungus native to the tropics, including Africa, Asia, and Australia. Experimental studies have shown that extracts of Pleurotus tuberregium caused a decrease in intraocular pressure in steroid induced ocular hypertension stimulating increasing interest in it as a potential anti-glaucoma drug. This study investigated the possible existence of similar active ingredients found in the antiglaucoma medications under study (2% Pilocarpine, 0.5 % Timolol, 0.5% Betaxolol and 0.005% Latanoprost) and the fractions of the mushroom extract. Column chromatography was performed using silica gel to isolate active compounds from the extract of Pleurotus tuberregium. Thin layer chromatographic analysis was then performed on the fractions alongside known anti-glaucoma medications to determine and compare their retardation factors and migration speeds. Further analytical study was carried out using UV-VIS spectrophotometry. Data obtained was presented in bar charts and graphs, and analyzed using one sample t-test in the Statistical Package for Social Sciences (SPSS) version 22.0. Thin layer chromatography showed comparative corresponding separation spots of the extracts with those of the antiglaucoma medication, and thus similar retardation factors. This study serves to further corroborate the postulated intraocular pressure lowering effect of P. tuberregium extract thereby contributing to the journey of the possible discovery of a potential anti-glaucoma medication.

Keywords: Anti-glaucoma medications, Bioactive Constituents, Column Chromatography, Pleurotus sp., Spectrophotometry, Retardation Factor.

INTRODUCTION
Glaucoma affects about 60.5 million people, leaving 8.2 million with bilateral blindness [1]. It is in fact the second leading cause of blindness in the world after cataract. Glaucoma is usually characterized by elevated Intraocular Pressure (IOP) and degeneration of the axons of Retinal Ganglion Cells (RGCs), which leads to visual field loss and if left untreated, ultimately blindness. The primary goal in the management of glaucoma is to prevent the risk factors, especially elevated IOP, using medications, laser therapy or conventional surgery. In recent times, there has been an increased interest in complementary/alternative medicine. But very little research has been done on the majority of herbal remedies, with regard to their effect on glaucoma. Gingko balboa extract, Coleus forskohlii, pueraria flavonoids, areca seed, Erycibe obtusifolia, Salvia miltiorrhiza are examples of herbs currently being used to treat glaucoma in various parts of the world [2]. Pleurotus tuberregium is a basidiomycete found in the tropical and subtropical regions of the world. It is also known as the “King Tuber Oyster Mushroom” [3]. Extracts of P. tuberregium have been studied for their ability to cause a reduction in IOP in dexamethasone – induced ocular hypertensive cats [4]. It is reported to heal eye diseases. There is therefore an emerging interest in Pleurotus tuberregium as a potential antiglaucoma drug. In this study, fractions from extracts of Pleurotus tuberregium are compared with other known antiglaucoma drugs on the basis of their retardation factor using Thin Layer Chromatography and then on the basis of their absorption spectrum from UV-spectrophorometry.
MATERIALS AND METHODS
This study was an experimental study. Column Chromatography and Thin layer Chromatography were carried out at the Department of Pharmacognosy, Faculty of Pharmacy, University of Benin, while UV-VIS Spectrophotometry was carried out at the Spectrophotometry/Chromatography center at the National Center for Energy and Environment (NCEE), University of Benin, Benin City, Edo State.

The study was performed in four different stages in order to complete the objectives. In the first stage, the *P. tuberregium* mushroom was cultivated. In the second stage, the aqueous extracts of *P. tuberregium* were separated into fractions using hexane and acetone as diluting solutions. The third stage deals with the comparison of the major active components present in the aqueous extract responsible for its antiglaucoma effect with those of the selected antiglaucoma medications. In the final stage, further comparison was carried out using UV-VIS Spectrophotometry.

Second stage: Fractions of 10ml were collected in thirty (30) numbered test tubes. The eluted compounds were monitored using TLC. A total of 30 fractions were collected from column chromatography. The fractions were pooled according to their spots on the TLC plates. The excess solvents in the pooled fractions were evaporated under reduced pressure using a rotary evaporator. The 30 fractions were pooled into five fractions labeled A1, A2, A3, A4 and A5 respectively.

Third stage: The prepared plates were spotted manually with the fractions of the mushroom extract and the known glaucoma medications used in the study using capillary tubes. The spots were labeled accordingly. Photographs of the plates were captured immediately after the plates have been removed from the iodine tank. The plates were studied and analyzed for identical sites of migration across the plates. The retardation factor was then calculated for the spots and comparisons made. Colours of the spots were noted and Retention factor (Rf) values were calculated using the following formula:

\[ Rf = \frac{\text{Distance Travelled by Solute}}{\text{Distance Travelled by Solvent}} \]

(Kanagasabapathy et al., 2011) [5]

Final Stage: On completion of the TLC, the analyte was scraped off from the TLC plates. Then diluted in the solvent system and filtered off. This was then sent to the Spectrophotometer/Chromatography research laboratory at the National Centre for Energy and Environment (NCEE), University of Benin, for spectrophotometric studies. The ultraviolet visible spectrophotometer was used to determine the absorption spectrum of fractions of *P. tuberregium* extract.

RESULTS
A total of five (5) fractions of mushroom extract (*Pleurotus tuberregium*) were obtained from column chromatography. These five fractions were spotted on the TLC plates with the aid of capillary tubes, however, A5 produced no spots and showed no migration of the sample. This means the fraction A5 was chemically empty therefore containing only the solvent system. It was thus excluded from further analysis. Two readings were taken and the mean of their migration distances and retardation factors recorded and presented in figures and bar charts below.
Figure 1: Thin layer chromatography (TLC) plate 1 showing separation spots of samples A1, A2 and A3.

Figure 2: Thin layer chromatography (TLC) plate 2 showing separation spots of samples A4, 2% Pilocarpine and 0.5% Betaxolol. P = 2% Pilocarpine and B = 0.5% Betaxolol.

Figure 3: Thin layer chromatography (TLC) plate 4 showing separation spots of samples preservative free 0.5% Timolol and fractions (A1, A2, A3 and A4) of 40mg/ml Pleurotus tuberregium extract. T = 0.5% Timolol.
Figure 4: A bar chart showing both migration distances and retardation factors of A1, 2% Pilocarpine, 0.5% Betaxolol, 0.5% Timolol and 0.005% Latanoprost.

The migration distances ranged from 3.2 (0.5% Betaxolol) to 8.2 (0.005% Latanoprost). A1 and 2% Pilocarpine have relatively similar migration distances and thus retardation factors. Retardation factor was calculated by dividing migration distance of the sample with the migration distance of the solvent. The migration distance of the solvent for plates 1 to 5 was 12cm.

ANOVA

Table 1: Table showing analysis of variance between groups and within groups.

|                | Sum of Squares | df  | Mean Square | F             | Sig.  |
|----------------|----------------|-----|-------------|---------------|-------|
| Between Groups | .110           | 3   | .037        | 2934.653      | .014  |
| Within Groups  | .000           | 1   | .000        |               |       |
| Total          | .110           | 4   |             |               |       |

Table 2: Table showing One Sample statistics of A1, 2% Pilocarpine, 0.5% Timolol, 0.5% Betaxolol and 0.005% Latanoprost.

|                | N   | Mean  | Std. Deviation | Std. Error Mean |
|----------------|-----|-------|----------------|-----------------|
| A1             | 2   | 4.00850 | 4.796305       | 3.391500        |
| PILOCARPINE    | 2   | 4.00600 | 4.799841       | 3.394000        |
| BETAXOLOL      | 2   | 16.13350 | 22.438619      | 15.866500       |
| TIMOLOL        | 2   | 4.00300 | 4.789941       | 3.387000        |
| LATANOPROST    | 2   | 4.44150 | 5.315322       | 3.758500        |
Table 3: Table showing the results of the student one sample t-test in comparing the means between the groups.

| Group              | t    | df | Sig. (2-tailed) | Mean Difference | 95% Confidence Interval of the Difference |
|--------------------|------|----|----------------|-----------------|------------------------------------------|
| 2% Pilocarpine     | -1.000 | 1  | .500           | -3.394000       | -46.51886 - 39.73086                      |
| 0.5% Betaxolol     | -3.864 | 1  | .161           | -5.666500       | -24.30015 - 12.96715                     |
| 0.5% Timolol       | -1.003 | 1  | .499           | -3.397000       | -46.43292 - 39.63892                     |
| 0.005% Latanoprost | -0.787 | 1  | .575           | -2.958500       | -50.71477 - 44.79777                     |

One Sample t-test were run to determine whether there was a statistically significant mean difference between the migration distance of the fractions of the extract and the migration distances of the drugs under study. Using the mean values of the migration distances, student one sample t-test were performed. Statistically, in all cases, the null hypotheses could not be rejected at level 0.05. This means that there is no significant difference between the migration distances of the fractions and the anti-glaucoma medications.

Decision Rule: Reject $H_0$ if $F_{cal} < P_{value}$, if otherwise do not reject.

Decision: Accept $H_0$.

Figure 5: A plot of the absorbance spectrum of A1

A1 recorded a wavelength of maximum absorption ($\lambda_{max}$) of 280nm.
Figure 6: A plot of the absorbance spectrum of A2 at varying wavelengths. A2 recorded a wavelength of maximum absorption ($\lambda_{\text{max}}$) of 220nm.

Figure 7: A plot of the absorbance spectrum of A3 at varying wavelengths. A3 recorded a wavelength of maximum absorption ($\lambda_{\text{max}}$) of 320nm.
DISCUSSION
The cost of glaucoma medications as well as their side effects has been reported as some of the major deterrents to drug adherence and compliance. These complaints can be effectively attended to by herbal remedies.
This study compared the migration distance (separation spots) and retardation factors of fractions of *Pleurotus tuberregium* extracts with those of drugs whose mode of action and pharmacodynamics are already known. The study took a step further by plotting the absorbance spectrum of the various fractions and comparing the wavelength of maximum absorption with those of the anti-glaucoma drugs under study whose $\lambda_{\text{max}}$ is already known from literature.

The results of this chromatographic and spectroscopic characterization of *Pleurotus tuberregium* and comparison with 0.5% Timolol, 0.5% Betaxolol, 0.005% Latanoprost and 2% Pilocarpine has helped to elucidate the pharmacodynamics of *Pleurotus tuberregium*. Pharmacodynamics is concerned with the effects of drugs and their mechanism of their action. This is directly related to the active ingredients found in the drug. Hence, the essence of this study was to determine the possible existence of similar active ingredients in the aqueous mushroom extract and the drugs under study.

The column chromatography yielded thirty fractions which were pooled together into 5 test tubes labeled A1, A2, A3, A4, and A5. However, only four fractions (A1, A2, A3 and A4) underwent further analysis. This is because A5 did not spot on the TLC plate. This means that it was chemically empty and only contained the solvent system.

The retardation factor of Latanoprost discovered by this study is 0.686. This is concurrent with the findings of a monograph by the United States Pharmacopeia (2012), which stated that the retardation factor of Latanoprost is about 0.7. The migration distance and retardation factor of A1 on the Thin Layer Chromatogram (7.30) was found to closely approximate that of 2% Pilocarpine and 0.5% Timolol, 7.33 and 7.41 respectively. This finding suggests that there is a possible existence of the active ingredients of these antiglaucoma medications in A1. This concurs with the study on Bovine Iris Sphincter muscle where it produced a contractile effect on the muscarinic receptors of the sphincter papillae [6].

The migration distance and retardation factor of fraction A2 and 2% Pilocarpine were discovered to be relatively similar. This study discovered the Rf value of Timolol to be 0.62. Another study placed the Rf value of Brimonidine and Timolol at 0.23 and 0.63 respectively. The accuracy of the method of the stated study was accessed by percentage recovery and found to be $99.77 \pm 0.71\%$ for brimonidine tartrate and $99.87\% \pm 0.86\%$ for Timolol maleate. This
study also concluded that the TLC method can be used for routine analysis of brimonidine tartrate and timolol maleate in pharmaceutical formulations [7]. However, the European Pharmacopoeia method for Timolol Identification Test C using silica gel G TLC plates reported the Rf value of Timolol to be 0.74 [8]. The disparity in value between this study and that is a result of the type of silica gel plates used.

Statistically, this study showed that there was no significant difference in the migration distances of the fractions and the anti-glaucoma medications indicating a comparative similarity in bioactive constituents as seen in the Figure 4.1 to 4.15, thus the alternate hypothesis, HAC is rejected. By implication, the null hypothesis, HO1 is accepted.

Furthermore, A1 recorded a wavelength of maximum absorption ($\lambda_{\text{max}}$) of 280nm. A2 recorded a wavelength of maximum absorption ($\lambda_{\text{max}}$) of 220nm. A3 recorded a wavelength of maximum absorption ($\lambda_{\text{max}}$) of 320nm. A4 recorded a wavelength of maximum absorption ($\lambda_{\text{max}}$) of 320nm. This connotes that the maximum absorbance wavelength of the extract is 320nm. Timolol maleate, a non-selective beta blocker reduces has an absorbance spectrum that falls within this range with a $\lambda_{\text{max}}$ of 295nm as determined by Isikhuehmen et al [9-12]. Though not a drug under comparison in this study, Brimonidine tartrate, a relatively selective alpha-2 adrenergic receptor agonist whose mechanism of action is to reduce aqueous humour production and increase uveoscleral outflow has a $\lambda_{\text{max}}$ of 247nm [8]. Latanoprost a prostaglandin analogue was reported by a monograph by the United States Pharmacopoeia as having an absorbance spectrum within the range of 200 – 400nm, with a $\lambda_{\text{max}}$ of about 200nm. Pilocarpine, as determined by Scott et al., (1981) has an absorbance spectrum that also falls within the range of that of the mushroom extract with a $\lambda_{\text{max}}$ of 215 – 220nm. Betaxolol, a selective beta blocker, has an absorption spectrum that falls within the UV range with $\lambda_{\text{max}}$ of 224nm as determined by Patil et al., (2013) [13].

The fractions of the Pleurotus tuberregium extract demonstrated corresponding spots to those of the antiglaucoma drugs used in this study. This suggests a possible existence of a combination of similar active ingredients in the extracts and the drugs. This could explain its intraocular pressure reducing properties as reported by Akinlabi et al., (2008)[4]. It is clear that the absorption spectrum of Pleurotus tuberregium falls within the range of the absorption spectra of the antiglaucoma drugs under comparison. It is therefore very likely that the mushroom extract has similar active ingredients as the drugs under study and hence similar pharmacodynamics activity.

CONCLUSION

From the analysis of variance above, we can conclude that there are similarities of migration distance and retardation factor between fraction A1 and 2% Pilocarpine, 0.5% Betaxolol, 0.5% Timolol and 0.005% Latanoprost. Summarily, from the experiment, we conclude that the separation spots of the fractions of mushroom extract in the thin layer chromatogram were similar to those of 0.5% Timolol, 0.5% Betaxolol, 2% Pilocarpine and 0.005% Latanoprost. All spots for this study are produced with a reproducibility better than 1.5% RSD. We can also conclude that Silica gel A plates can be used in the identification of the anti-glaucoma drugs under study.

Although the therapeutic activities exhibited by extracts, isolated compounds or fruiting bodies of Pleurotus sp. has been extensively documented, the biochemical mechanisms especially as related to its IOP reducing capabilities are still elusive due to poor characterization and identification of the bioactive components. In this experiment, Pleurotus tuberregium extract showed comparative corresponding absorbance spectrum with those of the antiglaucoma drugs. This information can be utilized in further studies and in the creation of an ecofriendly, economical and side effect minimized medication for combating glaucoma.

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