



Hair regenerative activities of flavonoid-rich extract of *Equisetum hyemale* L. (*Equisetaceae*) in chemically-induced alopecia in Sprague Dawley rats

[Actividad regeneradora del cabello de extracto rico en flavonoides de *Equisetum hyemale* L. (*Equisetaceae*) en alopecia inducida químicamente en ratas Sprague Dawley]

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Abstract

Context: The prevalence of alopecia around the world is high and awareness continuously increased due to social stigma. There are limited commercially-available medications for the management of different forms of baldness, most of which are prohibitively expensive and presents with various adverse effects.

Aims: To evaluate the flavonoid-rich ethyl acetate extract of *Equisetum hyemale* stem for its hair-regenerative properties in chemically-induced alopecia in Sprague Dawley rats.

Methods: Various concentrations of the flavonoid-rich extract of *E. hyemale* were applied in depilated areas in dorsal rat skin after chemical induction of alopecia. Evidence of hair growth was observed for 28 days in a weekly basis. Histopathological analysis of rat skin was performed to demonstrate evidence of follicular hair growth.

Results: The abundance of flavonoids in the ethyl acetate extract was established by total flavonoid contents and LC-MS analyses. The 2.5% *E. hyemale* extract exhibited hair-regenerative properties with high tensile strengths, combined masses and follicular growth, which was comparable to the positive control, 5% minoxidil ($p > 0.05$). The highest rate of hair follicular development was observed in the telogen phase in rat skin after 28 days of treatment.

Conclusions: The flavonoid-rich ethyl acetate extract of *E. hyemale* exhibits *in situ* hair-regenerative properties in chemically-induced alopecia in rats at 2.5% w/v concentration.

Keywords: alopecia; *Equisetum hyemale*; flavonoids; hair-regenerative; horsetail.

Resumen

Contexto: La prevalencia de alopecia en todo el mundo es alta y la conciencia aumenta continuamente debido al estigma social. Existen medicamentos disponibles pero limitados para el tratamiento de diferentes formas de calvicie, la mayoría de ellos son muy caros y presentan diversos efectos adversos.

Objetivos: Evaluar el extracto de acetato de etilo, rico en flavonoides, del tallo de *Equisetum hyemale* por sus propiedades regenerativas del cabello en la alopecia inducida químicamente en ratas Sprague Dawley.

Métodos: Se aplicaron diversas concentraciones del extracto rico en flavonoides de *E. hyemale* en áreas depiladas en la piel dorsal de rata después de la inducción de la alopecia. La evidencia de crecimiento del pelo se observó semanalmente durante 28 días. El análisis histopatológico de la piel de rata se realizó para demostrar la evidencia de crecimiento folicular del pelo.

Resultados: La abundancia de flavonoides en el extracto de acetato de etilo se estableció mediante el contenido total de estos y el análisis LC-MS. El extracto de *E. hyemale* al 2,5% mostró propiedades de regeneración del cabello con altas resistencias a la tracción, masas combinadas y crecimiento folicular, que fue comparable al control positivo, 5% de minoxidil ($p > 0.05$). La mayor tasa de desarrollo folicular del cabello se observó en la fase telógena en la piel de rata después de 28 días de tratamiento.

Conclusiones: El extracto de acetato de etilo, rico en flavonoides, de *E. hyemale* exhibe propiedades regenerativas del cabello *in situ* en la alopecia inducida en ratas a una concentración de 2,5% p/v.

Palabras Clave: alopecia; cola de caballo; *Equisetum hyemale*; flavonoides; regenerador del cabello.

ARTICLE INFO

Received: February 17, 2019.

Received in revised form: June 23, 2019.

Accepted: June 29, 2019.

Available Online: July 8, 2019.

Declaration of interests: The authors declare no conflict of interest.

Funding: The authors confirm that the project has not funding or grants.



INTRODUCTION

Alopecia is one of the most frequent dermatological disorders with universal prevalence, affecting more males than females at a ratio of 2:1 (Fricke and Miteva, 2015). The only FDA-approved medication available for the management of hair loss includes finasteride, dutasteride and minoxidil which are costly and presents with numerous adverse effects (Yamada et al., 2013). There was, therefore, a need to outsource naturally-derived drugs from plants which exhibit hair-regenerative properties. *Equisetum* is a genus of ferns known as horsetails, which consist of 15 species (Purcell, 2018). Several species were proven to have hair grower activities, e.g., *Equisetum arvense* (Pingale et al., 2016) whilst the Philippine horsetail plant, *E. hyemale*, is gaining attention to the hair-growth stimulatory properties of its flavonoid-rich extracts (Al-Snafi, 2010; Jiang et al., 2014). This research aimed to screen the abundance of flavonoids in ethyl acetate stem extract of *E. hyemale* and to screen if this extract demonstrates hair-regenerative properties in animal model of alopecia.

MATERIAL AND METHODS

Chemicals

Hexane, ethyl acetate, methanol and tween 80 were purchased from Belman Laboratories Quezon City, Philippines. Minoxidil 5% solution (Regrow, Derm Pharma Inc, Pasig City, Philippines) and Veet hair removal cream were procured at Mercury Drugstore Malolos City, Philippines. Quercetin, NaNO₂, AlCl₃, NaOH, formic acid and acetonitrile were provided by the University of the Philippines (UP), Manila.

Plant material and extraction

Whole plant of *E. hyemale* was randomly collected from Bulacan, Philippines (GPS 14.887058, 120.785283). A voucher specimen of the plant was verified by Mr. John Rey C. Collado, a botanist at the Botany Division of the Philippine National Museum with a control number of 15-07-124. The

stems were air-dried after removal of unwanted particles by hand picking, cut into small pieces and grounded to coarse powder using a blender (Magic Bullet 400W, Homeland Housewares, USA). About 250 g portions of the dried material were separately macerated with exhaustion with hexane, ethyl acetate, methanol and distilled water. Combined hexane, ethyl acetate and methanol extracts were concentrated *in vacuo* (Heodolph, Schwabach, Germany) and then air-dried while combine aqueous extracts were lyophilized (KD Freeze Dryer, Zhengzhou Keda Machinery, Mainland, China) (Kumar et al., 2011; Hossain et al., 2013).

Flavonoids evaluation

The presence and abundance of flavonoids in the ethyl acetate extract of *E. hyemale* was established and termed flavonoid-rich extract of *E. hyemale* (FREEh) by the quantification of total flavonoids and liquid chromatographic - mass spectral (LC-MS) analysis (Asha and Kumar, 2015; Khatiwora et al., 2010).

Analysis for total flavonoid was performed at the Institute of Pharmaceutical Sciences National Institute of Health UP Manila, Philippines with control number of IPSNP 18-139. This assay facilitates standardization of the FREEh for its total flavonoid contents in terms of the standard quercetin, based on the oxidative coupling and condensation of phenolic groups of individual flavonoid moieties (i.e., flavanols, proanthocyanin, anthocyanins, flavones, flavonones, chalcones and their glycosides) by trivalent aluminum. Briefly, exactly one mL of the FREEh, prepared as one mg/mL in methanol, and each of the standard solutions of quercetin in methanol (0 - 100 µg/mL), were diluted with four mL of distilled water in a 10-mL volumetric flask, followed by the addition of 0.3 mL of 5% NaNO₂; after five minutes, 0.3 mL of 10% AlCl₃ was added. After six minutes, two mL of 1 M NaOH was added and then water was finally added to volume and then mixed. Absorbance (Beckman Coulter DU 730 UV/Vis Spectrophotometer, China) were read at 510 nm and the total flavonoids were measured as quercetin in mg/g,

thus: total flavonoids = $C \times 10$, where C was the concentration of quercetin obtained by linear regression analysis of the calibration curve that plots quercetin concentrations and absorbance values while 10 was a dilution factor (Khatiwora et al., 2010).

The FREEH was submitted and analyzed at the Biochemistry Department, College of Medicine, UP Manila, Philippines for LC-MS (Waters Xevo G2-XS QToF, Massachusetts, USA) analysis using an ACQUITY HSS T3 C18, 1.8 μm , 2.1 \times 100 mm and a mobile phase consisting of phase A: water + 0.1% formic acid and phase B: acetonitrile + 0.1% formic acid at a flow rate of 0.4 mL/min, a voltage: 40 V Source at 120°C at a gas flow of 40 L/h; desolvation temperature was set at 550°C at a gas flow of 950 L/h and a scan range of 50 - 1,200 m/z; scan time was approximately 0.150 seconds at a collision energy of 15 to 50 eV. Leucine enkephalin was used as an internal reference compound for mass correction; data were processed using a Waters UNIFI Scientific Information System v1.8.1.073 Library and Waters Traditional Chinese Medicine Library, which allows for the putative identification of separated molecules based on their retention times and molar masses (Annex 1), which are monitored by a library fingerprint (Meng et al., 2018).

Evaluation of hair-regenerative properties

The protocol for screening of hair regenerative properties in rats was approved by the Institutional Animal Care and Use Committee (IACUC) with reference number of IACUC 2017-18/071. Healthy male Sprague Dawley rats, weighing at least 200 g, were purchased from the Department of Science and Technology (DOST) in Bicutan, Taguig City Philippines. Rats were acclimatized at the animal holding facilities of Centro Escolar University (CEU) Malolos City Bulacan Philippines for seven days at $22 \pm 3^\circ\text{C}$ and 55-75% RH with 10 - 15 cycles of air and an alternating 12-hour light and dark cycle. Rats were housed in rectangular transparent plastic cage with an adequate space and holes to breathe in and to allow *ad libitum* feeding with standard pellets and water. Soft wood shavings

(Chipsi, Rosenberg, Germany) were used to provide beddings, which were changed daily as the need arises.

The *in situ* screening method used in the study was a modification of the methods used by Adhirajan et al. (2003), Kurup et al. (2013), Allayle et al. (2012) and Lanzafame et al. (2013). Sprague Dawley rats were randomly distributed into five groups (n=5 rats per group); the first three groups were treated with 2.5%, 5% and 10% concentration of the predetermined flavonoid-rich extract in 0.5% Tween 80. Minoxidil 5% solution and 0.5% Tween 80 were used as positive and negative controls, respectively, and were administered to the 4th and 5th groups, respectively.

A commercially-available hair remover (Veet, Reckitt Benckiser, Maharashtra, India) was topically applied into the dorsal skin of the rats, covering an area of four \times four cm^2 where template was used to ensure uniformity of size of the bald areas. Exactly 0.5 mL portions of the samples were applied once daily, for 28 days, using an applicator. Caution was observed to ensure evenness of application in the bald areas. The growth of hair in each bald region was evaluated by visual observations and recorded using photographs (Vivo Y53, Manila, Philippines) at weekly basis after daily topical application of the samples (Young Oh et al., 2014).

On the seventh, 14th and 28th day, ten strands of hair were plucked randomly from each one cm^2 bald area for measurement of hair length using a digital micrometer (Mitutoyo, Illinois, USA) whereas the combined mass were weighed accurately using analytical balance (Denver Instrument SI-234 Colorado, USA). For tensile strength analysis, ten strands of hair per treatment group were randomly plucked after 28 days of treatment and mounted manually by supporting the ends of each hair with mechanical weights, which are incrementally added up to the maximum weight that can be held. The evaluation of hair regenerating effects was based on the scoring index used by Young Oh et al. (2014), as follows: one = 0 - 19% growth; two = 20 - 39% growth; three = 40 - 59% growth; four = 60 - 79% growth and five = 80 - 100% growth.

The percentage growth was calculated as followed:

$$\% \text{ Growth} = \frac{\text{Mean hair length (mm) or weight (g) in week 1}}{\text{Mean hair length (mm) or weight (g) in weeks 2 or 4}} \times 100 \quad [1]$$

Histopathological study

After 28 days, the rats were sacrificed and the dermal skin samples were fixed in 10% buffered formalin, followed by paraffin wax embedding and stained with hematoxylin-eosin (Belman Laboratories Quezon City, Philippines) and observed under digital microscope with LCD screen (Steindorff, New York, USA) at 400x magnification. The number, elongation and depth of hair follicular growth, graded according to the scale by Young Oh et al. (2014), thus: five = presence of fully developed hair with shafts, four is characterized by developing hairs in dermal, epidermal and slightly above epidermal layer; three noticed the presence of developing hair in both dermal and epidermal layers; two indicated presence of developing hair in the dermal layer, while one indicated absence of any hair.

Statistical analysis

Data are compared by the one-way analysis of variance (ANOVA), the two-tailed t-test and the 90% confidence interval using SPSS software (Statistical Package for the Social Sciences, version 20.0, SPSS Inc, Chicago, IL). Replicate analyses are expressed as means \pm standard error of the mean. $P < 0.05$ was considered significant level.

RESULTS AND DISCUSSION

From among the hexane, ethyl acetate, methanol and aqueous extracts of *E. hyemale* stem, the ethyl acetate extract was designated as the flavonoid rich extract of *E. hyemale* (FREEh) based on the quantification of total flavonoids registered quercetin equivalent of 57.14 ± 2.51 mg/g ($n = 5$), a quantity that is comparatively high based on the quercetin equivalent, at 17.47 ± 0.25 mg/g, of the ethanolic extract of *E. arvense*, another equisetum species with known hair grower properties (Mimica-Dukic et al., 2008).

LC-MS separation of the FREEh afforded the identification of 27 substances (Table 1). Most of these substances are flavonoids, mostly flavones and chalcones, as well as their derivatives that include two glycosides.

Chemically-induced alopecia in Sprague Dawley rats were used to screen the FREEh for its *in situ* hair regenerative properties after 28 days of treatment (Figs. 1 - 4). There were converse dose-response relationship responses when various concentrations of the FREEh were tested *in situ* in chemically-induced alopecia in rats based on mean tensile strengths (i.e., $r = -0.0567$) and hair mass (i.e., $r = -0.9588$). In this case, 2.5% concentration was significantly more effective than higher concentrations. This is due to the phenomenon called ceiling effect, where increasing dose or concentrations of a drug produces progressively small incremental therapeutic effects (Richardson and Raymond, 2018). This is possible when both agonists and antagonists are present in the sample. This mixed agonist-antagonist may affect 5 α -reductase inhibitory properties or any phases of the hair growth cycle in chemically-induced alopecia in rats (Bhasin et al., 2012). These can be due to saturation of receptors, which include the 5 α -reductase enzymes found within hair follicles in bald areas during alopecia, by bioactive secondary metabolites present in the sample, such as flavonoids (Hiipakka et al., 2002). The advantage of these findings is based on achieving optimum therapeutic effects at lower concentrations without posing risks of adverse effects associated with higher concentrations. Usually, these adverse effects occur long after the desired outcome from therapeutic ceiling concentrations are achieved (Gal, 2009). The popular tradition in the management of hair loss is by using botanical products as it lowers incidence of side effects as observed in synthetic products, recent studies showed that herbal and synthetic hair growers were combined producing synergistic effects (Keaney et al., 2016).

CONCLUSIONS

This study demonstrated the *in situ* efficacy of 2.5% of the flavonoid-rich ethyl acetate stem ex-

tract of *E. hyemale* as a hair-regenerative agent in chemically-induced alopecia in rats as observed in a weekly basis for 28 days, which showed evidences of higher hair tensile strengths, combined masses and follicular growth.

In the present study, ethnomedical knowledge on the use of *E. hyemale* as hair grower has been experimentally validated. Furthermore, the authors recommend a thorough study on the mechanism of action of FREEh on its hair growth activities.

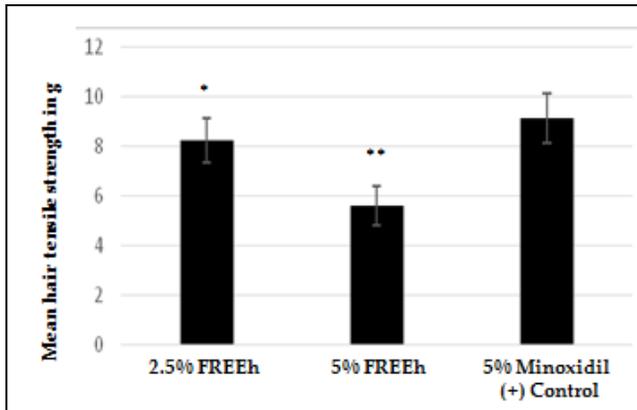


Figure 1. Mean hair tensile strength after 28 days of treatment with flavonoid-rich extract of *E. hyemale* in Sprague Dawley rats.

Data represent mean \pm SEM (n=5). Significant differences (**p<0.05) were detected with respect to 5% FREEh, 10% FREEh and the positive control 5% minoxidil where as a *p>0.05 were detected with respect to 2.5% FREEh and 5% minoxidil.

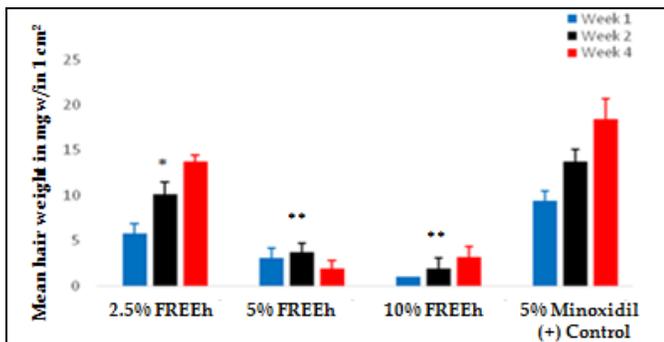


Figure 2. Mean hair weight in mg within 1 cm² after 28 days of treatment with flavonoid-rich extract of *E. hyemale* in Sprague Dawley rats.

Data represent mean \pm SEM (n=5). Significant differences (**p<0.05) were detected with respect to 5% FREEh, 10% FREEh and the positive control 5% minoxidil where as a *p>0.05 were detected with respect to 2.5% FREEh and 5% minoxidil.

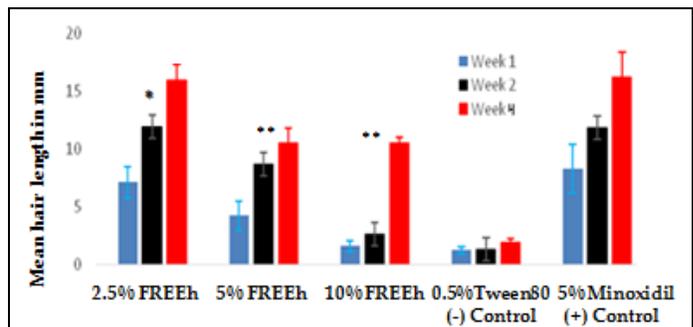
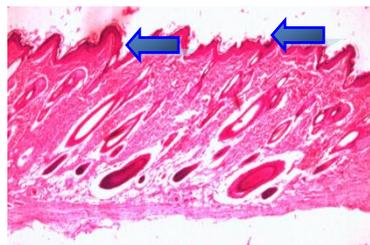


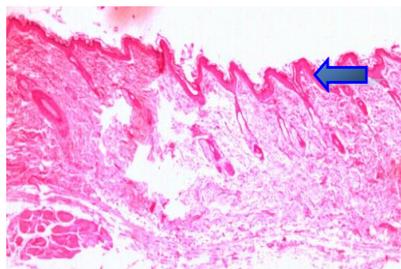
Figure 3. Mean hair tensile strength after 28 days of treatment with flavonoid-rich extract of *E. hyemale* in Sprague Dawley rats.

Data represent mean \pm SEM (n=5). Significant differences (**p<0.05) were detected with respect to 5% FREEh, 10% FREEh and the positive control 5% minoxidil where as a *p>0.05 were detected with respect to 2.5% FREEh and 5% minoxidil.

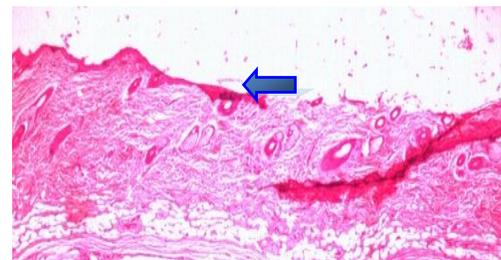
2.5% FREEh



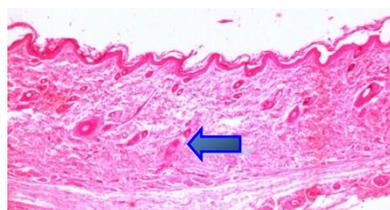
5% FREEh



10% FREEh



0.5% Tween 80



5% Minoxidil



Figure 4. Representative histological photomicrographs of depilated skin within 28 days of treatment with flavonoid-rich extract of *E. hyemale* in Sprague-Dawley rats.

The blue arrows are evidence of hair growth where the hair follicles and shaft are visible. Scale by Young Oh et al. (2014): 5 = presence of fully developed hair with shafts (e.g., 5% minoxidil or positive control and 2.5% FREEh); 4 is characterized by developing hairs in dermal, epidermal and slightly above epidermal layer (e.g., 2.5% FREEh); 3 notices the presence of developing hair in both dermal and epidermal layers (e.g., 5% FREEh); 2 indicates presence of developing hair in the dermal layer (e.g., 10% FREEh); while 1 indicates absence of any hair (e.g., 0.5% Tween 80 or negative control). Magnification 400x.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

This research work did not receive any grant from funding agencies in the public, commercial, or not-for-profit sectors. Authors wish to thank their families and school, Centro Escolar University, in their continued support and understanding in order for this study be materialized.

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AUTHOR CONTRIBUTION:

Contribution	David PP	Santiago CD	Castillo AL	De Guzman GQ
Concepts or ideas	x	x	x	
Design	x	x	x	
Definition of intellectual content	x	x	x	
Literature search	x	x		x
Experimental studies	x			x
Data acquisition	x	x		
Data analysis	x	x		x
Statistical analysis		x		x
Manuscript preparation	x	x	x	x
Manuscript editing	x	x	x	x
Manuscript review	x	x	x	x

Citation Format: David PP, Santiago CD, Castillo AL, De Guzman GQ (2019) Hair regenerative activities of flavonoid-rich extract of *Equisetum hyemale* L. (*Equisetaceae*) in chemically-induced alopecia in Sprague Dawley rats. *J Pharm Pharmacogn Res* 7(5): 323–330.

Annex 1. LC-MS chromatographic spectra of flavonoid-rich extract of *E. hyemale*.