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Formulation of granules from *Pleurotus ostreatus* mushroom with potentialities for developing solid dosage forms

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ABSTRACT

The study was aimed to evaluate the potential of *Pleurotus ostreatus* granules as a raw material for developing solid dosage forms. Drug-excipient compatibility was assayed using 1:1 binary mixtures and the ratio of formulation components was optimised with a D-optimal design considering flow properties as response variables. Three batches of granules were produced with the optimised mixture by the wet granulation method. The quality of the granules was evaluated based on physical, rheological, chemical, and microbiological parameters. The concentration of phenolic compounds in the binary mixtures remained unchanged at 30°C, but decreased at 45°C and 60°C. Moreover, a quadratic model was used to fit the response variables. Mixing design allowed selecting the best excipient ratio. The granules showed a residual moisture content and a particle size lesser than 5% and 350 μm, respectively, as well as, excellent flow and compressibility properties, and optimal microbiological quality.

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INTRODUCTION

Currently, there are numerous pharmaceutical and commercial products that utilise granules as a solid dosage form. Granules can serve as a precursor for other solid dosage forms, such as capsules and tablets [1]. They are composed of agglomerations of smaller particles with sufficient strength to allow their handling. Granules are primarily used when the Active Pharmaceutical Ingredient (API) is sensitive to moisture, has stability problems, or poor flow properties[2].

The powder derived from the fruiting body of the edible and medicinal mushroom *Pleurotus ostreatus* (oyster mushroom) is considered a nutraceutical preparation due to its abundance of bioactive compounds, including polysaccharides, proteins, amino acids, polyphenols, vitamins and fatty acids[3]. Pharmacologically, *P. ostreatus* has a wide range of activities, such as immunomodulatory[4], antioxidant[4], hypoglycaemic[5], anti-tumour[6] and antibacterial[7] properties.

In a previous publication, an evaluation of the technological, biochemical, and microbiological properties of *P. ostreatus* powder was performed to determine the quality parameters for its potential use as an API. The powder possessed good organoleptic properties, a rich nutraceutical composition, and adequate microbiological quality. However, rheological parameters indicated poor flowability, which negatively impacts in the development of solid dosage forms[8].

Preformulation studies are a critical stage in the drug development process. They involve the characterisation of physical, chemical, and mechanical properties to enable the design of dosage forms with greater stability, safety, and efficacy. Experimental designs and drug-excipient compatibility studies are useful tools to achieve a more stable formulation while reducing the investment of time, resources, and effort[9].

In view of the increasing prevalence of non-communicable diseases (NCDs), such as type II Diabetes Mellitus, the World Health Organization recommends exploring alternative therapies[10]. In this context, *P. ostreatus* extracts reduced the high blood glucose levels in hyperglycaemic rats[11], and in hyperglycaemic mice[5]. Moreover, ethanolic extracts of this mushroom exhibited an antihyperglycemic effect in high sucrose high fat diet streptozotocin induced diabetes in rats[12]. In this way, dietary phenolic compounds can be considered a potential

strategy in the development of pharmaceutical approaches that aim to reduce complications resulting from the progression of this metabolic pathology[13]. *Pleurotus florida* (=*P. ostreatus*) with a high concentration of total phenolics showed effective antioxidant and antidiabetic effects under *in vitro* conditions[14].

Moreover, most of the pharmaceutical formulations employed for animals closely resemble those utilised in human medication (including capsules, tablets, powders, and so on)[15]. Therefore, mushrooms granules can be considered in manoeuvring the innovative drug delivery systems for veterinary therapeutics.

This research was aimed to investigate the potential of *P. ostreatus* granules as a precursor for developing solid dosage forms with nutraceutical potential, including anti-diabetic effects in humans as well as veterinary applications. The study highlights research-development activities in the field of mushrooms natural products as an environmentally friendly, safe and viable alternative for third world countries. At least until we know, this is the first report of a comprehensive design and characterisation of mushroom granules with applications in food and pharmaceutical industries.

METHODOLOGY

Mushroom material

Pleurotus ostreatus CCEBI-3024 (Pleurotaceae) is a cultivated strain deposited in the Culture Collection of the Center for Studies on Industrial Biotechnology (CEBI, Universidad de Oriente, Cuba). Experts from the *Centro Oriental de Ecosistemas y Biodiversidad* (BIOECO, Santiago de Cuba, Cuba) confirmed the taxonomic identification. Slants with potato dextrose agar (PDA) solid medium, incubated at 37°C for 7 days, were used for strain conservation.

Excipients

Colloidal silicon dioxide (Aerosil 200, Evonik Resource Efficiency GmbH, Germany), microcrystalline cellulose (Avicel PH 101, JRS Pharma, Germany), magnesium stearate (Sudeep Pharma Pvt. Ltd, India), polyvinylpyrrilidone (K-25, O-BASF, Germany) and lactose monohydrate (Molkerei MEGGLE Wasserburg GmbH & Co. KG, Germany) were used as excipients. These Generally Accepted as Safe (GRAS) excipients were selected on the basis of their multifunctionality in the formulation of pharmaceuticals from natural sources[16]. All other chemicals and solutions used were of pharmaceutical grade.

Obtaining *Pleurotus ostreatus* powder

Powder preparation from the fruiting bodies of *P. ostreatus* mushroom was carried out as described by Arias-Ramos et al.[8]. Briefly, the fruiting bodies were harvested and cut into small pieces of approximately 1 cm^2. They were dried in an oven at 45°C for 24 h (VENTICELL, Spain). The dried material was ground in a blade mill (Retsch GM 200, Germany) to obtain a powder with a grain size <250 μm, and stored in plastic bags, protected from light and moisture, for further use.

Drug-excipients compatibility study

Binary mixtures of *P. ostreatus* powder with each of the excipients were prepared in a 1:1 ratio to assess compatibility. The substances were mixed in a mortar and pestle, and the resulting mixture was passed through a 350 μm mesh sieve (TSS-200, Utrecht, Germany) to homogenise the particle size. Then, 10 g of each mixture was placed in amber bottles with ground-glass stoppers. The mixtures were stored for 30 days at 30, 45 and 60°C in an oven (VENTICELL, Spain). Total phenolic compounds were quantified in the powder and in each mixture by the Folin-Ciocalteu method[17], after 0, 7, 15, 21 and 30 days of treatment.

D-optimal design

A D-optimal mixture design was used to determine the proportions of mixture components corresponding to the optimal rheological parameters of the granules (Design Expert 13.0 software, Stat-Ease, Inc., Minneapolis, MN, USA). The responses selected as indicative of the presence of drug-excipient interactions were Carr's index, Hausner's ratio, Flow rate and Angle of repose. The empirical models estimated to find the optimal formulation were plotted as contour plots.

A range of 0 to 46% of the independent variables (coded as 0 and 1) was used to optimise the composition of the excipient mixture. Five excipients, commonly used for preparing solid dosage forms from natural products were selected[18]. As previously mentioned, *P. ostreatus* powder was chosen based on its nutraceutical composition -both nutrients and mycochemicals, like phenolic compounds[3].

The granules were prepared for a total quantity of 100 g with a constant content of the active ingredient (50%), colloidal silicon dioxide (2%) and magnesium stearate (2%). Restrictions were applied to the remaining components in order to respect the actual amounts used in the pharmaceutical formulations

(Table 1). The evaluated responses were fitted to a quadratic model linking product properties with product composition (Equation 1):

$$Y = \beta 1xMCC + \beta 2xLM + \beta 3xPVP + \beta 12xMCCxLM + \beta 13xMCCxPVP + \beta 23xLMxPVP \quad (1)$$

where: Y represents the response; β1, β2 and β3 represent the effect relative to the concentrations of Microcrystalline Cellulose (MCC), Lactose Monohydrate (LM) and Polyvinylpyrrolidone (PVP) (coded values); β12, β13 and β23 represent the interaction effect between the three factors.

The best granules were selected taking into account the increase in flowability and compressibility, aspects related to the response variables evaluated.

Table 1. Experimental matrix of the D-Optimal Mixing Design used in the preparation of *P. ostreatus* granules (components levels are expressed in %)

Components	**Low levels (%)**	**High levels (%)**
A: Microcrystalline Cellulose (MCC)	0	1
B: Lactose Monohydrate (LM)	0	1
C: Polyvinylpyrrolidone (PVP)	0	1
Partial mixture	**46**	
Colloidal Silicon Dioxide (CSD)	2	
Magnesium Stearate (MS)	2	
Pleurotus ostreatus powder	50	
Partial mixture	**54**	
Total mixture	**100**	

Preparation of granules

The wet granulation method was used to produce the granules. All powders were first sieved through a 177 μm mesh (TSS-200, Utrecht, Germany). Then, *P. ostreatus* powder was mixed with CSD, followed by the addition of the remaining excipients in a horizontal laboratory mixer (Eureka AR-400, USA). PVP was used as a binder. The wet mass was dried in a vacuum oven (Sartorius, Germany) at 40°C for 12 h, and then, the dry mass was ground in a blade mill (Retsch GM 200, Germany) and sieved through a 350 μm mesh (TSS-200, Utrecht, Germany).

The best granules were selected considering fluidity and compressibility. Three batches of 200 g each were evaluated. The physical, technological, chemical and microbiological quality was assessed.

Quality evaluation of *Pleurotus ostreatus* granules

Moisture content

The moisture content was determined by the infrared gravimetric method using a thermogravimetric balance (model MB-110, MRC, Germany).

Scanning electron microscope - energy dispersive X-ray Spectrometer analysis

Surface morphology of the *P. ostreatus* granules and the chemical composition of the excipients were studied using a field emission Scanning Electron Microscope (SEM, FE, TESCAN Mira3, Brno, Czech Republic) coupled with Energy Dispersive X-ray Spectrometer (EDS, Oxford Instrument, INCAx-act, Abingdon, Oxfordshire, UK). The sample was coated with a 10 nm gold layer to ensure electronic conductivity. SEM images were taken with a view field of 1000, 200, 100 and 50 μm.

Particle size

The mean particle size of granules was studied using the vibration sieving method in a mechanical sieve (TSS-200, Utrecht, Germany)[8].

Rheological analysis

The following parameters were determined: Carr's index, Hausner's ratio, flow rate, and angle of repose[8,18].

Determination of total phenolic content and HPLC analysis of phenolic compounds

The determination of total phenolic content was carried out according to Beltrán et al.[19]. with slight modifications. Briefly, 1.5 mL of 10% Folin-Ciocalteu reagent was added to 1 mL of aqueous extracts of *P. ostreatus* granules and allowed to stand for 5 min at room temperature. Then, 2 mL of a saturated Na_2CO_3 solution was added. After one hour in the absence of light, the absorbance was measured at 765 nm in a UV-visible spectrophotometer (Genesis 10S, Thermo Fisher Scientific, Waltham, MA, USA). Tannic acid at concentrations of 6.25, 12.5, 25.0 and 50.0 μg/mL was used as standard (calibration curve $y=0.0076x - 0.4224$, $r^2=0.9917$). *The results were expressed as microgram tannic acid equivalents per mL of granules extract.*

Moreover, the HPLC analysis of phenolic compounds contained in mushroom granules was performed as a fingerprint and quality criteria. An aqueous extract of *P. ostreatus* granules was adjusted to a concentration of 5 mg/mL in 50% methanol and filtered through a 0.22 μm membrane filter before chromatographic

analysis (E-Merck, Darmstadt, Germany). The individual phenolic compounds were identified by using an Agilent 1200 series HPLC system with degasser, quaternary pump, automatic injection, thermostatic column compartment and a diode array detector (UV-DAD) (Agilent Technologies, Eindhoven, Netherlands). Detection was carried out at 280 nm as a preferred wavelength. Reverse-phase chromatographic analysis was carried out using a Phenomenex Luna C-18 column (250 × 4.6 mm i.d., particle size 5 μm, Phenomenex B.V., Utrecht, The Netherlands) at 26°C. Running conditions consisted of a gradient mixture of a solvent A (0.1% aqueous formic acid solution) and solvent B (acetonitrile), with a flow rate of 1 mL/min, and a run time of 65 min. The phenolic compounds were identified according to their UV spectra and by retention times (Rt), in comparison with authentic standards: gallic acid, pyrogallol, homogentisic acid, protocatechuic acid, chlorogenic acid, caffeic acid, vanillin, feluric acid, naringin, naringenin, hesperetin. Acetonitrile and formic acid were of HPLC grade and obtained from Fisher Chemical TM (Loughborough, UK), and the phenolic standard compounds were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Microbiological analysis

The microbiological stability of the *P. ostreatus* granules was evaluated to assess their susceptibility to the presence of filamentous fungi, yeasts, *Pseudomonas aeruginosa, Staphylococcus aureus, Enterobacteriaceae and Candida albicans* in accordance to the microbiological quality acceptance criteria[20].

Statistical analysis

The software Design-Expert version 13.0 (Stat-Ease, Inc., Minneapolis, MN, USA), the Microsoft Excel included in the Microsoft Office package, and the software Statgraphics Centurion XV version 15.2.14 (Statgraphics Technologies, Inc., The Plains, VA, USA) were used for the mathematical processing and statistical analysis of data.

The statistical parameters used to evaluate and select the best fitting model were the coefficient of determination(R^2), adjusted coefficient of determination (adjusted R^2), coefficient of variation (CV), standard deviation, predicted residual sum of squares (PRESS), and the lack of fit and regression data (p value and F value). The positivity of the coefficient in the equation of the best-fitting model represents the positive contribution to the response, and vice versa. For a better explanation, a contour plot and a three-dimensional response surface were additionally generated for each response.

The physicochemical, technological and chemical parameters of granules were expressed as mean ± standard deviation of each batch, and the means were compared with ANOVA coupled with the Tukey's Least and Maximum Significant Difference test in order to identify significant differences. In the case of the mean particle size distribution, the normality of the results was assessed by the Kolmogorov-Smirnov test. A significance level of 95% was considered in the analysis.

RESULTS and DISCUSSION

Preformulation of *Pleurotus ostreatus* granules

The selection of granulation technique depends on the characteristics of the product and its manufacture requirements. In the context of our investigation, the direct method is not considered a viable option, because of the *P. ostreatus* powder exhibits inadequate flow properties. Although dry granulation has a reduced production cost compared to wet granulation, it is an unfavourable alternative as judged by the high friability of the product and the presence of fines. On the contrary, wet granulation facilitates closed processing, enhances drying efficiency, and improves rheological properties, rendering a cost-effective process for applications in the pharmaceutical industry[1].

An optimal pharmaceutical formulation is achieved through the appropriate selection of excipients. Drug-excipient compatibility studies help to choose excipients that will not interfere with the drug. Incompatibilities can arise from a covalent chemical reaction between the API and excipients, resulting in an intrinsic degradation of the API. Among the extrinsic factors, temperature is one of the most affecting compatibility[21].

Phenolic compounds, which have been extensively studied for their pharmacological properties, are found in the fruiting bodies of the mushroom *P. ostreatus*[22,23]. Therefore, total phenolic content was selected as a chemical marker of API in the formulations.

The compatibility results for the five drug-excipient mixtures regarding phenolic concentration at different temperatures are shown in Figure 1. Phenolic content at 30 ºC remained stable between 97.36% and 98.43% of the initial values during the 30-day test period. However, the concentration of phenolic compounds varied between 93 to 86% and 77 to 70% of the initial concentration at temperatures of 45ºC and 60ºC, respectively.

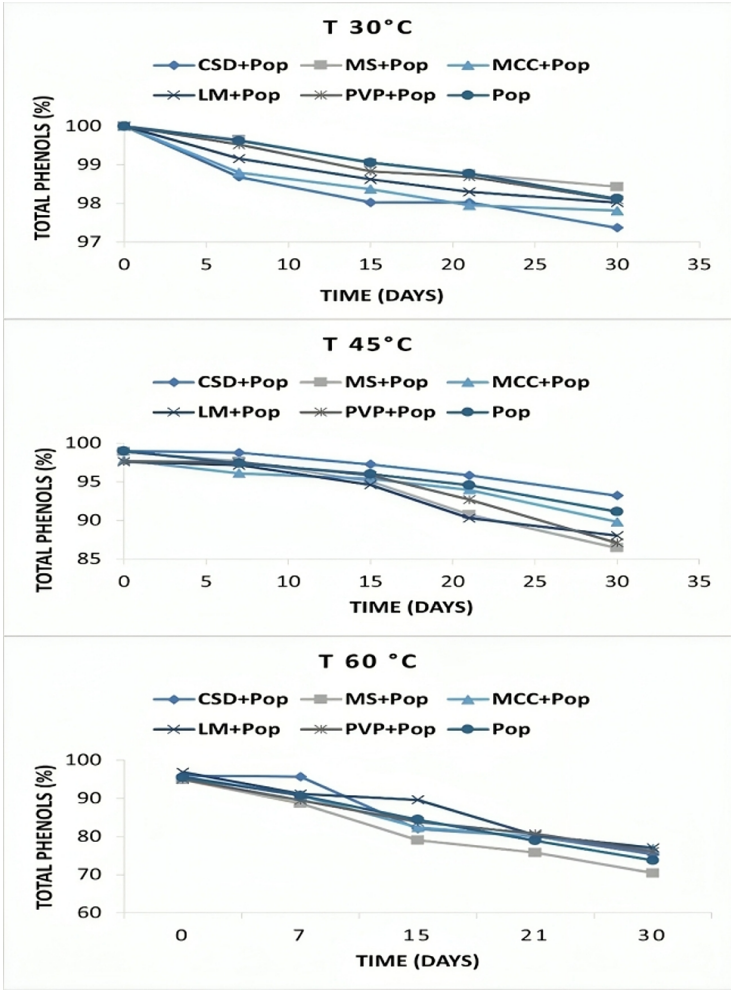


Figure 1. Concentration of total phenols at 30, 45 and 60 ºC for 30 days

Legend: *P. ostreatus* powder (Pop), colloidal silicon dioxide (CSD), magnesium stearate (MS), Microcrystalline Cellulose (MCC), Lactose Monohydrate (LM), Polyvinylpyrrolidone (PVP)

The decrease in the concentration of the bioactive compounds selected as chemical markers could be related to oxidation processes of phenolics. Though, it is important to note that the decrease in the concentrations was not significant when the mixtures were subjected to changing temperatures. Presumably, the decrease observed in phenol concentration may be due to oxidation reactions of phenolics rather than the presence of any other excipient, which should be confirmed using advanced analytical techniques such as HPLC or FTIR. However, the impact of excipients on the long-term stability of phenolic

compounds has been demonstrated. The carboxyl groups present in MCC and PVP allow the formation of esters, influenced by temperature and moisture. These esters can undergo hydrolysis, leading to a decrease in the concentration of phenolic compounds. This is supported by the fact that the process also occurred with powders treated individually at the same temperatures.

Rodriguez et al.[24], when evaluating the possible interactions between excipients and *Tamarindus indica* Soft Extract as the active ingredient, reported that the low differences in polyphenol content could be influenced by the sensitivity of the analytical method used in the determination.

Table 2 shows the results of the 15 runs according to the model describing the combination between different levels of the independent factors (MCC, LM and PVP). The experimental matrix showed the different combinations resulting from the D-optimal mixing design. The quality of each excipient combination was evaluated by considering the effect on rheological properties: Carr's index (CI), Hausner's ratio (HR), Flow rate (FR) and Angle of repose (AR).

Table 2. Experimental matrix of the mix design and results of the evaluation of the quality of the granules

Runs	Independent factors (%)			Response variables			
	MCC	LM	PVP	CI (%)	HR	FR (g cm^{-2} s^{-1})	AR (°)
1	1	0	1	20.26	1.25	6.16	34.68
2	0	1	1	10.25	1.1	8.77	24.98
3	1	0	1	17.96	1.2	6.25	34.94
4	1	1	0	17.86	1.22	6.99	32.98
5	0.83	0.58	0.58	17.35	1.21	6.46	34.03
6	0.5	0.5	1	10.12	1.1	8.77	25.3
7	0.5	1	0.5	20.89	1.26	6.93	31.46
8	0.33	0.83	0.83	12.82	1.14	8.36	25.47
9	1	1	0	17.8	1.22	7.03	33.02
10	0.58	0.83	0.58	20.9	1.26	6.65	31.12
11	0.83	0.83	0.33	20.08	1.25	6.3	33.86
12	1	0.5	0.5	20.76	1.25	6.86	33.94
13	0	1	1	10.27	1.11	8.76	25.16
14	0.83	0.33	0.83	20.02	1.25	6.84	33.11
15	0.5	0.5	1	15.42	1.19	7.96	28.42

In the statistical analysis, a quadratic model was suggested as the best model to analyse the flowability of the evaluated mixtures (Table 3) with p-values<0.05 in all the cases. Similarly, the lack of fit was negligible in all cases, thus indicating a low probability of error. The sum of squares and mean square ratio for the CI (10.71), HR (7.87), FR (19.81) and AR (26.12) showed values greater than 4.77 extracted from the Fisher-Snedecor law table at α=5% for (5.9) as degrees of freedom[25]. The coefficient of determination was 0.85, 0.81, 0.91 and 0.93 for CI, HR, FR and AR, respectively. The adjusted R-squared (R^2 -adj) for the evaluated responses were 0.97, 0.71, 0.87 and 0.89, respectively. Thus, there is evidence that all significant terms with values close to the R^2 values are part of the empirical models.

Table 3. Summary of Analysis of Variance (ANOVA) results for each response variable in the evaluation of granules flow properties in the optimisation process

	Carr's index (%)	**Hausner's ratio**	**Flow rate (g cm^{-2} s^{-1})**	**Angle of repose (°)**
Model	significant	significant	significant	significant
Coefficient of determination (R^2)	0.8561	0.8139	0.9167	0.9355
Adjusted coefficient of determination (adjusted R^2)	0.7762	0.7105	0.8704	0.8997
Coefficient of variation	11.48	2.68	4.81	3.95
Standard Deviation	1.93	0.03	0.35	1.22
Predicted residual sum of squares (PRESS)	83.73	0.02	3.44	35.53
Lack of fit	Not significant	Not significant	Not significant	Not significant
p-value	0.0014	0.0042	0.0001	<0.0001
F value	10.71	7.87	19.81	26.12

Figure 2a shows the influence of the evaluated excipients on the CI. The CI was favoured by the influence of PVP first, followed by LM. It was noticed that the higher the amount of these excipients, the lower the CI. This effect is largely due to the role of PVP as a binder in wet granulation process. In the case of LM, the fine particle size allows better mixing with other ingredients and helps to utilise the binder more efficiently[16]. The influence of these components and the interactions between them are also shown in the coded equation 2 for the CI.

The HR presented a similar pattern as the CI; this confirmed its relationship in predicting the flow properties of a powdery solid. The equation coded for the HR (3) showed a lower value for the influence of PVP and the interaction between LM and PVP in the evaluated granules as well as a negative coefficient for the interaction between MCC and LM. This result corroborated that LM and PVP were the excipients that most favoured the decrease in the HR, aided by MCC. The lowest values of the HR were obtained as the amount of PVP increased (Figure 2b)[18].

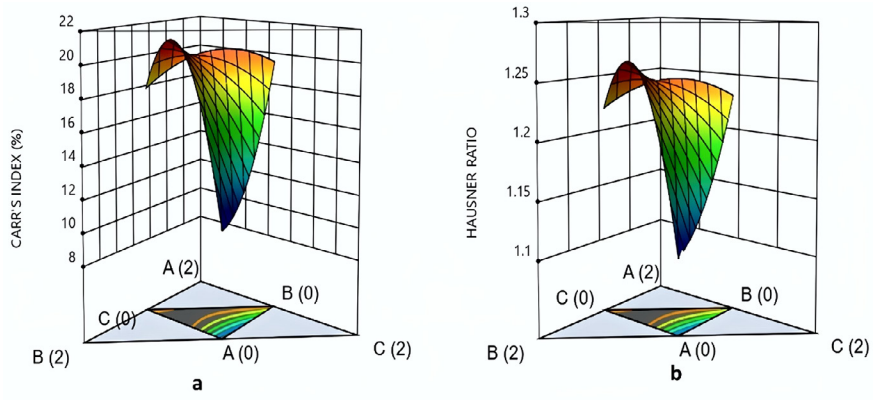


Figure 2. 3D diagram of the relationship between three variables, (*A*) MCC, (*B*) LM and (*C*) PVP, for the Carr's index (a) and Hausner's ratio (b) of granules.

For the FR (Figure 3a), the independent variable PVP showed the greatest influence, followed by MCC and LM (coded equation 4). In this case, the better the rheological properties of the granules and the rounder the shape of the particles composing them, the higher the values of FR.

$$HR = 1.14CM + 1.47LM + 0.56PVP - 0.35CMLM + 1.51CMPVP + 0.33LMPVP \quad (3)$$

The AR values obtained in the test runs allowed to classify the flow properties between excellent and passable (Figure 3b), probably due to that PVP, as a binder in the mixture, could be responsible for making the granules more spherical and homogenising the powder mass[18]. This implies that the lower values were favoured by increasing the amount of PVP (equation 5).

$$AR = 50.41MCC + 40.26LM + 0.57PVP - 48.91MCCLM + 37.79MCCPVP + 17.10LMPVP \quad (5)$$

The Design Expert software allowed the exploration of the experimental regions and the delimitation of the points where the response variables evaluated had the most fitted values. The evaluation of the best possible formulation

among those obtained in the factorial design yielded the following results: minimum MCC and maximum LM and PVP, with a desirability of 0.719 (Figure 4), which is the formulation selected for the preparation of *P. ostreatus* granules.

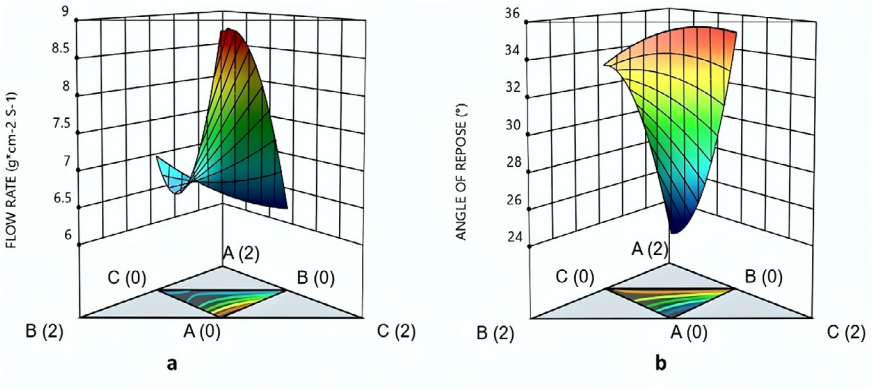


Figure 3. 3D diagram of the relationship between three variables, (*A*) MCC, (*B*) LM and (*C*) PVP, for the flow rate (a) and angle of repose (b) of granules

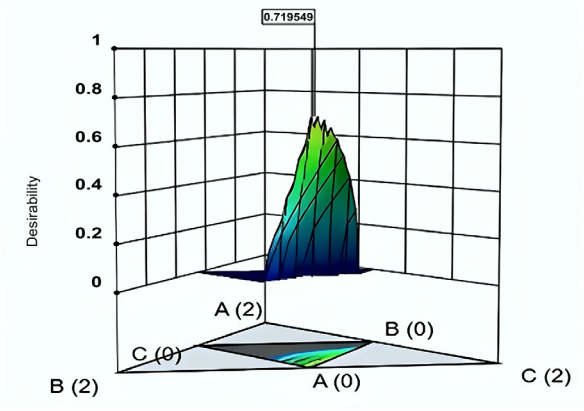


Figure 4. 3D diagram of the relationship between three variables, (*A*) MCC, (*B*) LM and (*C*) PVP, for desirability of granules

Quality of *Pleurotus ostreatus* granules

Moisture is a critical variable in the characterisation of materials such as powders and granules. There is a correlation between a higher amount of moisture absorbed or contained in the material and the increased difficulty in compression, mixing and other handling operations in the manufacture of pharmaceutical products, whether mechanical or manual, due to the increased cohesion forces between the particles of the material. According to the literature, the optimum residual moisture in pharmaceutical granules should be below 5%[26].

The results of the residual moisture in the three batches of *P. ostreatus* granules are shown in Table 4. A minimum value of 2.76% and a maximum value of 4.68% were obtained, and the differences between batches were no significant. The results obtained are within the range established for products of natural origin (maximum 10%)[24]. Taking into account that this product will act as an intermediate, the residual moisture values required are low. The moisture of a granule for use as an intermediate could not only affect the size of the granule, but could also exceed the desired size and lead to complications, such as, microbial contamination. Thus, the residual moisture in obtained granules was optimal and sufficient.

In this case, as the product is derived from the fruiting bodies of *P. ostreatus*, the drying process also increases the probability of degradation of the secondary metabolites responsible for the pharmacological action[27]. Moisture content is a critical property to consider when ensuring the shelf life and stability of nutraceutical/pharmaceutical products. An excess of water may result in physical or chemical instability resulting from microbial growth[28].

Table 4. Residual moisture of three batches of *P. ostreatus* granules

Batches	Residual moisture (%)
1	3.79 ± 0.76
2	3.39 ± 0.45
3	4.08 ± 0.44
F - Ratio	0.74
p-value	0.5158

Similarly, the efficiency of the drying process was confirmed by the moisture content of the granules, which ensures their microbiological safety. The link between the moisture content of natural products and the effectiveness of the drying process has been demonstrated in several studies[26,29].

The morphological characterisation of granules, as solid raw materials or intermediates in pharmaceutical formulations is critical, influencing many of their properties[30]. Figure 5 shows the results of scanning electron microscopy of *P. ostreatus* granules. Visible granules can be observed without dust particles, but with some degree of breakage (Figure 5A).

Figures 5B, 5C and 5D reveal the low surface porosity of the granules; this is an important parameter, as it affects the disintegration time, both of the granules as a solid dosage form and of tablets made from the intermediate product. For example, it can influence factors, such as, the flowability and dissolution rate of the product[31].

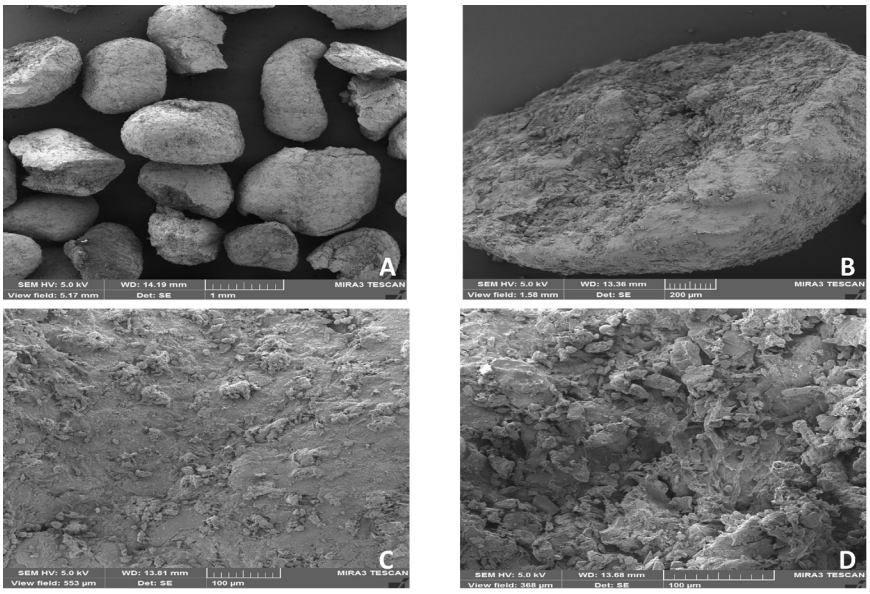
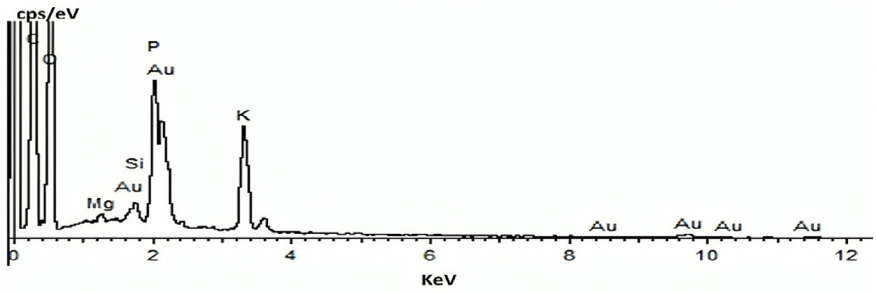


Figure 5. Scanning Electron Microscope Electronic images of *P. ostreatus* granules (A: 5.17 mm; B: 1.58 mm; C: 553 µm and D: 368 µm)

The composition of the granules was analysed by X-ray energy dispersive spectrometry (Figure 6). A certain amount of gold was found in the samples, which is the effect of sputtering with gold on the surface of the samples. With regard to the other constituents, no significant differences were observed in carbon and oxygen contents due to the different amounts of excipients (lactose monohydrate, magnesium stearate, polyvinylpyrrolidone and microcrystalline cellulose) used in granules preparation. The presence of silicon and magnesium is related to the concentrations of colloidal silicon dioxide and magnesium stearate. These agents are employed as moisture absorbers and flow enhancers, respectively, as well as lubricants. Potassium and phosphorus are derived from *P. ostreatus* mushroom biomass. Potassium was the main potential health element detected, followed by phosphorus[32]; mushroom powder could also contribute to magnesium content in granules.

The particle size of any material or substance has a direct effect on its rheological behaviour, modifies its properties, and increases or decreases its quality as a raw material or intermediate product for compression. In general, small particle sizes (<0.05 mm) have greater cohesiveness, which reduces their fluidity. However, with a smaller particle size, the surface area of the substance is increased and therefore, it would have greater solubility[33].



Element	Atomic number	series	App Conc	Intensity Corrn.	Weight %	Weight % Sigma	Atomic %
C	6	K-series	6.65	0.6288	47.71	0.56	56.75
O	8	K-series	5.09	0.5110	45.09	0.53	40.26
Mg	12	K-series	0.03	0.7968	0.16	0.04	0.09
Si	14	K-series	0.05	0.9257	0.25	0.04	0.13
P	15	K-series	0.88	1.3075	3.02	0.10	1.39
K	19	K-series	0.80	0.9627	3.76	0.09	1.38
Totals					100.00		100.00

Figure 6. X-ray diffraction analysis of the macro- and microelemental composition of *P. ostreatus* granules

An average particle size>0.1 μm was observed in the three batches of evaluated granules (Table 5). A particle size between 0.2 and 4 mm is commonly found in pharmaceutical granules. Small particle size can adversely affect flow characteristics[34]. In this case, the positive effect of the excipients used in the formulation of the granules, as a solid and/or intermediate dosage form for the production of capsules or tablets, can also be mentioned. When used as a filler, lactose monohydrate tends to increase particle size, but when combined with binders (e.g. polyvinylpyrrolidone), desiccants, and flow enhancers (e.g. colloidal silicon dioxide), and lubricants (e.g. magnesium stearate), it ensures further consolidation of granules[35]. These results are supported by the statistical analysis, which showed no significant differences among the three formulations.

Table 5. Mean particle size of *P. ostreatus* granules

Batches	Mean particle size (μm)
1	317.50 ± 14.03
2	326.18 ± 10.78
3	317.83 ± 5.09
F-Ratio	0.43
p-value	0.6702

The particle size was highest between the 350 and 177 μm sieves, with a distribution towards normality, proven by the Kolmogorov-Smirnov test (p-value of 0.53). These results corroborate those related to the particle shape, which showed that as the sphericity increased, the irregular particle shape and the roughness surface decreased[36].

Particle size distribution has a direct impact on the development and manufacture of dosage forms. It directly influences the efficacy, stability and safety of preparations[37].The drying method and type of raw material can both affect the particle size, and it is important to consider these factors to ensure optimal product quality during the processing, handling and storage of solid raw materials[29].

The results of the rheological evaluation of the granules of the three formulations studied are presented in Table 6. No statistical differences were observed in the formulations, which showed favourable flow and compressible behaviour, making them suitable for use in the production of solid dosage forms, as reported in the literature[21]. When a material is porous, it contacts more of the medium and is easier to disperse; therefore, less porous raw materials can cause problems when dispersing the product[38].

Table 6. Results of rheological properties of *P. ostreatus* granules

Batches	CI (%)	RH	FR (g cm^{-2} s^{-1})	AR (°)
1	11.20 ± 0.56	1.13 ± 0.01	8.72 ± 0.46	25.85 ± 1.08
2	11.23 ± 0.05	1.13 ± 0.00	8.21 ± 0.51	23.29 ± 2.75
3	10.45 ± 0.86	1.12 ± 0.01	8.76 ± 0.73	24.03 ± 0.76
p-value	0.3884	0.3906	0.6058	0.3888
F-Ratio	1.11	1.10	0.55	1.11

In this work, the composition of the particles influenced the results obtained for rheological properties. The cohesion between the particles lead to an adequate flow of the material, since a product composed of particles that tend to be spherical and without a high electrostatic charge between them and their walls, results in a material with adequate flow properties[30].

Particle size affects the flowability of powders derived from natural products, leading to problems with compaction, segregation and handling[39]. Juarez-Enriquez et al.[28] studied the structural changes in the matrix of pharmaceutical and food powders, resulting from water adsorption, particle agglomeration and powder caking, which are critical control points for improved flowability.

Varun and Ghoroi[40] reported that powdered solids are non-porous, and they tend to form spherical particles. On the other hand, Zolotov et al.[41] informed that solids derived from natural products have small and irregular particles, which can be reconstituted by the addition of excipients to fill the porous surface and ensure adequate fluidity. This is related with the fact that larger particle sizes result in lower cohesive forces between them.

Although the experimental conditions may affect the results obtained here, these tests allow the assessment of the appropriate use of selected excipients to improve the granulometric and rheological properties of the powdered solid from *P. ostreatus* through granule design. Successful use of lubricants can be indicated by a solid product with appropriate flow characteristics. These agents help to reduce cohesion and friction between particles[42].

Table 7 shows the total phenolic content in the three evaluated batches; no significant differences were found at 95% confidence level. Our previous studies reported that the total phenolic content of mushroom products is influenced by cultivation conditions and extraction solvents. These findings highlight the importance of the good cultivation practices and standardized extraction methods in the production of high-quality mushroom products. It is recommended to use solid-state fermentation for obtaining fruiting bodies under controlled cultivation conditions to ensure consistent and safe products for use as functional foods, nutraceuticals and bioactive compounds[4].

Table 7. Concentration of phenolic compounds in *P. ostreatus* granules

Batches	Total phenols (mg/100 g, d.w.)
1	34.52 ± 1.03
2	33.08 ± 1.01
3	34.23 ± 1.00
F-Ratio	1.69
p-value	0.2610

Aqueous extracts were prepared using water as the extraction solvent to quantify total phenolics in *P. ostreatus* granules. Compared to organic solvents, water was chosen because of its safety, economy and flexibility. Due to its polar nature, it is suitable for extracting compounds, such as polyphenols, which have a chemical structure based on aromatic rings substituted by hydroxyl groups. This structure facilitates their solubility in polar solvents, whether in free or conjugated form. This is particularly important for aqueous extraction of these compounds with applications in the development of functional foods, nutraceuticals and pharmaceutical formulations[43].

Beltrán et al.[44] reported the total phenolic content of *P. ostreatus* extracts obtained with solvents of different polarity, and the highest values were achieved with the most polar solvents (water and 50% ethanol) with values of 138.4 and 86.37 mg/100 g (d.w.), respectively. Peraza et al.[45] informed a total phenolic content of 73 mg/100 g in *P. ostreatus* extracts. The study also concluded that genetic differences between species and cultivation conditions, such as the use of commercial or wild substrates, could be responsible for the differences in phenolics concentration.

A number of phenolic compounds with anti-diabetic properties have been identified, including anthocyanins, ellagitannin, luteolin, rosmarinic acid, catechin, resveratrol, rutin, quercetin, diosmetin and myricetin. Therefore, phenolic compounds may be a promising therapeutic option for the treatment of type II Diabetes Mellitus[46].

The HPLC profile of the phenolic compounds identified in the aqueous extracts of mushroom granules as a fingerprint and quality criteria is shown in Figure 7. The five main peaks identified correspond to gallic acid, homogentisic acid, chlorogenic acid, caffeic acid and naringin. The differences in the structure of the phenolic compounds might have influenced the biological activities. For example, the antioxidant activity of the compound structure was reported to be dependent on the number and distribution (*ortho* and *para* positions) of the active group (OH)[47]. Therefore, correlation analysis between phenolic compounds and the potential anti-diabetic activities are worthwhile to be studied further.

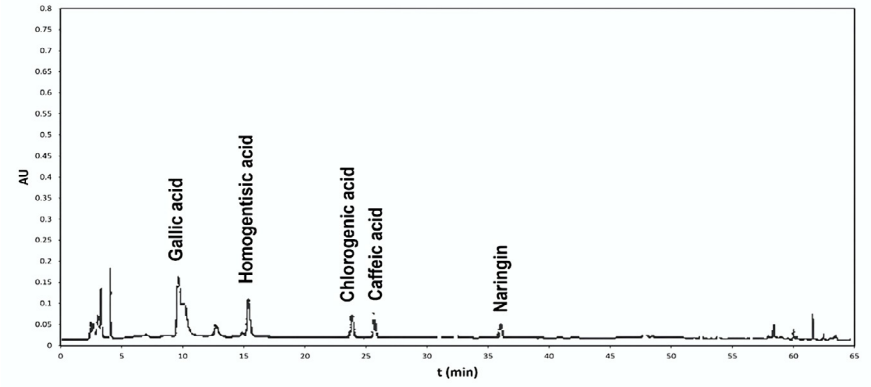


Figure 7. HPLC profile of the aqueous extract derived from P. *ostreatus* mushroom granules used as a fingerprint and quality criteria.

It is important to note that in a shelf stability study of granules from *P. ostreatus* mushroom, conducted over a 12-month period[48], no significant differences in the total phenolic content were shown up to the first six months, and a decreasing of only 5% was observed at the end of the experiment. On the other hand, the HPLC profile showed a similar pattern during the overall experimental time.

Contamination by microorganisms can alter the physical, chemical and therapeutic properties of a medicinal product and transform active ingredients into toxic substances[49]. Microbiological evaluation of *P. ostreatus* granules indicates the absence of pathogenic microbes which may hinder the use of granules in the development of nutraceuticals (Table 8).

Proper dehydration is crucial to prevent the growth of filamentous fungi and yeasts, which can grow in products with moisture levels below 50%. To ensure that the product is safe for human consumption, it is important to maintain low levels of bacteria, fungi and yeasts. It is also important to prevent the presence of harmful microorganisms such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterobacteriaceae* and *Candida albicans*[50].

The *in vitro* activation of the microbial autolytic system of the microorganisms tested was reported in a hot water extract of *Pleurotus sp*. mycelia[4]. In addition to mycelia, the antibacterial potential of *P. ostreatus* fruiting bodies was informed by Gutef et al.[51].

Table 8. Microbiological counts in *Pleurotus ostreatus* granules

TESTS	RESULT	LIMITS[20]
Total bacterial count	≤10 cfu/mL	$\leq 10^3$ cfu/mL
Total count of fungi and yeasts	≤10 cfu/mL	$\leq 10^2$ cfu/mL
Pseudomonas aeruginosa	Absence	Absence
Sthaphylococcus aureus	Absence	Absence
Enterobacteriae	Absence	Absence
Candida albicans	Absence	Absence

Recent studies have evaluated the phytochemical composition and *in vitro* antimicrobial activity of wild *P. ostreatus*. Results showed its antimicrobial effects against all tested microorganisms, and the aqueous extracts were more effective than methanol extracts, with different degrees of inhibition against both bacteria and fungi, including *Staphylococcus sp*., *Escherichia coli*, yeasts and moulds. The reported antimicrobial activity could be related to the presence of phenolic compounds, among other metabolites[7].

In the shelf stability study of granules from *P. ostreatus* mushroom, conducted over a 12-month period[48], no significant changes were found throughout the research period in the moisture content (2.8 and 4.7%) ($p<0.05$). Moreover, the microbiological analysis did not indicate the presence of pathogenic microorganisms.

Within the scope of this study, the potential of *P. ostreatus* for developing solid dosage forms with applications in food and pharmaceutical industries for human and veterinary applications, was demonstrated by obtaining mushroom granules with good flow and compressibility properties as well as appropriate chemical and microbiological quality.

Further *in vitro* and/or *in vivo* studies are needed to validate the anti-diabetic potential of the granules. Preliminary studies with the water-extract obtained from mushroom granules showed good activity in the α-glucosidase and α-amylase inhibition assays, as well as, in molecular docking studies (data not shown, VLIR – UOS Project CU 2019-2024 IUC 030A105). This approach would contribute to the diversification of mushroom commercial products with increased efficacy, stability and quality.

STATEMENT OF ETHICS

This study does not require any ethical permission.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication and dissemination of the information provided herein.

AUTHOR CONTRIBUTIONS

Conceptualization/ Design: HJ Morris, I Chil, P Cos; Acquisition of data: D Arias-Ramos, JA Rojas, Y Beltrán, Y Lebeque; Analysis of data/ Software: HJ Morris, I Chil, D Arias-Ramos, JA Rojas, Y Beltrán; Writing –Original Draft Preparation: D Arias-Ramos, I Chil, JA Rojas; Supervision: HJ Morris, P Cos; Financial support/ Project: HJ Morris, Y Lebeque, P Cos; Review & Editing: HJ Moris, I Chil.

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