IJBPAS, June, 2018, 7(6): 1121-1132

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

BENGUET PINE POLLEN (*Pinus kesiya*) AS NATURAL SOURCE OF PHYTOANDROGEN

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Received 28th Feb. 2018; Revised 20th March 2018; Accepted 25th March 2018; Available online 1st June 2018

DOI: https://doi.org/10.31032/IJBPAS/2018/7.6.4472

ABSTRACT

The result of the qualitative (colorimetric) phytochemical screening of pine tree indicated the presence of tannins (group of phenols), alkaloids (nitrogen containing compounds), steroids (groups of cholesterol, estrogen and testosterone), flavonoids (hydroxyl group), saponins (glycosidic/sugar compounds) and cardiac glycosides (secondary metabolites). All parts of pine tree (pine pollen, bark, twigs, roots, needles, branches) were tested positive for unsaturated steroids/sterols. The quantitative analysis of the pine pollen extract was performed using Gas chromatographic mass spectrometry. The compounds present in pine pollen were tricyclo (C_9H_{12}), pinene/careen/santolinatrienebicyclo (C10H16). These compounds are called terpenes, a hydrocarbon group. The testosterone content of pine pollen with an abundance of 1.25 x 10^6 was determined at 14 minutes. This testosterone content was confirmed when a standard 17- alpha methyltestosterone was also subjected to LCMS. Thin Layer Chromatography was also used to separate organic compounds. Under an ultra violet lamp, a change in the color of the yellow or brownish yellow spots in the developed chromatogram to red, violet, green or purple indicates the presence of phytochemicals.

Keywords: *Pinus kesiya*, colorimetric, phytoandrogen, steroids, LC50

INTRODUCTION

Pines are mostly large trees, generally long-lived over 100 years in suitable environments. *Pinus kesiya* of the family Pinaceae are common in northern Philippines, particularly in highland regions with an altitude of approximately 1,500 meters that provides optimum conditions for its growth [1]. It is commonly found in elevated areas of Baguio City, the mountain province, Zambales and Mindoro.

All pine species are evergreen, they keep their leaves for at least two growing seasons and up to about 30 years. Its narrow leaves or "needles" are arranged in bundles of two to five (2-5) and with a deciduous sheath at their bases. These bundles of needles are called fascicles [2]. They are monoecious, having both female (megasporangiate) cones which bear the ovules and male (microsporangiate) cones which shed the pollen. The pollen is carried by wind and gravity and none of the pines is pollinated by insects or birds.

The pollen of pine trees has been used for millennia in China and Korea as both food and a particularly powerful tonic and adaptogen for the elderly. Pine pollen, given its potency found to be similar to ginseng in some of its actions, and its status as, perhaps, the premier phytoandrogen.

Phyto-androgens are under class of phyto-compounds that mimic the effect of human androgens. Properties may include increasing strength, endurance, and stress tolerance, sense of well-being, mood, confidence, lean body mass, libido, and sexual response [3]. The ability of phytoandrogens to mimic the anabolic, strengthening, and stimulating effects of testosterone can support healthy brain, nerve, muscle, immune, cardiovascular, and other systems prone to atrophy, senescence or weakness. In our system, they become intimately involved in the biosynthesis of testosterone itself and does not interfere with body's own production. It is believed that it has the capacity to increase testosterone level [4].

The phyto-hormones like phytoandrogens are plant hormones but are not bio-identical to animal hormone. Pine pollen contains extremely high levels of steroid-like substances. The most potent of these are the brassinosteroids [5]. These compounds act as powerful growth stimulants in plants. In pine forests, surrounding plants feed off from these pine brassinosteroids in order to promote rapid growth [5]. These plant steroids are very similar in structure to the steroids found in humans and other mammals [6]. Pine pollen actually contains significant amounts of bio-active de hydro epiandrosterone (DHEA), androsterone, and testosterone [7]. The total amount of testosterone in the

pine species *Pinus tabulaeformis* is 27 nanograms per 0.1 grams of dry weight.

Materials and Methods

Collection of pine tree parts

The collection of pine tree parts such as bark, needles, branches and roots was coordinated with the Ecosystems Research and Development Services (ERDS) of the Department of Environment and Natural Resources (Philippines).

The parts of pine trees were air-dried for two weeks After air-drying, the pine tree parts were chopped. The chopped parts were further reduced into smaller particles using the hammer mill (Asahi P211 and Kubota diesel engine). The basic principle of grinding increases the surface area and thereby increased the rate of extraction.

Aqueous extract

Distilled water served as the dissolving agent. Water is an excellent solvent but it has some limitations on its selectivity. It is a polar solvent (hydrophilic) that dissolve substances that are water soluble but do not dissolve the oily substances.

Serial exhaustive extraction

This involves successive extraction with solvents of increasing polarity from a non-polar to a more polar solvent to ensure that a wide polarity range of compounds can be extracted.

Ethanolic extract

The higher activity of the ethanolic extracts as compared to the aqueous extract can be attributed to the presence of higher amounts of polyphenols as compared to aqueous extracts. It means that they are more efficient in cell walls and seeds degradation which have unipolar character and cause polyphenols to be released from cells.

Thin Layer Chromatography

This is a semi quantitative method of analysis that was used to identify the components in a compound mixture like alkaloids, phospholipids, amino acids and other phytochemicals.

Samples of two (2) grams each were defatted in 10 ml hexane (the mixture of hexane and samples were heated for 3-5 minutes). The remaining solvent was decanted and discarded and the remaining residue was treated with 9.9 ml chloroform and 0.1 acetic acid and heated for 3-5 minute-water bath. After bath, the mixture was filtered and the filtrate was labelled Solution A. The resulting residue was treated with a mixture of 4.95 ml chloroform, 4.95 ml ethanol and 0.1 ml of acetic acid. Then heated for 3-5 minutes in water bath. The mixture was filtered and the filtrate was labelled Solution B.

The capillary tube was dipped onto the sample (needles, twigs and roots) one at a time, rinsing the tube with distilled water. Each spot 1 cm apart was made on the

drawn line. Solution A/B was poured into the chamber A/B around 10 mm height (i.e. solution A samples soaked into Solvent A and solution B soaked into Solvent B. Using a forcep, the TLC plates were placed in the developing chamber in an upright position. The solvent slowly traveled through the plate until it reached the top mark. The plate was removed and air dried. After air drying, the TLC plates were placed in the iodine chamber (iodine crystals + silica gel contained in bottle and shaken until iodine became in powder form). The plate was shaken and observed with yellow color. From the iodine chamber, the dark spots on the TLC plate were marked and viewed under the ultraviolet lamp. The UV showed the fluorescence, indicating the presence of phytochemicals.

$$R_f \text{ values} = \frac{\text{Distance travelled from point of origin application}}{\text{Distance of solvent from point of application}}$$

Quantitative Screening

This method is the best method for detection of steroid drugs and the profiling of endogenous steroids. Two methods are used: Gas chromatography mass spectrometry (GSMS), combined gas chromatograph and mass spectrometry to identify different substances or trace elements while Liquid chromatography mass spectrometry (LCMS) was the technique for general identification of chemicals in the presence of other chemicals.

Proximate Analysis

A proximate analysis, as defined by ASTM (American Society for Testing and Materials, is the determination by prescribed methods of moisture, volatile matter, fixed carbon (by difference) and ash. Each sample parts contained in the crucible was weighed and dried in a convection oven (Blue M B-2729 Q) at 100 ^{0}C for two hours. Three replicate or preparations were made to ensure that weight is constant. After heating, the sample were cooled in a desiccator for 30 minutes. The heated samples were weighed. Then 2nd and 3rd heating ensued for 30 minutes and cooling followed for another 30 minutes. The average weights of the heated samples were computed by getting the mean of the 1st, 2nd and 3rd heating. The moisture weight, percentage moisture and percentage dry matter were computed using the formula:

Moisture weight = (total wt of crucible & sample) – average of heating weight

$$\% \text{ Moisture weight} = \frac{\text{Moisture weight}}{\text{weight of sample}} \times 100$$

% Dry matter=100 - % moisture

The ash content was determined using the Furnace (Type 47900, Thermolyne Sybron). The 1st replicate of samples were heated at 550^{0}C for two hours then cooled in a desiccator for one hour and weighed for the percentage ash content.

$$\% \text{ Ash} = \frac{(\text{weight of crucible \&Ash} - \text{weight of crucible})}{(\text{weight of sample})} \times 100$$

Determination of Nitrogen Content

The Kjeldahl method was developed over a hundred years ago for determining the nitrogen content in organic and inorganic substances. The method is broken down into three main steps: digestion, distillation, and titration.

Digestion

Digestion was accomplished by boiling the homogeneous sample in sulfuric acid (0.1 molar sulfuric acid: 5.55 ml sulfuric acid+ 994 ml distilled water). The end result was an ammonium sulfate solution. The general equation for the digestion of an organic sample is shown below:

Organic N + H_2SO_4 → $(NH_4)SO_4$ + H_2O + CO_4 + other sample matrix by products

One (1) gram of each part was weighed [pine pollen= 1.034 g; bark=1.171 g; needles=1.040 g; roots=1.057 g; twigs=1.119 g]. The sample was transferred to the volumetric flask and a catalyst was added [(5 grams' anhydrous copper sulfate + 100g anhydrous K2SO4 (potassium sulfate)].

Another 40 ml of concentrated sulfuric acid was added to each sample in the flask. The sample turned black and was burned. The digestion process ran for 1.5-2 hours; after digestion, the color of each sample became crystal blue.

Distillation

Excess base was added to the digestion product to convert NH_4 to NH_3as indicated in the following equation. The NH_3 was recovered by distilling the reaction product.

$$(NH_4)_2SO_4 + 2NaOH + 2NaOH \rightarrow 2NH_3 + Na_2SO_4 + 2H_2O$$

The running time for distillation was one (1) hour or more to reach the 75 ml mark on the receiving flask. The preparation of distilling apparatus included: A 5 ml boric acid (4 g boric + 100 ml Distilled water) + 3 drops of methyl red indicator were poured into the receiving flask. The methyl red indicator was prepared (0.25 g Bromocresole green + 125 ml distilled water. Another mixture was done (0.05g methyl red + 25 ml distilled water) and the two mixtures were combined.

The crystal blue samples of each part was transferred to the distilling apparatus, after which 200 ml distilled water +40 ml NaOH was added to the crystal blue samples.

Titration

Titration quantifies the amount of ammonia in the receiving solution. The amount of nitrogen in a sample can be calculated from the quantified amount of ammonia ion in the receiving solution.

$$(NH_4)_2SO_4 + H_2SO_4 + 2NaOH \rightarrow Na_2SO_4 + (NH_4)_2SO_4 + 2H_2O$$

The Liebermann-Burchard's test [8] used: 0.5 ml crude extract, 1 ml chloroform, 2ml acetic anhydride and 1 ml sulphuric acid. The positive results gave the colors ranging from blue to green, red, pink, purple or violet due to the presence of steroid/triterpenoid skeleton. All parts of pine tree (pine pollen, bark, twigs, roots, needles, branches) were tested positive for unsaturated steroids/sterols.

RESULTS AND DISCUSSION

Phytochemical Screening

Phytochemical screening of the different parts of pine tree using different solvents were shown on table 5. Aqueous extract of twigs contained flavonoid but ethanolic, dichloromethane and petroleum extract showed negative results for flavonoids.

Quantitative Analysis

The analysis of the extracts was performed using Gas chromatographic mass spectrometry, the single most important tool for the quantitation of volatile and semi volatile organic compounds in complex mixtures. It was found useful for the determination of molecular weights and elemental compositions of unknown organic compounds in complex mixtures [9].

The chemical compound pinene is a bicyclic monoterpene chemical compound. There are two structural isomers found in nature: a-pinene and ß-pinene in which both forms are important constituents of pine resin and both were also used by many insects in their chemical communication system [10].

Another chemical compound present in pine pollen is Carene, a bicyclic monoterpene which occurs naturally as a constituent of turpentine, with a content as high as 42% depending on the source. Carene has a sweet and pungent odor. It is not soluble in water, but miscible with fats and oils [11].

Identification of volatile components was confirmed by comparison of collected mass spectra with those of standards and spectra at the De La Salle University instrument room.

This is a preliminary experiment of a Benguet pine pollen subjected to Liquid Chromatography Mass Spectrometry. The testosterone content of pine pollen with an abundance of 1.25×10^6 was determined at 14 minutes. This testosterone content was confirmed when a standard 17 alpha methyltestosterone was also subjected to LCMS.

The phyto-hormones, like phytoandrogen found in pine pollen is not identical to animal hormone however, the effects of phytohormones are very similar. Some plants produce plant sterols, a type of plant testosterone that is beneficial to the human body. Plants create chemicals that are similar to the testosterone found in the

human body. Most of these plant sterols cannot be converted by the human body, but they can be engineered in a lab. Some plants contain a bioflavonoid known as chrysin, which is extracted from plants and used by bodybuilders as a testosterone-boosting agent. Other plants contain chemicals that help produce and increase testosterone in the body (Morgan, 2012).

Fernando, S. I. et al., 2017 cited that there were evidences that inclusion of pine pollen in a rat diet may affect fecal dry matter, crude protein and crude ash concentration as well as nutrient digestibility. These data implicate the use of Pine pollen as a feed additive that may help enhance dietary fiber supply [12].

Thin Layer Chromatography

This technique was used to separate organic compounds. Rf is also known as the Retention factor, with value close to 1.0. The Rf value was calculated by taking the distance travelled by the compound divided by the distance travelled by the solvent front. Table 5 shows the different Rf values in two solutions (A and B). Solution B shows larger distance travelled ranging from 0.7142 (pollen) to 94 (roots) as compared to solution A with distance travelled from 0.4630 (twigs) to 0.94 (pine pollen). The solvents in B were chloroform, acetic acid and distilled water. The larger an Rf of a compound, the larger the distance it travels on the TLC plate. When comparing two different compounds run under identical chromatography conditions, the larger Rf means that the compound is more soluble in the given solvent system. Or simply, the pigments that are strongly attracted to the solvent (mobile phase) move faster while pigments that are more strongly attracted to the silica gel move slower.

Proximate Analysis

The moisture weight of pine pollen was revealed highest at 0.14 g, followed by roots, needles and bark (trunk and branch) at 0.1 g while the twigs indicated a moisture weight of 0.09 grams. The percentage moisture ranged from 9.57-12.46% with pine pollen with the highest moisture followed by bark (trunk), 11.14%, needles with 10.96%, bark (branch), 10.96%; twigs, branches and roots with 9.57%,9.92% and 9.94 % respectively. The dry matter (DM) varied from 87.53-90.42, with twigs, the most dried part. DM is the percentage of the feed that is not water (moisture). The lower the DM, the more moisture is present, and the lower is the nutrient density.

The percentage ash ranged from 0-2.99 %. Ash is the total inorganic matter. Ash is a measure of the total mineral content of the feed, but it does not tell us how much of each mineral is present.

Nitrogen and Crude Protein Analysis

The pine pollen had the highest nitrogen content of 2.24%, followed by needles, 1.76%, twigs, 0.82%, bark, 0.60% and roots, 0.38%. Nitrogen is an essential nutrient for plants and animals because it is a building block for proteins and amino acids. The crude protein (CP) content ranged from 2.62% (roots)-13.98% (pine pollen). CP is an estimate of the level of protein in the feed based on the amount of nitrogen present. Not all of the nitrogen is in the form of true protein, thus it is termed "crude" protein. CP also does not give individual amino acid profiles.

Figure 1: Beguet pine and the catkin containing its pollen

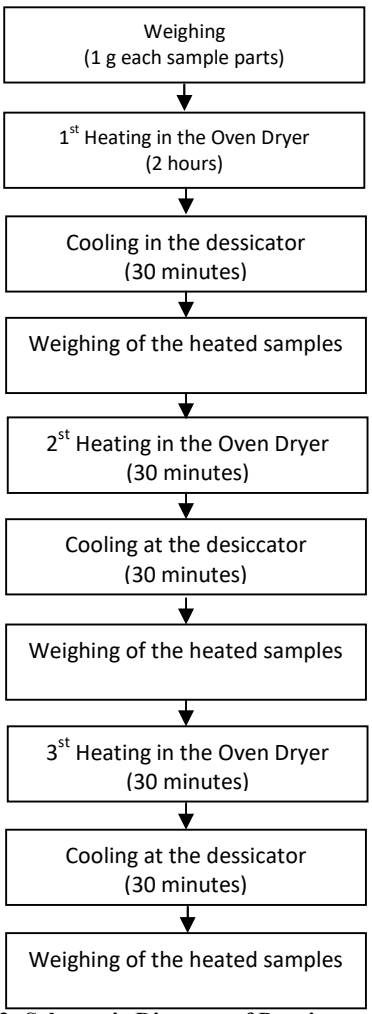

Figure 2: Schematic Diagram of Proximate Analysis

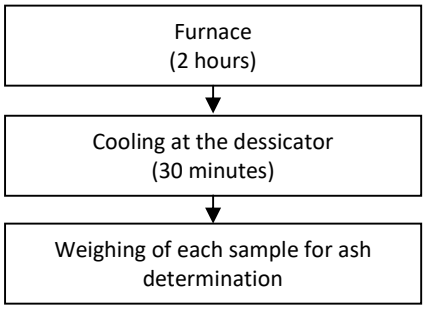

Figure 3: Ash Content Analysis

Table 1: The phytochemicals and reagents in aqueous extract

Phytochemical	Reagent	Remarks
Tannin	1 ml aqueous extract + 5 drops Ferric chloride ($FeCl_3$)	Greenish/black/mosh green/brown
Flavonoid	1 ml aqueous extract NH3 solution + sulfuric acid (H_2SO_4)	Yellow color
Saponin	Shaking	Frothing/foaming of sample

Table 2: The phytochemicals and reagents in petroleum ether, dichloromethane and serial extract

Phytochemical	Reagent	Remarks
Unsaturated steroids	0.5 ml extract + 1 ml chloroform + 2ml acetic anhydride + 1 ml sulphuric acid	Purple coloration due to steroid triterpenoid skeleton; Dark green
Cardiac glycosides	2 ml extract + 1 ml glacial acetic + 2 drops $FeCl_3$ + 3 drops H_2SO_4	Brown ring interface

Table 3: The phytochemical and its reagent in ethanolic and chloroform extract

Phytochemical	Reagent	Remarks
Alkaloids	Pine tree parts + 15 ml 10% ethanol in chloroform	Formation of precipitate

Table 4: The phyto-hormone/phytochemical tested in LC-MS and GC-MS

Phytochemical/Phytohormone	Method
Testosterone- like Terpenes (tricyclo, Carene, Pinene, Santolinatriene)	LC-MS/GC-MS

Table 5: The pine tree parts, needles, bark (branches) and phytochemical contents in various dissolving agents

Parts	Extraction Method	Tannin	Saponin	Flavonoid	Alkaloid	Sterol	Cardiac Glycoside
Twigs	Aqueous	+	-	+	-		
	Ethanolic	+	-	-	-		+
	Dichloromethane	-		-		+	-
	Petroleum Ether	-		-			
	Serial (PE-DCM)					+	
	10% Ethanol in Chloroform	+			+		
Pine pollen	Aqueous	-	-	-	-		
	Ethanolic	-	-	+			+
	Dichloromethane						
	Petroleum Ether						
	Serial (PE-DCM)					+	-
	10% Ethanol in Chloroform	+			+		
Needles	Aqueous	+	-	+	-		
	Ethanolic	+	-	+	-		
	Dichloromethane	-		+			-
	Petroleum Ether	-		+		+	
	Serial (PE-DCM)						
	10% Ethanol in Chloroform				+		
Bark branches	Aqueous						
	Ethanolic	+	-	-			-
	Dichloromethane						
	Petroleum Ether	-		+			
	Serial (PE-DCM)					+	
	10% Ethanol in Chloroform				+		
Roots	Aqueous	+	-	-	-		
	Ethanolic	+	-	+			-
	Dichloromethane	-		+		+	-
	Petroleum Ether	-		+			
	Serial (PE-DCM)						
	10% Ethanol in Chloroform				+		
Bark (trunk)	Aqueous						
	Ethanolic						
	Dichloromethane						
	Petroleum Ether	-		-			
	Serial (PE-DCM)					+	
	10% Ethanol in Chloroform				+		

Table 6: The qualitative and quantitative tests for pine tree parts.

PART	LC-MS	GC-MS
Pine pollen	+ for testosterone	+ for tricycloheptane; Tricylohexane

Table 7: Results of the thin layer chromatography.

PARTS	R_f (Solution A)	R_f (Solution B)
Roots	0.52	0.94
Needles	0.51	0.90
Twigs	0.46	-
Pollen	0.94	-
Bark (trunk)	0.73	0.82
Branch	-	.91
Pine Pollen	.57	.71

Table 8: The nitrogen and crude protein content of the different parts of pine tree

Parts	%Nitrogen	% crude protein
Pollen	2.24%	13.98%
Bark (branch & trunk)	0.60%	3.78%
Needles	1.76%	10.97%
Roots	0.38%	2.62%
Twigs	0.82%	5.13%

CONCLUSION

Different parts of pine trees exhibited vast amount of phytochemicals. Pine pollen displayed the highest number of phytochemicals. The testosterone content of Pine pollen was found with an abundance of 1.25 x 10^6 determined at 14 minutes and confirmed with the standard 17- a-methyltestosterone. Pine pollen also has the highest amount of crude nitrogen. Thus, Benguet pine pollen can be a source of phytoandrogen.

ACKNOWLEDGEMENT

This is the initial information on the phytoandrogen research of the Central Luzon State University and the authors are grateful to the Philippine Department of Science and Technology for funding this research study, GIFT Foundation facility, Department of Chemistry, Central Luzon State University, Small Ruminant Center, Ecological Research Development Station of DENR and De lasalle University Manila.

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