Administration effects of four psilocybin mushroom extracts on serotonin levels and endothelial nitric oxide synthase activity levels in vivo and in vitro after one hour

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Research Article

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Abstract

Background

Psilocybin-containing mushrooms induce antidepressant and momentary increase in blood pressure (BP) with potential risk to users with cardiovascular diseases. Irregularities in nitric oxide (NO) levels play a key role in endothelial dysfunctions leading to increases in BP. Mushrooms species show large variation in potency which may potentially induce different outcomes and mechanisms of action. Effects of the mushrooms on the endothelial nitric oxide synthase activity is not known.

Aim

To investigate safety and effects of administration of four psilocybin-containing mushroom species, *Panaeolus cyanescens, Psilocybe natalensis, Psilocybe cubensis* and *Psilocybe cubesis leucistic A+ strain*, on acute haemodynamic and LV parameters in normal Wistar rat and on serotonin, NO levels and endothelial NO synthase (eNOS) activity *in vivo* and *in vitro* on H9C2 cardiomyocytes.

Methods

Mushrooms were extracted with hot-boiling water and administered (5 mg/kg) through a direct catheterization in anaesthetized rats. Nuzak (0.2 mg/kg) and Nω-Nitro-L-arginine methyl ester hydrochloride (LNAME) were used as positive controls and negative control group given saline. Levels of serotonin, NO and eNOS activities were measured after 1-hour treatment.

Results

Mushroom treatments incited non-significant increase in LV parameters peaks only after 20 minutes and not immediate like with LNAME. Mushrooms induced a significant increase in serotonin levels and a suppressing effect on the eNOS activity *in vivo* in rats and *in vitro* in cardiomyocytes.

Conclusion

Mushroom treatments were safe on the LV function and induced a significant serotonin level with the concentration investigated. Disturbance in eNOS pathways may be the underlying mechanism involved in the psilocybin-mushroom extracts to inducing temporary BP increase. The four mushrooms exhibited different cardiac effects indicating variations depending on mushroom species.

1. Introduction
Psilocybin-containing mushrooms, commonly known as magic mushrooms, have antidepressant effects that has attracted a lot of attention and the use of the magic mushrooms have increased [1, 2]. Psilocybin, the primary psychoactive compound found in the magic mushrooms is known to induce its effects by mimicking the serotonin and acting on its receptors [3]. Psilocybin-containing mushrooms are considered safe with very rare fatalities and the mushrooms are also reported to induce slight increase in blood pressure (BP) [4]. Tachycardia is also known to be common with patients intoxicated with psilocybin mushrooms [4]. Mechanisms that these mushrooms use to increase BP are not fully known and this elevation may be a potential risk especially to the users suffering from cardiovascular diseases such as hypertension and heart failure [4].

Nitric oxide (NO) is one of the well-known facilitators of blood pressure regulation. Nitric oxide is produced from L-arginine by the enzyme NO synthase (NOS), in the presence of several co-factors such as heme, flavin adenine dinucleotide, mononucleotide and tetrahydrobiopterin (BH$_4$) in response to various factors including shear stress [5, 6]. There are three well-studied isoforms of NOS, which are endothelial-derived NOS (eNOS), induced NOS (iNOS) and neural NOS (nNOS) [7]. In particular, eNOS is responsible for the production of NO within the endothelium [8]. Upon its release in the endothelium NO diffuses through the smooth muscle, stimulates guanylate cyclase and causes an increase in production of cyclic guanosine monophosphate (cGMP) leading to vasodilation [9].

Irregularities in NO levels play a significant role in endothelial dysfunctions leading to increases in BP as it related to diseases such as hypertension. Changes in NO levels can be either through its production (with NOS dysfunctions or disturbances in the availability of the substrate L-arginine) or through the inhibition of its availability via irregularities in reactive oxygen species (ROS) also known as oxidative stress [10]. Besides its direct inhibition on NO activity, ROS can also activate inhibition of eNOS mRNA expression and eNOS activity via MAPK pathway [10]. Additionally, ROS may oxidise BH$_4$ to induce eNOS uncoupling which will lead to eNOS producing more ROS than NO [11]. As a result, the increased reduction in NO production and/or availability will cause vasoconstriction leading to an increase in BP. Use of pharmacological inhibitors of NO synthesis, such as $\text{NG}$-monomethyl-L-arginine and $\text{N\omega}$-Nitro-L-arginine methyl ester hydrochloride (LNAME), have resulted in significant BP increases and are firmly established to produce both acute and chronic hypertension in many animal species [12;13,14]. Moreover, administrations of 7 to 10 mg/kg LNAME doses in rats induced tachycardia at the time when BP reached the peak [13, 15].

This study aimed at investigating the one-hour acute effects of *Panaeolus cyanescens*, *Psilocybe natalensis*, *Psilocybe cubensis* and *Psilocybe cubensis leucistic A + strain* magic mushroom extracts on the aortic and on the left ventricular blood pressure parameters in a normal Wistar rat model and on the production of serotonin, NO and activity of eNOS *in vivo* and *in vitro* on H9C2 cardiomyocytes. We extracted the mushrooms with hot-water, which is one of most commonly used method of mushroom consumption by magic mushroom users. In a previous study the 25 $\mu$g/mL of the hot-water extracts of the four magic mushrooms showed potential anti-inflammatory properties on lipopolysaccharide-induced human macrophage cells [16]. Consequently, this concentration was also used to investigate the effects
of the magic mushrooms on eNOS activity in the cardiomyocytes. The results from this study may indicate the safety of mushrooms on LV function and mechanism of action used by these magic mushroom extracts to induce the temporary blood pressure increase.

2. Materials and Methods

2.1. Ethical clearances

The protocol for this study was submitted to the University of Pretoria faculty research committee and approved with the number REC045-18. In South Africa psilocybin mushrooms are schedule 7 substances, and approval by the South African Department of Health Medical Control Council (MCC) was also applied for, and a permit license POS 223/2019/2020 was granted for the project. The protocol was also submitted to the University of Pretoria Animal Ethics Committee (UPAEC) and approved with the number V101-18.

2.2. Growing mushrooms and making extracts

The spore prints of Psilocybe nataleases (P. natalensis), Psilocybe cubensis (P. cubensis), and Psilocybe cubensis leucistic A + strain (P. A + strain) and Panaeolus (Copelandia) cyanescens (Pan cyanescens) mushrooms were verified with SKU number TEA-1, NSS-1, AAP-1 and TBMN-1, respectively, together with the growing sterile substrate kit (SSK-2) and were all purchased from Spore Spot Company, Durban, South Africa. As soon as they arrived the spores were inoculated in the sterile substrate, grown, harvested and extracted with hot boiling water as described elsewhere [17]. The extracts were then kept in the dark in a refrigerator until use.

2.3. Animal protocol

Forty-two 4 months old normal male Wistar rats (body weights between 325 to 425 g) were purchased from Wits central animal service, (Parktown, Johannesburg) and housed in a ventilated room at 24–26°C with humidity of 30–70% in a room with a dark and light cycle of 12 hours. The animals were housed in pairs (2 rats) per cage equipped with environmental enrichment in the form of tunnels and they have free access to standard rodent chow and water. The animals were housed and cared in accordance to the AEC regulation and were allowed one-week acclimatization prior to commencement of the experiment. The rats were randomly divided into seven experimental groups of 6 rats per group. The different treatments were administered via a catheter whilst under anaesthesia: Group one: Control, were given only saline, the vehicle; Group two given 5 mg/kg of Pan cyanescens; Group three: given 5 mg/kg of P natalensis Group four: given 5 mg/kg of P. cubensis; Group five: given 5 mg/kg of P. A + strain, Group six: given 0.2 mg/kg Nuzak (positive control 1: prescription drug used to treat depression) and Group seven: given 10mg/kg LNAME (Sigma-aldrich) (positive control 2: NOS inhibitor and induce high BP and heart rate

2.3.1. Surgical procedure and administrations of drugs and mushroom extracts
On the day of surgery, each rat was given 1.3 ml/kg.bw of anaesthesia intraperitoneally. The anaesthesia was prepared with xylazine 20 mg/ml (Centaur Labs, SA) and Anaket-V ketamine HCl 100 mg/ml (Kyron laboratories, SA) administered at a ratio of 3:1. After the rat was sedated and its mass determined, the rat was placed in the supine position on a pre-warmed operation table to help maintain a constant body temperature. A transverse incision was made on the skin of the neck region, to expose the underlying muscle and trachea. The muscle was separated in order to locate the right common carotid artery, which was then separated from the vagus nerve. A small incision was then made and a tip of a 2F catheter was carefully inserted into the opening made in the artery, and pushed forward into the aorta and left ventricle respectively. The catheter was quickly connected via a stopcock to the rest of the fluid filled monitoring system and care was taken to minimize blood loss throughout the procedure. By manipulating the catheter back and forth, aortic and LV pressure curves were respectively monitored on the “strip chart” oscilloscope (Lasec) and when stable, the baseline values were recorded. Then the drugs and extracts were administered slowly to the respective groups and both aortic and LV pressure parameters were recorded over 60 minutes and the recorded data was stored on the PC for off-line analyses.

The following indices were measured from each recorded minute of the 60 minutes recordings (1, 5, 10, 20, 30, 40, 50 and 60 minute) of all the rats; the mean arterial blood pressure (MAP) calculated from systolic blood pressure (SBP measured in mmHg) and diastolic blood pressure (DBP, measured in mmHg), peak left ventricular systolic pressure (LVSP, measured in mm Hg), an index of cardiac work (CW) calculated by multiplying LVSP with HR, the maximum rate of rise in left ventricular pressure (dP/dt$_{\text{max}}$, measured in mmHg/s) and heart rate (HR, measured in beats per minute (b/min)).

### 2.3.2. Blood sample collection plasma storage

After the aortic BP and LV pressures were successfully recorded at the end of an experiment, blood was collected from each rat using pre-cooled ethylenediamine-tetra acetic (EDTA) coated tubes and immediately placed on ice in a cool bag. The blood samples were centrifuged at 3000 x g for 10 minutes at 4°C (using a Heraeus Sepatech, 17RS centrifuging machine) and the plasma stored at -80°C in a freezer until use.

### 2.4. Cell culture

#### 2.4.1. Culturing of cells

The rat H9C2 cardiomyoblast cells were purchased from American Type Culture Collection and the cells were maintained using Dolbecco Modified Earlage media (DMEM) (Pan, Separations Scientific) supplemented with 10% fetal bovine serum (FBS) (Gibco, Sigma Aldrich) and 1% of 100 IUunits/mL penicillin and 100 µg/L streptomycin (Pan, Celtics diagnostic) in 75 cm2 tissue culture treated flasks (NEST, Whitehead Scientific). The cells passaged 17 were grown at 37°C in a 5% CO$_2$ balanced air environment in an incubator (HERAcell 150, Thermo Electron Corporations, USA).

#### 2.4.2. Treatment of cells
As soon as the H9C2 cardiomyoblast cells reached 70% confluence they were passaged, counted and 5 x $10^4$ cells seeded and grown in 24 well plates (NEST, Whitehead scientific). After 24 hours medium was removed, the cells in the 24 well plates were deprived of serum for 18 hours. After 18 hours sera-free media was removed, and some of the cells were induced for 45 minutes with 100 µM of Nω-nitro-L-arginine methyl ester (L-NAME) (Sigma-aldrich), a NOS inhibitor and S-(2-Boronoethyl)-L-cysteine hydrochloride (BEC) (Sigma-aldrich), an arginase inhibitor while other set of cells were not induced. The cells were then treated with the extracts (25 µg/mL) with and without LNAME and BEC for 1 hour in the media supplemented with 1% FBS and 1% penicillin-streptomycin. This medium was used to prepare and dilute all the treatment and drugs. After 1 hour the medium was collected and stored in -80°C freezer until use. The experiments were repeated in three different times.

### 2.4.3. Mitochondrial activity

To test for toxicity, $10^4$ cells were seeded in 96 well plates (NEST, Whitehead scientific), deprived of serum the same way as above with serum-free DMEM over 18 hours prior to inducing the cells with LNAME and BEC 45 minutes. Then cells were treated with the four hot-water extracts with and without LNAME and BEC for 1 hour. Then mitochondrial activity was measured using the Resazurin assay kit AR002 (R & D, Whitehead scientific) according to the manufacturer’s manual. Viability of cells was calculated using the formula: % Viability= ((Sample Absorbance/control Absorbance) x 100). The experiments were repeated in three different times.

### 2.5. Nitrite content measurements

The nitric oxide production after 1-hour treatment in cardiomyocytes and recording in animals were measured in cell culture supernatant and blood plasma as an indication of NO production using Nitrite Colorimetric Assay kit (E-BC-K070-S, Elabscience, Biocom Africa) according to the protocol manual. The concentration of nitrite was determined from the serially diluted standard curve.

### 2.6. Measurement of eNOS concentrations

The effects of the extracts on eNOS levels after 1-hour treatment were determined on the blood plasma and H9C2 cardiomyocyte culture media using the rat endothelial nitric oxide synthase (eNOS/NOSIII) ELISA kit (E-EL-R0019, Elabscience, Biocom Africa) according to the manufacturer manual protocol. Concentrations of rat eNOS in the plasma and cell culture media samples were calculated from the standard curve.

### 2.7. Measurement of serotonin concentrations

The effects of the extracts on the levels of serotonin after 1-hour treatment were determined on the blood plasma of the rats using a ST/5-HT (Serotonin/5-Hydroxytryptamine) ELISA Kit (E-EL-0033, Elabscience, Biocom Africa) according to the manufacturer manual protocol. Concentrations of serotonin levels in the plasma samples were calculated from the standard curve.

### 2.8. Statistics analysis
Statistically significant values were compared using one-way ANOVA analysis of variance using an interactive statistical program (Sigmastat, SPSS version 26, USA). Normality test was performed using Shapiro-Wilk and equal variance test of Brown-Forsythe. Results are expressed as mean ± standard deviations and the p-value of ≤ 0.050 was considered statistically significant.

3. Results and Discussion

The study showed an increase with all the groups including the saline-control in both the aortic and LV pressure parameters on the 1st minute after the drugs and mushroom extracts administrations through direct catheterization in anaesthetized rats in the study, and this effect was expected as it resulted from the volume of the vehicle injection (Fig. 1, 2, and 3). Treatment with saline, the control vehicle showed a reduction from minute 5 with all the direct aortic and LV haemodynamic pressures and cardiac work which was maintained until the 60th minute (Figs. 1, 2 and 3). The same effects were observed with the 0.2 mg/kg dose of Nuzak, the positive control 1 (which is one of the common and current used antidepressant drugs) on the treated rats. Nuzak administration did not increase either aortic nor LV pressures and parameters in the study.

Treatment with the positive control 2, LNAME (a NOS inhibitor), increased the aortic pressures (MAP) as expected significantly from minute 1 to 20 and the pressures started dropping drastically after the 40th minute, Fig. 1. The study showed that LNAME administration generally peaked in the 5th and 10th and this effect agreed with previous studies where the peak of LNAME was observed in 7 to 10 min after administration of 10 mg/kg in rats [18]. This study however, also showed that the acute effects of 10 mg/kg single dose of LNAME on aortic BP lasted for 30 minutes period after which the effects started to fade away from minute 40 where we observed the pressures dropping to the lower levels same as saline and falling even lower than the pressures of the control group by the 60th minute. The same respond was observed with the contractility as indicated by dp/dt\textsubscript{max} (Fig. 2) as well as CW parameter (Fig. 3) which was sustained significantly higher than saline until the 40th minute also followed by a sharp drop to levels under control on the 60th minute. As a result, these LNAME drop effects suggests that the physiological compensatory mechanisms which probably involved up-regulating the activity of eNOS were in operation as the LNAME suppressing effects were worn out after the 40th minute and hence decreases was recorded for SBP, DBP, LVSP, dp/dt\textsubscript{max} and CW levels towards control values on the 60th minute in the study.

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observed with the contractility as indicated by $\text{dp/dt}_{\text{max}}$ (Fig. 2) as well as CW parameter (Fig. 3) which was sustained significantly higher than saline until the 40th minute also followed by a sharp drop to levels under control on the 60th minute. As a result, these LNAME drop effects suggests that the physiological compensatory mechanisms which probably involved up-regulating the activity of eNOS were in operation as the LNAME suppressing effects were worn out after the 40th minute and hence decreases was recorded for SBP, DBP, LVSP, $\text{dp/dt}_{\text{max}}$ and CW levels towards control values on the 60th minute in the study.

The four magic mushroom treatment induced a non-significant decrease in aortic (MAP, Fig. 1), $\text{dp/dt}_{\text{max}}$ (Figue 2) and CW (Fig. 3) from the 10th minute below control and treatment where *Pan cyanescens*, *P. cubensis* and *P. natalensis* had increasing trends with peaks between the 20th to the 40th minute observed within each treatment recording period. The *P A + strain* treatment on the other hand induced a further drop from minute 10 until the 60th minute same as Nuzak under control, without any increasing trends in between during the entire recordings Many studies have reported these increases in MAP with magic mushrooms consumptions [4] which are generally experienced from 20 to 40th minute after consumption and not immediate as we observed with LNAME. However, in our study, the increasing trends were not statistically significant when compared with the saline group with 5mg/kg dose that was used indicating safety in the LV function.

After the 60th minute, analysis of the blood plasma for NO production was performed and revealed that the rats treated with Nuzak produced a non-significant slightly increase in NO concentrations compared to the saline levels, Fig. 4A. Administration of LNAME also induced non-significant NO production very close to the saline levels after 60 minutes. The measurement of eNOS concentration in plasma of rats also showed the same response with regards to the controls where the eNOS concentrations of rats treated with Nuzak were non-significantly slightly higher than saline levels while the levels of LNAME treated rats displayed the same eNOS concentration same as the saline control group, Fig. 4B. Although LNAME is a non-selective NOS inhibitor which will be expected to inhibit and reduce NO product and eNOS concentrations, these opposite findings were not surprising. Based on the haemodynamic finding, it became evident that the LNAME administration was only effective before the 40th minute and its effect were worn out by the 60th minute and hence the pressures dropped to the control levels. The findings with plasma NO production and eNOS concentrations suggest that physiological compensation effects were present and involved eNOS pathways as speculated before which evidently brought concentrations levels to normal or control levels. As a result, the blood plasma taken from rats after1 hour after will reflect exactly what we observed with the NO and eNOS concentrations with the LNAME treatment.

In order to further establish the effects of the four magic mushrooms on the NO production, and eNOS activity following 1-hour treatment *in vivo* in normal condition, the mushrooms were also tested on the H9C2 cardiomyocytes over the same 1-hour period with and without the LNAME, NOS inhibitor and BEC, arginase inhibitor stimulations (Figs. 5 and 6) and they are discussed in synergy below.
There was no significant difference in the NO levels of the cells treated *Pan cyanescens* mushroom over 1-hour after mushroom treatment while the eNOS concentration was significantly decreased ($p = 0.001$) compared to the control cells, suggesting a suppressive eNOS effect by the extract. Moreover, the NO concentration was decreased significantly ($p = 0.025$) in the presence of NOS inhibitor and associated with a significant suppression of eNOS levels ($p = 0.017$) as expected. Also, the NO levels were significantly increased ($p = 0.006$) however, with a non-significant increase in eNOS in the presence of the arginase inhibitor. These finding suggested that the significant increase observed with BEC was partially due to production via eNOS activity, suggesting that arginine suppression availed more L-arginine which was used by eNOS as expected. The initial effects with non-stimulated cells above where eNOS were significantly suppressed, supported the plasma evaluation results where the rats treated with *Pan cyanescens* produced significantly lower plasma eNOS concentrations ($p = 0.037$)) compared to the control saline group. These lower eNOS levels were in line with the plasma NO concentrations which were also significantly lower ($p < 0.001$) than observed in the saline control plasma. Since the aortic and LV pressures of this mushrooms were very close to the saline levels on the 60th minute when blood was collected, it would be expected to find similar or close to the control eNOS concentrations however, the opposite was true with this mushroom. As a result, the significantly low plasma eNOS and NO concentrations findings suggested the presence of compounds in this mushroom extract with possible suppressing effects on the eNOS activity and/or pathway.

When it comes to *P. natalensis* mushroom treatment, treatment of the H9C2 cardiomyocytes produced a significant increase in NO concentration ($p = 0.013$) which was associated with a significant ($p = 0.02$) suppression in eNOS concentrations. The levels of NO were decreased significantly ($p = 0.005$) in the presence of LNAME, and increased significantly in the presence of BEC ($p = 0.033$) as would be expected. However, the treated cells also decreased eNOS concentration significantly ($p < 0.001$) in the presence of both LNAME and BEC, suggesting that the significant increase in NO levels observed in non-stimulated cells and in the BEC-stimulated treated cells were not due to NO production via the eNOS activity and/or pathway. As a result, the study proposed that the 1-hour treatment with *P. natalensis* mushroom increased NO level of the treated cells by mechanisms that increase availability of NO and not through its production via eNOS pathways. Analysis in the plasma showed a non-significant lower plasma NO levels with *P. natalensis* mushroom administration which was associated with a significantly suppressed plasma eNOS levels ($p = 0.015$), further agreeing with the *in vitro* cardiomyocytes results with the suppressed eNOS findings. The pressures of the *P natalensis* were also around the control BP values on the 60th minute, yet the plasma eNOS concentration was significantly lowered compared to the control. Previously we have demonstrated significant anti-oxidant and anti-inflammatory potential of *P. natalensis* mushrooms, and we speculate presence of these compounds to also play a part maintaining the haemodynamic of the treated around the saline levels even though the plasma NO was non-significantly lowered with the significantly suppressed eNOS as revealed in the study [17]. Furthermore, the study also proposed possible suppression of eNOS activity and/or pathway by *P. natalensis* mushroom after 1st hour treatment which was also observed in the plasma of the rats.
When it comes to the *P. cubensis* analysis, treatment on the H9C2 cardiomyocytes with *P. cubensis* mushroom extracts induced no change regarding the NO levels of the treated cells compared to the controls while eNOS concentrations were significantly high (p < 0.001) after 1-hour treatment. In the presence of NOS inhibitor, the cells increased NO levels significantly (p = 0.011) while the eNOS concentration was non-significantly affected and in the same level as the LNAME-control. In the presence of arginase inhibitor, where high levels of NO and eNOS may be expected, it was not the case with *P. cubensis* treatment. The NO levels were significantly increased (p = 0.011) while the eNOS concentrations significantly (p < 0.001) lowered. This finding that NO levels were only increased significantly where eNOS was the same as LNAME-control and where eNOS was significantly suppressed as observed in the presence BEC, suggested that the increased NO was not produced from eNOS pathway but rather from mechanism that increase NO availability. Our previous study has shown the significant anti-ROS effects of *P. cubensis* mushroom extracts displayed in hypertrophy-induced cardiomyocytes conditions and we speculate the presence of compounds with these effects to also play a part with the high NO levels observed in the presence of a suppressed eNOS concentration in the cardiomyocytes [19]. Analysis from the rats administered with *P. cubensis* conversely produced significantly (p = 0.001) low plasma NO levels with non-significant low plasma eNOS concentrations which were however higher than the other three mushrooms. This finding agreed with the BP observations from this mushroom where the pressures were generally higher than all the other mushrooms especially on the 60th minute with a sharp increased observed with the LVSP, dp/dt\text{max} and CW recordings. However, it was also interesting to note that in the non-induced cells, NO levels were not increased even though eNOS was significantly high and also in the blood analysis, plasma NO levels of *P. cubensis* treated rats were not increased compared with the other three mushrooms even though its levels of eNOS was noticeably higher than these mushrooms. As result, the rats that were treated with *P. cubensis* in general displayed higher pressures and their hearts were also subjected to a greater cardiac work than the others despite their better plasma eNOS levels which were low yet better than all the other mushrooms in the study. These findings suggested the possibility of other compounds as well with possible inhibitory effects on the eNOS pathways or activity that hindered it to produce the required NO after 1-hour consumption.

Analysis of *P. A + strain* administration on the other hand, revealed that treatment of the H9C2 cells with the mushroom increased significantly (p < 0.001) the levels of NO of treated cells compared to the control cells while the eNOS concentration was significantly lowered (p = 0.001). Levels of NO were lowered significantly (p = 0.030) in the presence of LNAME with significantly lowered eNOS concentrations (p = 0.002) with *P. A + strain* mushroom treatment while the NO levels were increased (p = 0.002) significantly in the presence of the arginase inhibitor with very significantly (p < 0.001) low eNOS concentrations. These findings suggested that the high NO levels induced with *P. A + strain* mushroom treatment was not due to NO production via eNOS activity and/or pathway but rather due to mechanisms that increased NO availability. The study also proposes from these findings that there are compounds in *P. A + strain* mushrooms with suppressing effect on the eNOS activity and/or pathway. These findings on cardiomyocytes also support the findings in eNOS activity observed in the *in vivo* study in rats where the rats administered with *P. A + strain* produced the highest level of NO with significantly lower levels of
eNOS concentration (p < 0.001) compared to Saline, Nuzak and LNAME. Further suggesting that NO was not produced from eNOS instead the findings support mechanisms that increases NO availability to be in place. The high plasma NO was also in agreement with the lower aortic and LV haemodynamic pressures observed with the $P. A + strain$ mushroom treatments throughout the entire recording which were lower than saline and close to Nuzak treatment even though the eNOS concentrations were so significantly suppressed in these rats. As a result, the finding suggested presences of compounds in the $P. A + strain$ mushroom extract with biological ROS suppressing activities that lead to increase NO availability and concentrations in the study. This finding also indicated possibilities that the $P. A + strain$ mushroom may also have compounds that suppressed the eNOS activity and/or pathway.

The plasma serotonin levels of the rats treated with the four magic mushrooms showed a significant increased levels (p < 0.001) same as with Nuzak treatment (p < 0.001) after 1-hour of administration in comparison to the saline group, Fig. 4. The study also showed moreover, that the mushrooms treatments induced acute levels of serotonin which were higher than that of Nuzak. Treatment with $P. A + strain$ produced the highest significant levels (p < 0.001) of plasma serotonin followed by $Pan cyanescens$ (p < 0.001) and $P. natalensis$ (p < 0.001) in comparison to the levels produced by the rats treated with Nuzak. The antidepressant effects of psilocybin-containing mushrooms are known to occur via their binding to serotonin receptors and generally exhibiting their agonist properties [3, 20]. The serotonin 2A receptor subtype has been found to be the key proteomic binding site of serotonergic psychedelic compounds like psilocybin by many researchers [21, 3, 20]. The therapeutic mechanism of action of SSRIs such as Nuzak involves alteration in the serotonin system [22]. They inhibit neuronal uptake pump for serotonin and increase its availability and are commonly used as first-line antidepressants [21, 22].

Our study showed a significant effect of the 5mg/kg dose of the four extracts administrations on the level of serotonin within the first hour which was associated with non-significant increasing peaks in aortic and LV pressure parameters indicating safety in the LV function. Mitochondrial activity as indicated by % cell viability of the H9C2 cardiomyocytes treated with the four magic mushrooms $Pan cyanescens$, $P. natalensis$, $P. cubensis$ and $P. A + strain$ extracts revealed that the effects and finding observed in the study were not due to toxicity, Fig. 8.

4. Conclusion

In conclusion, the study showed that the single dose of 10 mg/mL LNAME concentration significantly increased the CW of the rats and these effects faded away after 40 minutes. The study also revealed for the first time that $Pan cyanescens$, $P. natalensis$ and $P. cubensis$ had increasing peaks in aortic and LV pressure parameters peaks which were not significant while treatment with $P. A + strain$ mushroom lowered these pressures suggesting safety in the LV functions of the treated rats with the dose used in the study. The increase peaks only happened after 20 minutes of mushroom extracts administration and not immediate as observed with LNAME. These effects were associated with different significant increase in serotonin levels of the treated rats which were even higher than the Nuzak, a well-known SSRI drug, suggesting possible significant serotonergic effect with the dose used in the study. The four
mushrooms also displayed different cardiac effects indicating variations depending on mushroom species in support of projections by previous studies.

The study also showed for the first time that the four psilocybin-containing magic mushrooms induced an acute suppressing effect on the eNOS activity and/or pathway in vivo in normal Wistar rats and in vitro in H9C2 cardiomyocytes following 1-hour administration. The study proposes that this effect could be the underlying mechanism implemented by the mushrooms to induce temporary increase in BP. Further investigations into the eNOS pathway following magic mushroom administration in vivo and in vitro is recommended.

Declarations

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Conflicts of interest:

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Availability of data and materials:

Data for this research will be obtained upon request from the corresponding author.

Code availability:

Not applicable.

Author contributions:

S.M.N.: Conceptualization of the study, S.M.N., L.H. Methodology S.M.N.: Preparation of all the figures, S.M.N.: Writing the original manuscript, S.M.N., L.H., C.M.L.S., J.N.E.: Writing review and editing of the
manuscript. All authors reviewed the manuscript. Authors have read and agreed to the published version of the manuscript.

**Ethical approval:**

The protocol for this study was submitted to the University of Pretoria faculty research committee and approved with the number REC045-18. In South Africa psilocybin mushrooms are schedule 7 substances, and approval by the South African Department of Health Medical Control Council (MCC) was also applied for, and a permit license POS 223/2019/2020 was granted for the project. The protocol was also submitted to the University of Pretoria Animal Ethics Committee (UPAEC) and approved with the number V101-18 for animal use.

**Consent to participate:**

Not applicable.

**Consent for publication:**

Not applicable.

**References**


Figures

Figure 1

Effects of the hot-water extracts of *Pan cyanescens, P. natalensis, P. cubensis*, and *P. A+ strain* mushrooms and positive controls; Nuzak, LNAME and saline on the % MAP of rats over 60 minutes (n= 5/6 rats per group). (*: significant).
Figure 2

Effects of the hot-water extracts of *Pan cyanescens*, *P. natalensis*, *P. cubensis*, and *P. A+ strain* mushrooms and positive controls; Nuzak, LNAME and saline on the % dp/dtmax rats over 60 minutes (n=5/6 rats per group). (*: significant).
Figure 3

Effects of the hot-water extracts of *Pan cyanescens, P. natalensis, P. cubensis,* and *P. A+ strain* mushrooms and positive controls; Nuzak, LNAME and saline on the % CW of rats over 60 minutes (n= 5/6 rats per group). (*: significant).
Figure 4

The effects of the hot-water extracts of *Pan cyanescens*, *P. natalensis*, *P. cubensis*, and *P. A+ strain* mushrooms and positive controls; Nuzak, LNAME and saline on the plasma nitrite production (µmol/L) and eNOS production (pg/mL) after 1-hour treatment (n= 5 rats per group). (*: significant).
Figure 5

The effects of the hot-water extracts (25 µg/mL) of *Pan cyanescens*, *P. natalensis*, *P. cubensis*, and *P. A+ strain* mushrooms on nitrite concentrations after 1-hour treatment in H9C2 cardiomyocytes without (A) and with LNAME (B) and BEC (C) stimulation, measured in three different occasions. (*: significant).
Figure 6

The effects of the hot-water extracts (25 µg/mL) of *Pan cyanescens*, *P. natalensis*, *P. cubensis*, and *P. A+ strain* mushrooms on eNOS concentrations after 1-hour treatment in H9C2 cardiomyocytes without (A) and with LNAME (B) and BEC (C) stimulation, measured in three different occasions. (*: significant).
Figure 7

Effects of the hot-water extracts of *Pan cyanescens*, *P. natalensis*, *P. cubensis*, and *P. A+ strain* mushrooms and Nuzak (positive control) and saline on the plasma serotonin levels (ng/mL) of rats over 60 minutes (n= 5/6 rats per group). (*: significant).
Figure 8

Effects of the hot-water extracts of *Pan cyanescens, P. natalensis, P. cubensis,* and *P. A+ strain* mushrooms on the viability of cells after 1 hour treatment without (A) and with LNAME (B) and BEC (C) stimulation.