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RESEARCH ARTICLE

Synthesis and Characterization of Phytoandrogen from Different Species of Pine Trees and its Potential Use for Aquaculture

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ABSTRACT

Pine pollen is an example of phytoandrogen that has been utilized as a feed additive for all animals worldwide and used as an alternative to hormone replacement therapy that can safely restore healthy testosterone levels in men. Previous on-farm trials demonstrated the effectiveness of Benguet pine pollen (*Pinus kesiya*) as an alternative hormone source for the sex redirection of tilapia. The study characterized phytoandrogen four types of pine pollen, coded as PPL1 (Benguet 1), PPL2 (Benguet 2), PPC1 (commercially available in cracked form), and PPC2 (commercially available in whole form). The characterization showed the differences in morphology and particle size in micrometer scale under the scanning electron microscopy (SEM) analysis. Meanwhile, Energy dispersive X-ray (EDX) analysis determined that the Benguet and commercial pine pollen contains carbon, oxygen, nitrogen, and potassium. All samples have strong elemental bonding based on FTIR analysis. Pine pollen with cracked cell walls has the smallest particle, around 35.48 μm, and it was significantly different among all other samples at $p<0.05$. The particle size of characterized pine pollen ranged from 35.48±0.87 to 46.72±3.67 μm, which can be used for an efficient drug delivery system. Additionally, a stable particle was produced after intercalation was applied. Moreover, the study found that all pine pollen samples were good sources of phytoandrogen levels 1.25×10^6, comparable to testosterone; this can be a good replacement for 17 α -MT in aquaculture, making it sustainable and safe for the environment.

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1. INTRODUCTION

Aquaculture is the production of fish and other aquatic products in all types of water environments (NOAA 2023). It aims to produce fish sustainably to eliminate the negative impacts to wild stocks in any culture environment (Logan 2010). However, artificial reproduction and sex reversal in aquaculture are commonly administered through the use of synthetic hormones, which has been under public criticism due to its harmful effects on the environment and human health, as well as facing issues with legal regulations in some countries (Hoga et al. 2018). Due to the adverse effects of synthetic hormones, the use of plant materials has been investigated (Tadese et al. 2022). Different parts (e.g., leaf, flower, and rhizome) and forms (e.g., crude, extract, and active ingredient) of plants were observed as safe and eco-friendly substances to regulate immune status, improve growth performance, prevent the occurrences of fish diseases, and sex reversal (Baluran et al. 2018; Tadese et al. 2022). As Chinese traditional medicine, pine pollen has been utilized as a feed additive for all animals worldwide (Nian et al. 2017) as it carries high levels of steroid-like hormones where brassinosteroids are found to be the most potent of them (Dinan et al. 2001).

Plant hormones, particularly phytoandrogen, are not bio-identical to the animal hormone, which have been used for food and serves as tonic and adaptogen for adult persons (Karslı 2021; Velasco et al. 2018). Additionally, phytoandrogen is found to increase muscle mass and strengthen bones (Karslı 2021). However, little is known about the use of pine pollen in aquaculture (Baluran et al. 2018).

In 2012, the Freshwater Aquaculture Center at Central Luzon State University conducted its first research study on phytoandrogen-containing phytohormones. These are plant hormones but not bio-identical to animal ones (Darvin 2012). Earlier studies on phytochemical screening, thin-layer chromatography, and gas chromatography-mass spectrometry showed that pine pollen contains phytoandrogen, a hormone found in plants comparable to testosterone (Velasco et al. 2018) that when used as a sex-redirection agent, the sex distribution is more inclined to the male group. The complete inversion is best observed within 28 days of the culture period. (Velasco et al., unpublished). The study was limited to the characterization of local and commercially available pine pollen. Correspondingly, this study has been executed to further characterize the phytoandrogen from local and commercial pine pollen in terms of surface morphology, particle size, and chemical properties and to identify its potential use for aquaculture.

2. MATERIALS AND METHODS

2.1 Collection of samples

The two local pine pollen samples, PPL1 and PPL2, were collected from male catkins of pine trees in Baguio City, Benguet, Philippines. These samples were analyzed to determine differences between the two locally available pine pollen samples. Collected samples were air-dried, segregated, and vacuum-sealed until used to preserve their natural chemical components. On the other hand, two commercially available pine pollen samples, PPC1 and PPC2, were imported from Yunnan Province, China. The PPC1 was whole pollen, while PPC2 was broken pollen.

2.2 Characterization of phytoandrogen

The local and commercially sourced phytoandrogen were characterized. Physical and chemical properties such as composition, structure size, morphology, surface area, and functional groups were determined using Energy dispersive x-ray (EDX) analysis, scanning electron microscopy (SEM) analysis, and Fourier transform infrared spectroscopy (FTIR), respectively.

2.2.1 Scanning electron microscopy (SEM)

The SEM evaluates the surface of a particular material with high-energy electron beams (Titus et al. 2019). SEM uses light waves to generate a magnified image that differentiates it from ordinary light microscopes (Titus et al. 2019). SEM analysis was done by coating the pine pollen with Au-Pd using Ion Sputter (E1010, Hitachi, Japan). SEM images were taken at a voltage of 5 kV under magnifications 100x and 500x for PPC1, PPC2, PPL1, and PPL2. Meanwhile, intercalated pine pollen was observed at 15 kV under magnifications 1,000x and 10,000x. Higher magnifications were used for intercalated pine pollen due to its smaller sizes.

2.2.2 Energy-dispersive X-ray (EDX)

Surface compositions of samples were also investigated through Energy-dispersive X-ray spectroscopy (EDX) with Hitachi Flex SEM 1000 Oxford EDX System. EDX is connected to electron microscopy and utilized with the use of X-ray (Scimeca et al. 2018). EDX is a significant tool in element determination, endogenous or exogenous, in the tissue, cell, or several types of samples (Scimeca et al. 2018). EDX was used to evaluate and determine the elemental composition of all pine pollen samples (PPC1, PPC2, PPL1, and PPL2).

2.2.3 Particle size analysis

For particle size analysis, the images generated in SEM were used. A combination of the SEM and the Image J software (version 1.53t) was used to measure the size of PPC1, PPC2, PPL1, and PPL2.

The chemical structure and presence of functional groups of the porous particles were investigated using Fourier transform infrared analysis (FTIR Horizon MB300). This analysis is a widely used analytical tool in evaluating a broad spectrum of materials (Haas and Mizaikoff 2016), such as pure substances, mixtures, impurities (Kumar Pandey et al. 2016), and compositions of substances (Ghica et al. 2019), particularly for characterization of unknown materials (Kowalczuk and Pitucha 2019). Besides, this technique can characterize polymeric and biopolymeric materials (Escobar Barrios et al. 2012), making it a valuable tool in screening applications (Kowalczuk and Pitucha 2019). In this method, the dried sample was placed in a sample holder, and the spectra were recorded between 4000 cm^{-1} to 450 cm^{-1}.

2.3 Intercalation of pine pollen

To improve pine pollen samples, cracked and whole pine pollen underwent an intercalation process to reduce the size further and determine the stability of pine pollen particles after size reduction. Intercalation is a process that is found to be essential for the preparation of an improved barrier property and the ability of polymer composites to hold other substances (Boo et al. 2007). Ion exchange intercalation was used due to its wide range of available intercalants for synthesizing compounds (Zhou et al. 2021).

Intercalation was done following the modified method of Pagtalunan et al. (2018), wherein the process was achieved by creating two mixtures. The first mixture comprised 50 g of pine pollen and 1,000 mL of hot distilled water, while the second mixture comprised 1.0 g of 12-aminolauric acid, 40 mL of hydrochloric acid, and 200 mL of hot distilled water. The second mixture was then added to the first mixture with continued stirring for 24 hours. After stirring, the mixture was filtered using filter paper (Wattman no. 1, 10–500 mm mesh). Filtered pine pollen was oven-dried at 100°C for 3 hours. The dried sample was ground and sent to a laboratory for analysis.

2.4 Data and statistical analysis

The data on the particle size of phytoandrogen (pine pollen) were subjected to a one-way analysis of variance (ANOVA). Further analysis was conducted using Post Hoc, particularly Tukey's Honest Significant Difference test, to assess significant differences in particle size. The data were the means of the measurement and its corresponding standard error.

3. RESULTS

3.1 Scanning electron microscopy (SEM)

The appearance of PPC1 was flat-shaped (Figure 1), while other samples, such as the PPC2 (Figure 2), PPL1 (Figure 3), and PPL2 (Figure 4) showed similar appearance and surface texture, are

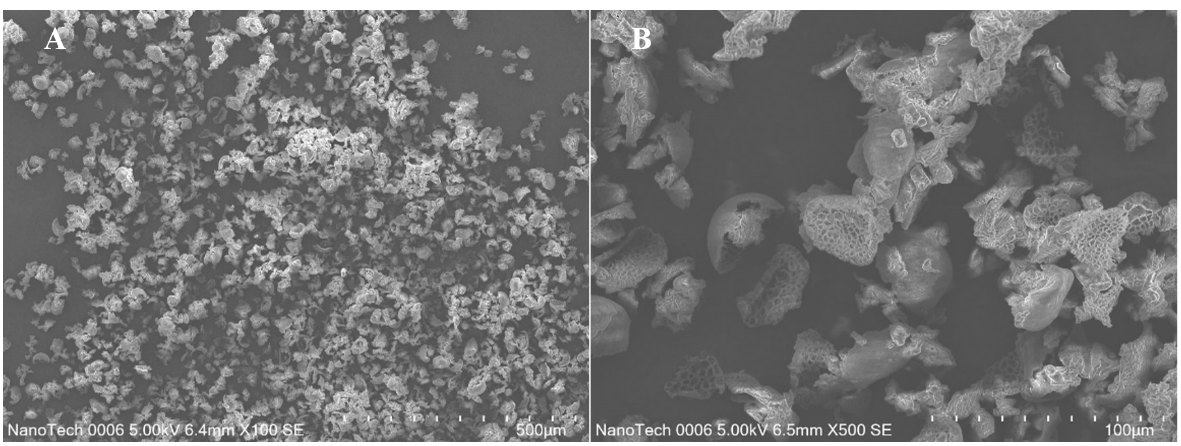

Figure 1. Image of commercial pine pollen 1 in SEM at magnifications (a) 100x and (b) 500x.

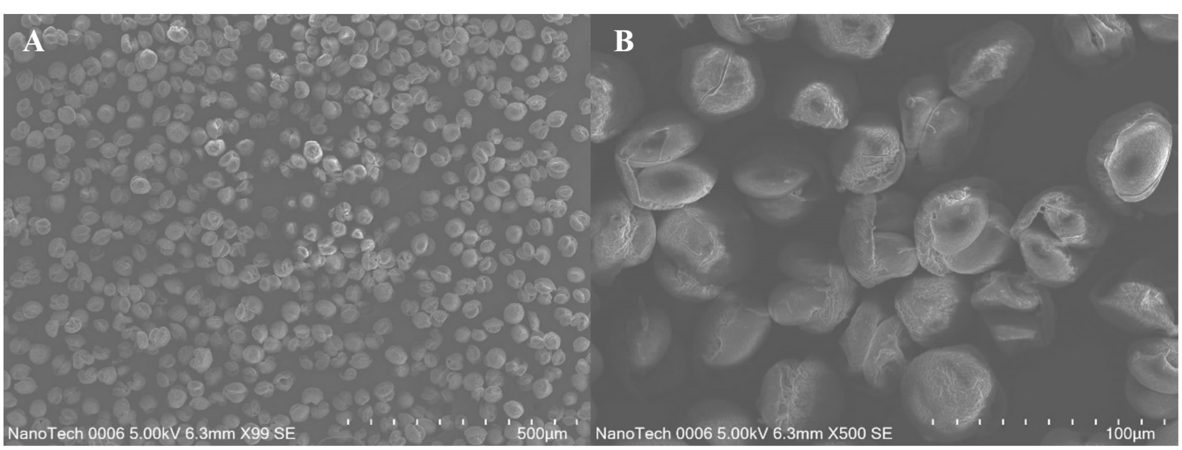

Figure 2. Image of commercial pine pollen 2 in SEM at magnifications (a) 100x and (b) 500x.

bean-shaped, indicating the whole appearance of the pine pollen. Moreover, smooth surface texture was observed in the intercalated samples (Figure 5).

3.2 Particle size analysis

The particle size of the different pine pollen samples PPL1, PPL2, PPC1, and PPC2 were recorded at 35.48 ± 0.87 µm (PPL1), 46.72 ± 3.67 µm (PPL2), 41.08 ± 1.24 (PPC1), and 43.74 ± 0.89 (PPC2) (Table 1), respectively. The smallest particle was observed in the PPC1 (35.48 µm), and the largest is the PPC2 (46.72 µm). There are significant differences ($p<0.05$) in the particle size of PPC1 compared to the rest of the pine pollen samples (Table 1).

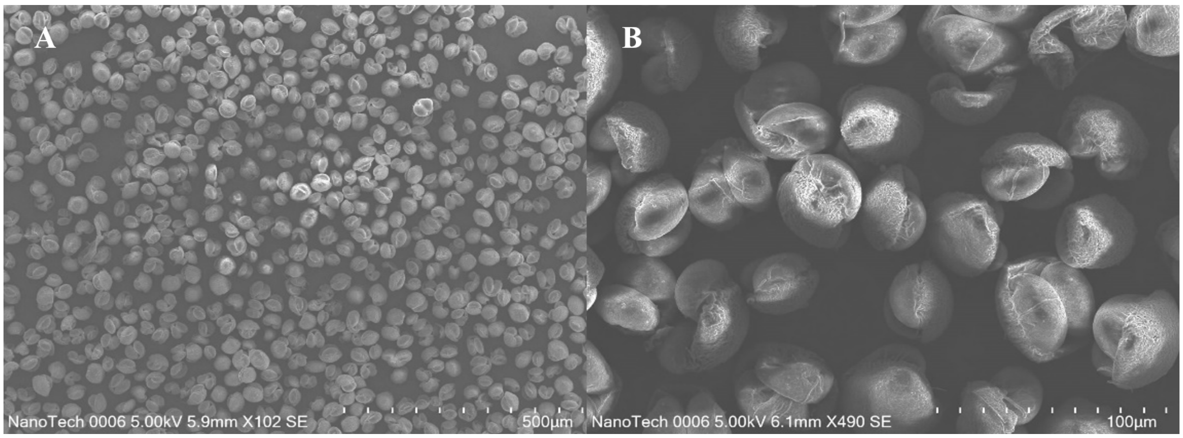

Figure 3. Image of local pine pollen 1 in SEM at magnifications (a) 100x and (b) 500x.

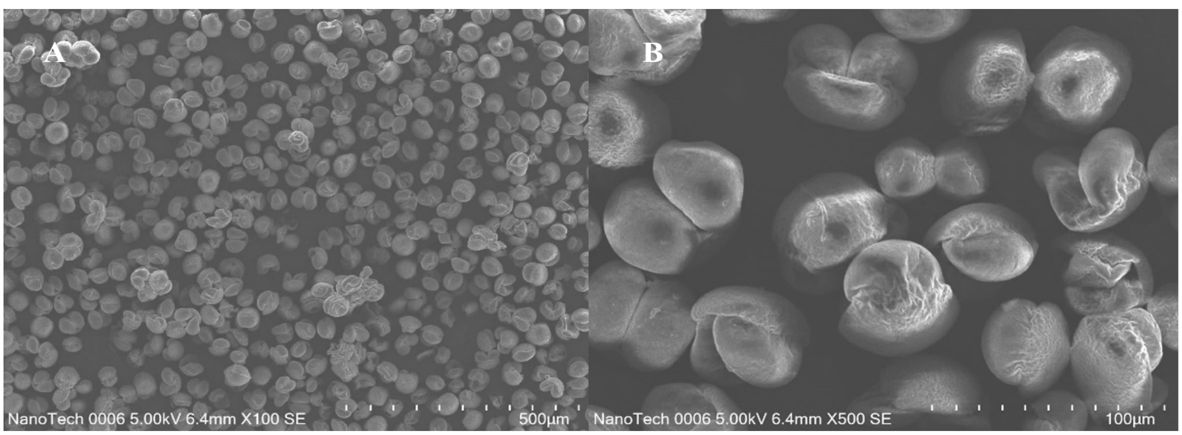

Figure 4. Image of local pine pollen 2 in SEM at magnifications (a) 100x and (b) 500x.

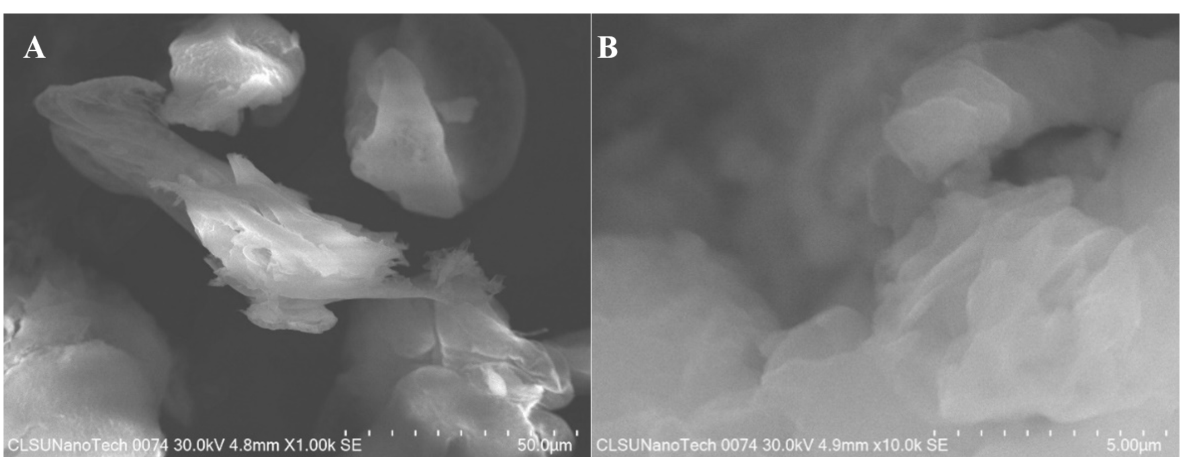

Figure 5. SEM analysis of intercalated pine pollen at magnifications 1,000x and 10,000x.

Table 1. The particle size of the different pine pollen samples.

Pine Pollen	Size (μm)
PPC1 (Commercial 1)	35.48 ± 0.87[c]
PPC2(Commercial 2)	46.72 ± 3.67[a]
PPL 1 (Local 1)	41.08 ± 1.24[b]
PPL2 (Local 2)	43.74 ± 0.89 [ab]

Note: Mean (± SD; n = 4) with different superscripts show significant differences using independent sample ANOVA at $p<0.05$.

3.3 Energy-dispersive X-ray (EDX)

Based on the EDX, the different elements found in the samples were carbon, oxygen, nitrogen, and potassium (Table 2), wherein the highest concentration of carbon was found in PPL1 (67.60%), followed by PPC2 (65.12%), PPC1 (63.16%) and the lowest was in PPL2 (62.92%). Oxygen concentrations of PPC1, PPC2, PPL1, and PPL2 are 33.13%, 32.74%, 29.31%, and 33.69%, respectively. The highest concentration of oxygen was found in PPL2 (33.69%), followed by PPC1 (33.13 %), PPC2 (32.74 %), and PPL1 (29.31 %) being the lowest. The nitrogen content of pine pollen samples was 2.92% and 1.84% in PPC1 and PPC2, while 2.30% and 2.95% in PPL1 and PPL2, respectively. The highest nitrogen concentration was found in PPL2 (2.95 %), followed by PPC1 (2.92 %), PPL1 (2.30 %), and lowest in PPC2 (1.84 %). The highest potassium contents were found in PPC1 (0.79 %) and PPL1 (0.79 %), followed by PPL2 (0.45 %) and lowest in PPC2 (0.3 %).

Table 2. Percentage of different elemental compositions of pine pollen samples found on Energy-dispersive X-rays (EDX).

Element	Atomic No.	Mass Norm. (%)	Atom (%)	abs. error (%) (1 sigma)	rel. error (%) (1 sigma)
Commercial Pine Pollen 1					
C	6	63.16	69.58	7.04	11.14
O	8	33.13	27.4	4.07	12.3
N	7	2.92	2.75	0.69	23.63
K	19	0.79	0.27	0.05	6.81
		100	100		
Commercial Pine Pollen 2					
C	**6**	65.12	71.28	7.21	11.08
O	**8**	32.74	26.9	4	12.22
N	**7**	1.84	1.72	0.5	27
K	**19**	0.3	0.1	0.04	12.61
		100	100		
Pine Pollen from Local Source 1					
C	**6**	67.6	73.63	7.52	11.13
O	**8**	29.31	23.96	3.69	12.6
N	**7**	2.3	2.15	0.61	26.53
K	**19**	0.79	0.26	0.05	6.87
		100	100		
Pine Pollen from Local Source 2					
C	6	62.92	69.24	6.98	11.09
O	8	33.69	27.83	4.1	12.16
N	7	2.95	2.78	0.67	22.85
K	19	0.45	0.15	0.04	9.58
		100	100		

3.4 Fourier transform infrared spectroscopy (FTIR)

Based on FTIR analysis, pollen samples have similar vibrational peaks and functional groups (Figure 6 and Table 3). N-H stretching was assigned at 3,297 cm^{-1}, and C-H stretching was assigned at 2923–2853 cm^{-1}. Both N-H and C-H stretching have a medium peak appearance, indicating the strength of their hydrogen bond. The C=C stretching (conjugated alkene) was assigned at 1606 cm^{-1}. Recorded N-O stretching was found in 1516 cm^{-1}. Moreover, at 1439–1241 cm^{-1}, O-H bending was observed. S=O stretching was at 1038 cm^{-1}. While C=C bending is attributed at the peak of 832 cm^{-1}. All these vibrational peaks have been found in all PPL1, PPL2, PPC1, and PPC2.

3.5 Zeta potential analysis

Zeta potential analysis resulted in values approximately between -54.5 to -52.6 mV for the cracked pine pollen and -55.6 to 50.6 mV for the whole pine pollen; this indicates a highly stable particle system (Figure 5). Furthermore, particles with less than -30 mV zeta potential values are strongly anionic (Bhattacharjee 2016). The intercalation was successful as it was able to synthesize the larger pine pollen into smaller and more stable particles ranging from 46.77 µm down to 35.48 µm.

4. DISCUSSION

The study was able to characterize pine pollen. SEM analysis and particle size analysis identified that the smaller particle size of PPC1 and its significant difference was due to its broken core and cell wall compared to PPL1, PPL2, and PPC2, which have not undergone grounding and size reduction process. Pawlik and Ficek (2023) found that the range of pine pollen grains ranges from 1.25 to 250 µm, which corresponds to the study's findings wherein the sizes fall within the range of 35.48–46.72 µm.

EDX results in the study showed the presence of different elements of the pine pollen containing carbon, oxygen, nitrogen, and potassium, which are usually found in organic materials, including the pine pollen, as reported by Yao et al. (2020) and Filipiak (2016). The concentration of carbon was the highest, which is in support of the previous study (Pawlik and Ficek 2023) wherein they found that the investigated pine pollen was a good source of carbon as it contains

Table 3. Infrared (IR) vibrations and functional group assignments of all the pine pollen samples.

Vibrational Frequency (cm^{-1})	Functional Groups
3297	N-H Stretching
2923-2853	C-H Stretching
1606	C=C Stretching
1516	N-O Stretching
1439-1241	O-H Bending
1038	S=O Stretching
832	C=C bending

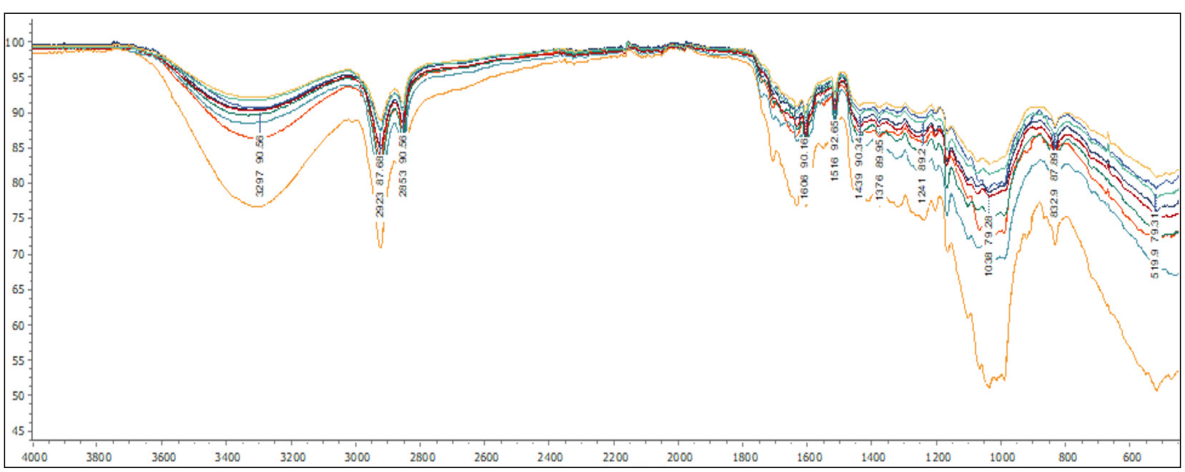

Figure 6. Fourier Transform Infrared (FTIR) bands of all pine pollen samples.

47.66% to 50.79% C, which is the highest among the present elements. The range of C found by Pawlik and Ficek (2023) was lower (47.66% to 50.79%) than the present study's findings (62.92% to 67.60%). High concentrations of carbon can be utilized in the marine environment as a water enhancer (Kerienė et al. 2023; Zhang et al. 2018). Furthermore, nitrogen concentrations found by Pawlik and Ficek (2023) (2.00% to 2.50%) were within the range of the current study (1.84% to 2.95%). Among these elemental compositions, potassium was found to be the lowest, which supports the previous findings of Cheng et al. (2023) and Sardans and Peñuelas (2021). The elemental composition available in pine pollen could have a major impact on the macro- and microelements of a body of water if added as fertilizer that could increase the productivity of ponds (Filipiak 2016; Pawlik and Ficek 2023; Rösel et al. 2012).

Based on the FTIR analysis of PPL1, PPL2, PPC1, and PPC2, the C-H stretching corresponds to the presence of alkane compounds (Merck 2023), a functional group that can also be found in testosterone that is used for sex redirection of fish. N-H and C-H stretching indicate the strength of hydrogen bonds (Hansen et al. 2021). The C=C bond showed less stiff bonds with lower stretching frequencies (Tzvetkov et al. 2017; Swiatly-Blaszkiewicz et al. 2021), whereas the N-O stretching indicates strong bands of nitroalkenes (Orgchemboulder 2023). According to Yu et al. (2020), the O-H stretching represents alcohols present in organic compounds, the S-O stretching denotes a strong peak of sulfoxide compounds (Unitechlink 2022), and the C=C bending represents the alkene group present in the samples (Tzvetkov et al. 2017).

Several investigations have been carried out about the potential application of pine pollen in aquaculture. According to Baluran et al. (2018), this plant material has 1.25×10^6 level of testosterone, or about 0.8 μg of testosterone in 10 g of raw pollen according to Luo and Seladi-Schulman (2019). This makes it a good source of phytoandrogen for aquaculture.

The efficacy of Benguet pine pollen (*Pinus kesiya*) in sex redirection of Nile tilapia was studied in a farm setup and found that it can generate 88% male percentage and was comparable to the effects of 17-α-methyltestosterone (Nieves 2017). Pine pollen contains phytoandrogen that can serve as an alternative hormone for the sex redirection of tilapias (Nieves 2017). In another study by Nian et al. (2017), the phytoandrogen exhibited a high sex conversion of 81.0% and 89.1% from fish fed with pine pollen supplemented diets. Additionally, Baluran et al. (2018) investigated the effects of pine pollen in the body of Nile tilapia, and it was found that it can be used as an immunostimulant to improve fish health status, particularly in controlling and combating pathogenic bacterial diseases in Nile tilapia. Likewise, Abaho et al. (2022) revealed that pine pollen enhances growth performance, altered sex ratio, and masculinized Nile tilapia (*O. niloticus*) at concentrations of 1.28 g kg-1. These abilities of pine pollen may be associated with its components, such as some nutrients like proteins, amino acids, sugars, fats, and minerals, as well as its vitamins, phospholipids, and enzymes, making it known as a "natural micro-nutrient bank" (Cheng et al. 2021a; Cheng et al. 2021b). These nutrients and vitamins can deliver essential support for normal functioning and metabolic regulation of the body of fish (Cheng et al. 2021a; Cheng et al. 2021b). However, the different chemical and nutritional compositions of pine pollen may be affected by several factors, such as source, cultivar, and post-harvest processing (Cheng et al. 2021a; Cheng et al. 2021b).

5. CONCLUSION

The study's results revealed the potential use of pine pollen in aquaculture, specifically in sex redirection process. Pine pollen is a good source of phytoandrogen and one of the organic plant materials that can be used in the sex redirection of Nile tilapia. Phytoandrogen can potentially replace the use of 17α-methyltestosterone, which is under public criticism due to its negative impact on the environment and human health. Using pine pollen instead of 17 α-MT in aquaculture can potentially reduce or eliminate the adverse impact of synthetic hormones in the environment, thus enhancing fish growth and providing a sustainable and safe approach to aquaculture. In conclusion, based on the characterized commercially and locally sourced pine pollen, this can be used for aquaculture in increasing pond productivity, enhancing fish growth, sex redirection process, enhancing waters, and other potential applications for aquaculture.

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AUTHOR CONTRIBUTIONS

Gagelonia JD: Methodology, Visualization, Investigation, Writing – Original draft preparation, Writing – Reviewing & Editing. **Suyom JCP:** Methodology, Data curation, Software, Investigation, Formal analysis. **Monserate JJ:** Methodology, Supervision, Resources. **Velasco RR:** Conceptualization, Methodology, Validation, Investigation, Resources, Data curation, Writing – Original Draft, Writing – Reviewing & Editing, Visualization, Supervision, Project administration, Funding acquisition.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

No animal or human studies were carried out by the authors.

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