Gamma Irradiation Response of Phytochemicals in Pine Needles (Pinus kesiya): In Ovo Determination of the Angiogenic Activity of its Crude Extract

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ABSTRACT

Pinus kesiya needles are valued in traditional medicine for their diverse phytochemical profile, including phenolics, terpenoids, and alkaloids. This study investigated how gamma irradiation modulates these compounds and their subsequent angiogenic effects using the Chick Chorioallantoic Membrane (CAM) assay. One hundred grams of powdered *P. kesiya* needles were macerated to produce crude extracts, which were then exposed to gamma irradiation at three dose levels: 0 kGy (control), 4 kGy, and 8 kGy. Phytochemical screening revealed that irradiation induced compound-specific, dose-dependent changes, notably enhancing the detectability of alkaloids, cardiac glycosides, and saponins at higher doses. To evaluate vascular response, sixty 10-day-old Philippine duck (*Anas luzonica*) embryos were treated with the irradiated extracts for 48 hours. Results showed persistent vascularization across all groups, with the highest neovascular density and branching complexity observed in the irradiated samples, particularly at 8 kGy. Statistical analysis via one-way ANOVA indicated a significant difference in mean vessel counts ($F(2,57)=13.76$, $p<0.001$). Mean counts increased from 1.70 (control) to 3.75 (4 kGy) and 4.00 (8 kGy). Post-hoc Tukey HSD testing confirmed that both irradiation doses significantly outperformed the control, though the difference between 4 kGy and 8 kGy was not statistically significant ($p=0.862$). These findings suggest that gamma irradiation enhances the angiogenic activity of *P. kesiya* extracts, likely by altering phytochemical structures, with a potential plateau effect beyond 4 kGy. This demonstrates that controlled irradiation is a viable strategy for optimizing the therapeutic potential of plant-based bioactive compounds.

Keywords: *Pinus kesiya, gamma irradiatiom, phytochemicals, CAM assay, angiogenesis, radiation dose-response relationship*

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INTRODUCTION

The pine needles have been traditionally used to treat varied conditions (Rose, 2024). Its medicinal value is due to its rich phytochemical compounds, which have been the focus of numerous studies. In the study of Weerapreeyakul et al. (2016), a significant amount of phenolic compounds in pine needles *(Pinus kesiya)* was found to exhibit antioxidant and anti-inflammatory properties. These biological activities are primarily attributed to the presence of gallic acid, chlorogenic acid, caffeic acid, vanillin, and coumaric acid. In addition to phenolic compounds, Govindarajan et al. (2016) reported the presence of a range of terpenoids, including monoterpene and diterpene derivatives, in *P. kesiya* needles. Pocasap et al. (2021) further revealed that the phytochemical profile of *P. kesiya* includes alkaloids, steroids, xanthones, reducing sugars, and saponins, with α-pinene-containing compounds demonstrating low cytotoxicity against HepG2 cells.

These findings suggest that the terpene derivatives in *Pinus kesiya* may collectively induce apoptosis, reinforcing the plant's potential as a natural source of anticancer agents. The presence of phenolics and other bioactive compounds enhances the medicinal potential of *P. kesiya*, making it a promising subject for further research, particularly in the context of inflammation and cancer. Phenolic compounds, in particular, are known for their potent antioxidant properties and significant biological activities. They can be classified into simple phenols (e.g., gallic acid, salicylic acid) and complex polyphenols containing multiple aromatic rings (Kulbat, 2016). Their ability to prevent or repair oxidative damage—commonly associated with carcinogenesis—highlights their therapeutic prospects. Bhuyan and Basu (2017) demonstrated that phenolics can modulate gene expression related to cell proliferation and apoptosis, both in vitro and in vivo. Similarly, Vuong et al. (2014) examined catechin and epigallocatechin-3-gallate (EGCG), which exhibit anticancer properties against various cancer cell lines.

Moreover, phenolic compounds are highly sensitive to radiation, with gamma rays known to affect their stability, bioavailability, and pharmacological efficacy (Benjedi et al., 2015). Their radical-scavenging capacity plays a vital role in stabilizing harmful free radicals within the body (Vuong et al., 2014). According to Belcadi et al. (2023), gamma irradiation can significantly alter the chemical structure of plant-derived compounds, either enhancing or reducing their therapeutic properties. Although previous studies have reported that gamma irradiation can improve the extractability and activity of phytochemicals, there remains a gap in understanding how varying doses of irradiation specifically affect the yield and functional properties of phenolic compounds in *Pinus kesiya*.

In embryonic development, the fusion of mesodermal layers of the allantois and chorion leads to the formation of the chick chorioallantoic membrane (CAM), a highly vascularized and immunodeficient structure (Ribatti, 2016). The CAM is commonly used in experimental oncology and pharmacology due to its ability to support grafts and tumors without eliciting an immune response. It has become an established in vivo model for assessing angiogenesis and metastasis due to its real-time vascular visualization and cost-effectiveness (Ribatti, 2021).

Angiogenesis—the formation of new blood vessels from pre-existing ones—is a fundamental process in both health and disease. It is triggered by hypoxia-induced growth factors such as VEGF, which stimulate endothelial migration and

proliferation (Mentzer & Konerding, 2014). This process is critical not only in embryonic development and wound healing but also in cancer progression, where tumors exploit angiogenesis to sustain growth and metastasize (Rajabi & Mousa, 2017; Eelen et al., 2020). Al-Ostoot et al. (2021) even described angiogenesis as a hallmark of cancer, emphasizing its role in the transition from benign to malignant tumors. Ricciuti et al. (2019) further investigated this role in the development of anti-angiogenic therapies.

Given this background, the present study aimed to evaluate the angiogenic activity of gamma-irradiated Pinus kesiya crude extracts using the duck CAM assay model. Specifically, the study examined the qualitative phytochemical changes induced by varying doses of gamma irradiation (control, 4 kGy, 8 kGy) and correlated these changes with the observed angiogenic responses in the CAM model.

This research employed an experimental study design with descriptive phytochemical screening and statistical validation of biological activity. Gamma irradiation treatment and an in ovo CAM assay were used to explore the phytochemical and angiogenic responses of Pinus kesiya crude extracts at varying radiation doses. Through a combination of controlled experimentation (gamma irradiation and CAM application), descriptive analysis (phytochemical screening and visual vascular pattern assessment), and quantitative evaluation (vessel counts and statistical testing), the study captured the differential and compound-specific effects of irradiation on plant bioactivity. While qualitative methods revealed compound-specific responses, statistical tools such as one-way ANOVA and Tukey HSD were used to validate observed angiogenic trends across treatment groups.

METHODOLOGY

Sample Preparation

Pine needles were gathered from Camp John Hay, Baguio City. Some of the collected samples were sent to Jose Vera Santos Memorial Herbarium at University of the Philippines Diliman for authentication, while most of the collected samples were sun dried for five (5) days followed by oven-drying to remove the residual moisture, and then ground into fine powder for further processing.

Sample Extraction and Irradiation

One (1) liter of 70% ethanol was added to one hundred (100) grams of powdered *Pinus kesiya* needles and left to macerate for forty-eight (48) hours. The resulting extract was filtered using a coffee filter and concentrated using a rotary evaporator for sixteen (16) hours at a temperature below 40°C. The crude extract was then transferred into 50 mL amber glass bottles, sealed with parafilm, and stored at a temperature not exceeding 10°C. Gamma irradiation was applied at three (3) different absorbed dose levels: control, 4 kGy, and 8 kGy, using the Co-60 Ob-Servo Sanguis Self-Shielded Gamma Irradiator at the Philippine Nuclear Research Institute. The irradiated extracts were stored in a cooler, placed within a secondary compartment containing dry ice as the primary coolant. This was done maintain a stable, controlled temperature environment and prevent fluctuations that may cause degradation.

Phytochemical Screening

Phytochemical screening was performed at the Natural Science Research Unit (NSRU) Laboratory at Saint Louis

University to test the presence of the following compounds:

Test for Alkaloids

In preparation, 5 mL of each sample from a different dose group was diluted with 5 mL 1% aqueous HCl. For Mayer's test, 1 mL of the filtrate was treated with 2 mL Mayer's reagent. The formation of white or pale precipitate confirms the presence of alkaloids. As confirmatory tests, Wagner's and Dragendorff's tests were performed. In Wagner's test, 1 mL of the filtrate was treated with 2 mL Wagner's reagent. Then each of the samples from different dose groups was observed for any reddish-brown precipitate to confirm the presence of alkaloids. For Dragendorff's test, 1 mL of the filtrate was treated with Dragendorff's reagent, similarly each sample from different dose groups were observed for the formation of orange or red-orange precipitate to confirm the presence of alkaloids.

Test for Steroids

The Liebermann-Burchard test was used for the detection of steroids. 1 mL of the extract was diluted with a few drops of chloroform, 3 mL of glacial acetic acid and 3 mL of acetic anhydride. The solution was warmed for a few minutes and was cooled under running water before adding a few drops of concentrated sulfuric acid. The samples were observed for the appearance of reddish violet color at the junction between the 2 layers while the appearance of bluish-green color acetic acid layer to confirm the presence of unsaturated sterols and/or triterpenes.

Test for Anthraquinones Glycosides

5 mL of the stock plant extracts from each sample were evaporated. The residues were dissolved with 10 mL distilled water and were filtered with 5 mL of benzene twice. Each sample was divided into 2 portions where the 1st portion was designated as control, while the second portion was treated with 5 mL of ammonia solution. It was then observed for the formation of a red color at the alkaline layer for confirmation of the presence of Anthraquinones glycosides.

Test for Cardiac Glycosides

Cardiac glycosides were tested using the Keller-Killiani test. 1 mL of the crude extract was hydrolyzed with HCl. The samples were then mixed with 5 mL 70% alcohol. After 2 minutes, each filtrate was mixed with 5 mL of chloroform and was evaporated in a porcelain dish to separate the chloroform layer and the solvent. After cooling, it was dissolved in 3 mL glacial acetic containing 2 drops of 5% Ferric chloride solution before transferring to the surface of 2 mL concentrated sulfuric acid. Each of the samples from different dose groups were observed for the appearance of a reddish-brown layer formed at the junction of the 2 liquids and the upper layer that slowly became bluish-green and darkening with standing confirms the presence of cardiac glycosides.

Test for Flavonoids

The presence of flavonoids was tested using the Shinoda's and Lead Acetate tests. For Shinoda's test, approximately 1 gram of extract per treatment group was dissolved in 5 mL of 98% ethanol. A small piece (1) of magnesium foil metal was then added, followed by the dropwise addition of concentrated hydrochloric acid (HCl). After allowing the mixture to stand for a few minutes, the intense cherry red color developed, indicating the presence of flavonoids. The lead acetate test was also performed to confirm the result. In this test, 1

mL of the ethanolic extract of the treatment groups was mixed with three drops of 10% lead acetate solution. The sample was observed for the formation of a yellow precipitate in confirming the presence of flavonoids.

Test for Saponins

The presence of saponins was evaluated using the Froth Test. In this method, 2 mL of the ethanolic extract was mixed with 10 mL of distilled water in a test tube and shaken vigorously for one minute. It was then observed for the appearance of a persistent foam layer that should be approximately 1 cm thick as an indication for the presence of saponins.

Test for Phenolics

The presence of phenolic compounds was determined using the ferric chloride test (*for phenolics)*. In this test, 1 mL of 10% ferric chloride solution was added to 1 mL of the ethanolic extract from each treatment group. The sample was then observed for the appearance of a dark green coloration or a bluish-black precipitate as an indication of the presence of phenolics.

Test for Tannins

The presence of tannins was assessed using both the gelatin test and the ferric chloride test *(for tannin*). The plant extract was first dissolved in 5 mL of distilled water for both tests. In the gelatin test, four drops of a gelatin salt solution (prepared using 1% gelatin and 10% NaCl) were added to the mixture. The formation of a white precipitate indicated the presence of tannins. For the ferric chloride test, 1 mL of the ethanolic extract was mixed thoroughly with 5 mL of distilled water. A few drops (3–4) of 10% ferric chloride solution were then added, followed by further mixing. The samples were then observed for the appearance of a dark green or black precipitate for the confirmation of the presence of tannins.

CAM Assay

An Institutional Animal Care and Use Committee (IACUC) form was submitted to the BSU - Cordillera Center for Animal Research & Development (CCARD) in La Trinidad, Benguet for ethical review and approval of procedures involving Philippine duck (*Anas luzonica*) embryos. After the certification from IACUC was granted, a total of sixty (60) fertilized duck eggs were collected from a producer in San Jose, Nueva Ecija at zero (0) days old and were incubated for eight (8) days at a temperature between 38 and 40 °C. On the eighth day of incubation, the eggs were transported to Baguio City and further incubated until they reached ten (10) days of age. Prior to the experiment, the eggs were cleaned using seventy percent (70%) isopropyl alcohol to eliminate surface contaminants. A candling procedure was performed to assess embryological development. The position of the air cells within each egg was marked. The embryos were divided into three (3) treatment groups based on the irradiated ethanolic plant extract: the control group, which received the unirradiated (control) extract; the low-dose group, which received a four (4) kGy dose; and the high-dose group, which received an eight (8) kGy dose. Sixty (60) paper discs were prepared, sanitized, and soaked in plant extracts that had been subjected to their respective radiation doses. Twenty (20) eggs were assigned to each treatment group. The marked area of each egg was carefully cracked open using sterile tweezers, taking care not to damage the integrity of the inner membrane. The exposed membrane was gently moistened with distilled water using a cotton ball to aid adhesion of the paper discs.

The soaked discs were then placed at the center of the exposed membrane using sterile tweezers. Following application, the eggs were covered with cling wrap to prevent contamination and labeled according to the radiation dose of the *Pinus kesiya* (pine needle) extract applied. The eggs were then incubated at a temperature above 38°C but not exceeding 40°C for 48 hours at the DOST Tuklas Lunas Laboratory, Saint Louis University, Baguio City. After the incubation period, the eggs were removed from the incubator, and the cling wrap was carefully removed. A second candling procedure was performed to observe and qualitatively record variations in neovascular density and branching patterns across treatment groups. The embryos were then properly disposed of by placing them in a container and discarding them in designated biohazard waste bins to prevent contamination.

Qualitative & Quantitative Analysis

This study employed an experimental design incorporating both qualitative and quantitative analysis methods to assess the phytochemical composition and angiogenic activity of *Pinus kesiya* extracts subjected to gamma irradiation. Phytochemical screening utilized colorimetric assays (e.g., Mayer's, Dragendorff's, and Ferric Chloride tests) to detect compound presence, with results categorized using intensity grades: negative (–), weakly positive (+), moderately positive (++), and strongly positive (+++). These classifications reflected relative compound detectability but did not quantify exact concentrations.

RESULTS/FINDINGS

For the CAM assay, vascular responses were initially evaluated qualitatively based on observable features such as vessel density, branching complexity, and orientation relative to the extract-soaked paper disc. These visual criteria were used to assess pro- or anti-angiogenic trends across dose groups.

To strengthen the reliability of these observations, quantitative data analysis was also performed. Mean vessel counts were recorded per embryo for each treatment group (control, 4 kGy, and 8 kGy). The data were subjected to one-way Analysis of Variance (ANOVA) to determine whether radiation exposure had a statistically significant effect on angiogenesis. Tukey's Honestly Significant Difference (HSD) test was then applied for post-hoc comparison between groups. A significance level of $p < 0.05$ was used to determine statistical relevance. This combination of qualitative and quantitative approaches provided a more comprehensive understanding of the biological responses induced by gamma-irradiated extract

(-) = negative (compound not detected) (+) = Weakly positive (++) = Moderately positive (+++) = Strongly positive

Table 1 *Phytochemical Screening Result*

Compound	Test	control	4 kGy	8 kGy	Main Properties
Alkaloids	*Mayer's Test*	(-)	(+)	(++)	Anti-proliferative, anti-bacterial, anti-oxidant
	Wagner's Test				
	Dragendorff's Test				
Steroids	*Liebermann-Burchard Test*	(+)	(+)	(+)	Anti-tumor, antibacterial, antihelminthic, cytotoxic
Anthraquinones Glycosides		(+)	(+)	(+)	Laxative, Anti-inflammatory, Anti-microbial, Anti-angiogenics
Cardiac Glycosides	*Keller-Killiani Test*	(+)	(++)	(+++)	Cardiotonic effect, Anti-angiogenic
Flavonoids	*Shinoda's Test*	(+)	(+)	(+)	Anti-angiogenic, antioxidant, anti-inflammatory, antiviral
	Lead Acetate Test				
Saponins	*Froth Test*	(-)	(+)	(++)	Anti-angiogenic, anti-inflammatory, antioxidant
Phenolics	*Ferric Chloride Test for Phenolics*	(+)	(+)	(+)	Anti-angiogenic, anti-inflammatory, antioxidant
	Gelatin Test				
	Ferric Chloride Test for ***Tannins***	(+)	(+)	(+)	Anti-inflammatory, antioxidant, antiproliferative, anti-angiogenic

Table 1 reveals the phytochemical screening results for *Pinus kesiya* extracts subjected to gamma irradiation at control, 4 kGy, and 8 kGy. Several classes of secondary metabolites were assessed using standard qualitative tests. The presence and relative reactivity of each compound class are indicated using a grading system: negative (-), weakly positive (+), moderately positive (++), and strongly positive (+++).

However, it is important to note that not all tests used in this screening provide a semi-quantitative basis for interpreting compound concentration. In particular, some tests only yield a presence/absence result based on color change or chemical reactivity, without observable variation in precipitate formation or intensity. As a result, some compound classes show consistent results across all irradiation doses (e.g., flavonoids and phenolics), not necessarily because their concentration is unchanged, but because the test method does not allow finer differentiation. Therefore, consistent positive results across doses (e.g., (+), (+), (+) should be interpreted as presence only, not necessarily stability in quantity.

Alkaloids

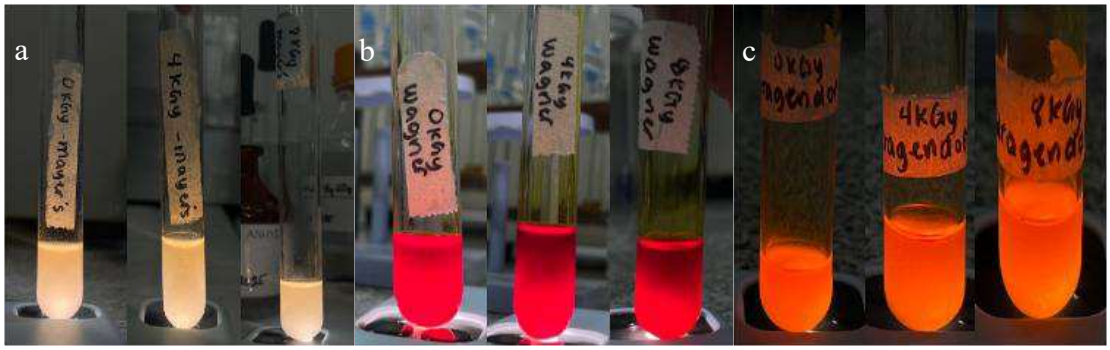

Fig 1: *Mayer's (a); Wagner's (b); Dragendorffs Test (c)*

Alkaloids were not detected in the control extract but were present at 4 kGy and further increased at 8 kGy, as evidenced by progressively stronger reactions in Mayer's ***(figure 1a)***, Wagner's ***(figure 1b)***, and Dragendorff's ***(figure 1c)*** tests. The increasing intensity, particularly in these tests which indicate precipitate formation, suggests that gamma irradiation may enhance the extractability or biosynthesis of alkaloids in *Pinus kesiya* extracts.

Steroids and Anthraquinones Glycosides

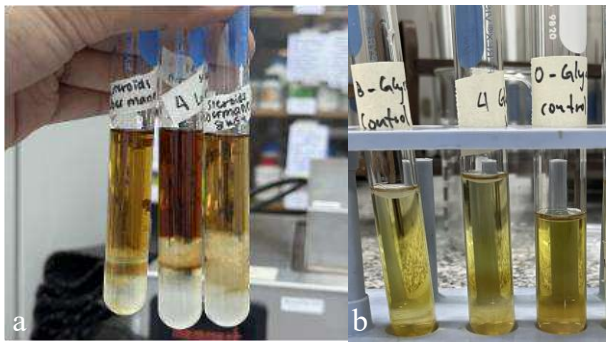

Fig 2: *Liebermann-Burchard test for Steroids (a); Test for Anthraquinone Glycosides (b)*

The presence of steroids, as determined by the Liebermann-Burchard tests (***figure 2a)***, and anthraquinone glycoside ***(figure 2b)***, remained constant across all irradiation doses. Since the test does not provide gradation in intensity, while these compounds are clearly present, no conclusion can be drawn regarding changes in concentration.

Cardiac Glycosides

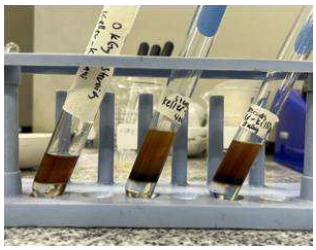

Figure 3: Keller-Killiani's test for Cardiac Glycosides

A dose-dependent increase in cardiac glycoside presence, with a weakly positive result at the control group that intensified to strongly positive at 8 kGy, as shown in the Keller-Killiani test ***(figure 3)***. This suggests that gamma irradiation may enhance the concentration or promote the transformation of precursors into active cardiac glycosides.

Flavonoids

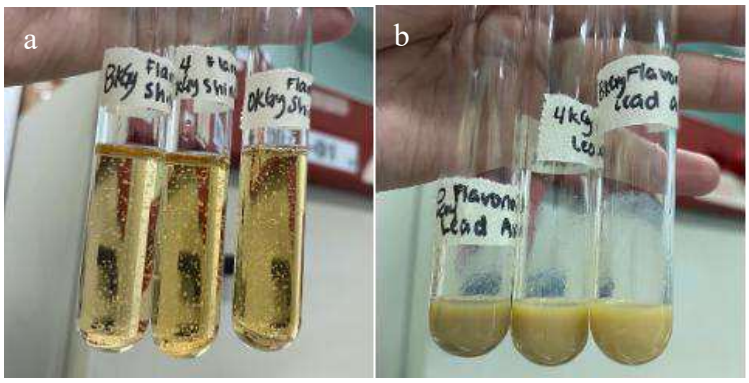

Figure 4: Shinoda's (a) and Lead Acetate test for flavonoids (b)

Flavonoids were detected at all doses using the Shinoda ***(figure 4a)*** and lead acetate tests ***(figure 4b)***, with no apparent change in intensity. As these methods are based on color change without precipitate formation, they confirm the presence of flavonoids but do not indicate differences in concentration across doses.

Saponins

Figure 5: Froth test for Saponins

Saponins tested negative at the control group, with foam formation not exceeding 1 cm in the Froth test ***(figure 5)***. However, they were detected at 4 kGy and more strongly at 8 kGy, suggesting that irradiation may facilitate their release or conversion into detectable forms.

Phenolics and Tannins

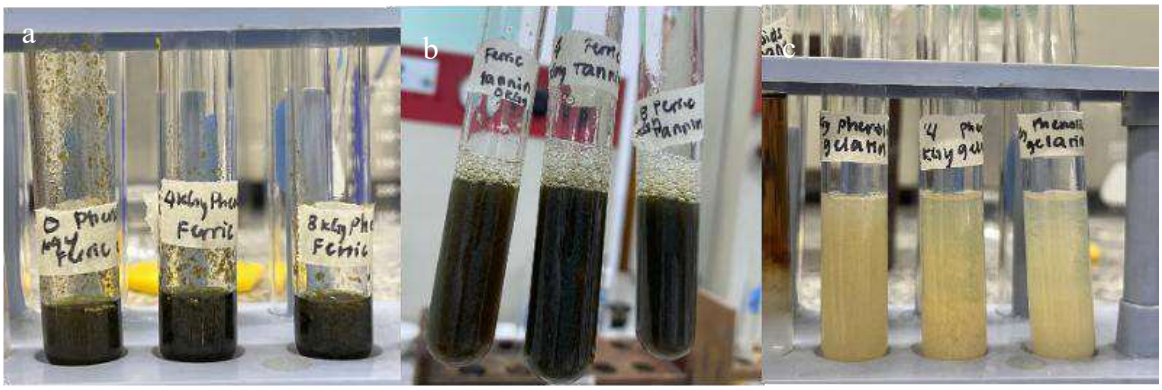

Figure 6: phenolics (a); tannins (b); Gelatin test for phenolics (c)

Phenolic compounds, including tannins ***(figure 6b)***, were confirmed at all irradiation levels using ferric chloride (***figure 6a)*** and gelatin tests ***(figure 6c)***. These tests indicate the presence of phenolics but do not provide a measure of concentration, so while the compounds are present, no conclusions can be made regarding variation in their levels.

Table 2 *CAM Assay Result*

control	4 kGy	8 kGy

1	One long vessel with branching around the disc; three thin vessels; one directed toward disc	5	One thick vessel with visible branching and four additional thin vessels spread near the disc.	5	One thick vessel directed towards the disk, four branching out underneath.	5
2	One long vessel encircling the disc; one thin vessel near the disc	2	One thick, straight, long vessel extends from the disc with two thin vessels.	3	Three vessels of the same origin feeding directly on the disc, with one major and some thin, minor branching within the surrounding.	4
3	Two branched vessels directed toward the disc	2	One thick vessel with branching (most prominent vessels are straight), with three thin vessels surrounding the disc.	4	One mother vessel with four thick, major branches surrounding the disc.	5
4	Embryo is dead	0	Four thick straight vessels projecting from the disc and with two thin vessels.	6	One circular vessel with two thick, major vessels connected to the disc.	3
5	Embryo is dead	0	One thick vessel with branching and four thin vessels surrounding the membrane.	5	One mother vessel with a network of thick vessels within the surrounding of the disc.	3
6	One thick, long vessel encircling the disc	1	Three thick, straight vessels extend from the disc.	3	Three thick, major vessels directed toward the disc, one branching out the disc crossing through the midline.	4
7	One long vessel around the disc with thin branching	2	Three thick, straight vessels with branching.	3	Two thick vessels, not passing through the disc, with two minor thin branches.	4
8	One large vessel directed toward disc; one large vessel encircling the disc	2	Three thick vessels with visible branching and one thin vessel extending around the disc.	4	Two thick vessels directed towards the disc, with one minor branching.	3
9	One large vessel with branches directed toward the disc	2	One thick vessel with multiple branches around the cracked area of the shell.	1	One mother branch of vessel with two minor branching in a circular direction around the disc.	3
10	Embryo is dead	0	Two thick vessels converging near the disc and three thin vessels.	5	One mother vessel seeping directly to the disc, with six major and minor branches.	6
11	One long vessel; two branches extending away from the disc	3	Two thick vessels with moderate thickness and two thin vessels.	4	Four thick vessels surrounding the disc.	4
12	Embryo is dead	0	Two thick vessels with multiple branches surrounding the disc and three thin vessels.	5	Embryo is dead.	0
13	One long vessel directly hitting the disc; small, faint branching	2	One thick, long vessel with multiple branches and one thin vessel.	2	One mother vessel branch with cited thickness directed to the disc, with four major branches.	5
14	One thick, long vessel near the disc; one thin vessel directed toward the disc	2	One thick vessel with multiple branches and three thin vessels around the disc.	4	One thick, major vessel feeding straight towards the disc, with two major and other minor branches.	5
15	Embryo is dead	0	One thick, straight, long vessel with multiple branches extending along the disc.	1	Two thick vessels with major branching around the disc.	4

16	Three thin vessels directed toward the disc, faint and unnoticeable	3	Three thick vessels extending radially from the disc, without thin vessel presence noted.	3	One thick vessel surrounding the disc in a circular direction, with four major branches.	5
17	One thick, long vessel encircling the disc	1	Six thick vessels clearly emerge and radiate across the disc membrane.	6	Embryo is dead.	0
18	Three thin vessels directed toward the disc	3	Three thin vessels are visible, with minimal branching.	3	One thick vessel directed towards the disc, with five major branches also connected.	6
19	Four thin vessels; one directed to disc, two surrounding, one extending far from the disc	4	One thick vessel near the disc, along with three thin vessels.	4	One circular vessel around the disc with five other thick vessels connected.	6
20	Embryo is dead	0	Three thick vessels emerged from the disc, and one thin vessel.	4	One mother vessel feeding directly towards the disc, with four other connected branches.	5
	(34/20=) **1.70**		(75/20=) **3.75**		(80/20=) **4.00**	

Table 2 presents the results of the CAM assay, conducted under the supervision of the FRIP and authorized by a licensed veterinarian. A clear trend was observed in the control, the control group exhibited minimal vascularization, with a mean vessel count of 1.70. The vessels observed were generally thin and sparsely distributed around or toward the disc. Several embryos in this group were non-viable.

Vascular development was more noticeable in the 4 kGy group, with a mean vessel count of 3.75. Most embryos displayed thicker vessels with visible branching patterns. These vessels were quite thick—though not excessively so—and extended around or near the disc. The number of viable embryos was also higher compared to the control group.

At 8 kGy, vascularization was most prominent, with a mean vessel count of 4.00. The vessels were thicker and more numerous, often forming intricate networks around the disc area. Extensive branching and multiple major vessel structures were commonly observed. Additional representative CAM images are provided in ***Appendix J*** for visual confirmation of vessel branching.

The descriptive statistics of CAM assay results across the radiation exposure groups are presented in ***Table 3***

Table 3 *Descriptive Statistics for CAM Assay Across Radiation Exposure Groups*

		CAM Result	
Exposures	*n*	*M*	*SD*
Control	20	1.70	1.455
4 kGy	20	3.75	1.410
8 kGy	20	4.00	1.686

M = Mean

SD = Standard deviation

A one-way analysis of variance (ANOVA) was performed to examine the effect of radiation exposure on CAM assay outcomes (**Table 4**).

Table 4 *One-Way ANOVA Summary for the Effect of Radiation Exposure on CAM Assay Results*

Source	*SS*	*df*	*MS*	*F*	*p*
Between groups	63.7	2	31.85	13.75862	1.33196E-05
Within groups	131.95	57	2.314912		
Total	195.65	59			

SS = Sum of squares
df = Degrees of freedom
MS = Mean square
F = F value
p = p-value

A one-way analysis of variance (ANOVA) was conducted to examine the effect of radiation exposure on CAM assay outcomes across three groups: Control (not exposed), 4 kGy, and 8 kGy. The results revealed a statistically significant effect of exposure level on CAM values, $F(2, 57) = 13.759$, $p < .001$. This indicates that there are significant differences in CAM assay outcomes among the exposure groups

Post-hoc comparisons using the Tukey HSD test indicated significant differences between groups, as shown in ***Table 5***.

Table 5 *Tukey HSD Post-Hoc Comparison of CAM Assay Results Between Exposure Groups*

Comparison (Exposure 1 vs. Exposure 2)		**Test Statistic**	**p value**	**Interpretation**
Control vs.	4 kGy	-2.05	**<.001**	**Significant**
	8 kGy	-2.30	**<.001**	**Significant**
4 kGy vs.	8 kGy	-0.20	.862	Not significant

Post-hoc analysis using Tukey's Honestly Significant Difference (HSD) test revealed that both the 4 kGy and 8 kGy groups demonstrated significantly greater vascularization compared to the control group ($p < 0.001$). However, the difference between the 4 kGy and 8 kGy groups was not statistically significant ($p = 0.862$), suggesting that the angiogenic effect may plateau beyond a 4 kGy radiation dose.

DISCUSSION

The findings of this study highlight a multifaceted interaction between gamma irradiation, phytochemical transformation, and angiogenic outcomes as evaluated through the CAM assay. These outcomes underscore the importance of understanding both the biochemical and physiological factors that influence vascular development. Gamma irradiation, known for generating reactive oxygen species (ROS) and inducing structural changes in bioactive compounds, can enhance the solubility, extractability, and biological activity of phytochemicals (Kavita et al., 2025; Zaib et al., 2018). Such changes may either potentiate or suppress angiogenic effects, depending on the type of compound and the radiation dose applied. In the present study, phytochemicals like alkaloids, cardiac glycosides, and saponins exhibited dose-dependent increases in detectability post-irradiation, which may explain the enhanced neovascularization observed in the CAM model. However, this angiogenic effect plateaued between 4 kGy and 8 kGy, indicating a potential biological threshold. Additionally, extrinsic factors such as hypoxia, mechanical stress, and microenvironmental pH within the embryo system may have further influenced endothelial behavior and vessel formation. The CAM assay, therefore, not only serves as a model for angiogenesis but also reflects the integrated impact of phytochemical modulation and embryonic physiological conditions. The following sections examine these findings in detail, structured according to the study's objectives, to elucidate the mechanisms driving the angiogenic response and the broader therapeutic potential of gamma-irradiated *Pinus kesiya* extracts.

Effect of gamma radiation on phytochemicals

The phytochemical screening results of *Pinus kesiya* ethanolic extracts subjected to increasing gamma irradiation doses (control, 4 kGy, and 8 kGy) reveal a compound-specific and dose-dependent response to ionizing radiation. Gamma irradiation acts primarily through the radiolysis of water, generating reactive oxygen species (ROS), along with direct ionization and excitation effects that can cleave covalent bonds, oxidize functional groups, and promote structural rearrangements (Zaib et al., 2018; Naveed et al., 2018; Nuclear Regulatory Commission, 2023). These physicochemical changes collectively influence the solubility, extractability, and biological activity of plant-derived compounds. Among the most notable alterations was the emergence of alkaloids, which were absent in the control extract but became detectable at 4 kGy and intensified further at 8 kGy. This pattern may reflect a radiation-induced transformation or increased extractability of nitrogen-containing precursors. Alkaloids often contain labile bonds especially susceptible to gamma irradiation, leading to structural rearrangements and increased bioavailability (Wieczorek et al., 2015; Jo et al., 2022). Their progressive appearance across increasing doses likely reflects improved detectability may be due to bond cleavage and oxidative modification. Moreover, alkaloids are known for their antibacterial, antioxidant, and antiproliferative properties, which may influence angiogenic activity observed in bioassays (Khattak & Rahman, 2016).

Similarly, cardiac glycosides displayed a clear dose-dependent intensification from weakly positive at control to strongly positive at 8 kGy. These compounds often exist in bound forms, and

gamma irradiation may enhance their detectability by hydrolyzing glycosidic linkages or degrading glycone moieties (Xu et al., 2022). Saponins, which were undetectable at control but appeared after irradiation—with visible foam formation—follow the same pattern. These surface-active compounds are sensitive to irradiation-induced oxidative stress and bond cleavage, which may either activate biosynthesis or release them from bound forms (Saeid et al., 2023).

In contrast, flavonoids, phenolics, tannins, steroids, and anthraquinone glycosides remained qualitatively constant across all irradiation doses. However, this does not necessarily indicate a lack of response. Many of these compounds are chemically stable due to their conjugated aromatic rings or rigid tetracyclic structures, which resist radiation-induced breakdown (Elmehy, 2018; Silva et al., 2019). For instance, phenolics and flavonoids can retain their antioxidant activity post-irradiation due to resonance stabilization, while steroids are stabilized by their fused ring systems. Though qualitative tests such as the ferric chloride test for phenolics and the Liebermann–Burchard test for steroids did not reveal changes, such methods are limited in sensitivity and may not detect subtle shifts in concentration or structure (Shaikh & Patil, 2020).

These observations are consistent with the concept of radiation hormesis, wherein low to moderate doses of ionizing radiation elicit beneficial biological effects, such as enhanced phytochemical activity or increased compound yield. Conversely, excessive doses may lead to degradation of bioactive constituents (Jo, An, & Kim, 2022). In the present study, irradiation at 4 kGy appeared sufficient to induce compound transformation without causing significant degradation, while exposure to 8 kGy further intensified these effects, indicating a positive dose-dependent relationship between gamma irradiation and the release or activation of bioactive phytochemicals.

Additionally, while not directly tested in this study, compounds such as coumarins, phytosterols, terpenoids, lignins, xanthones, and quinones—reported in the phytochemical profile of *Pinus kesiya* (***Table 6)***—may also contribute to the observed biological activity. These metabolites are known to exert anti-inflammatory, antioxidant, anticancer, or angiogenesis-modulating effects (Zhao et al., 2020; Ullah et al., 2022). Their potential synergistic or antagonistic interactions with detected compounds should be considered when interpreting CAM assay results, and their profiling is recommended in future work.

Table 6 *Other Compounds that are known to be present in P. kesiya*

Compounds	Main Properties
Coumarin	Anti-microbial, Anti-cancer, Anti-mitotic, Anti-oxidant, Anti-inflammatory, and Anti-coagulant
Phytosterols	Anti-inflammatory, anti-cancer
Quinones	Anti-angiogenic, anti-neoplastic, cytotoxic agents, anti-microbial
Terpenoids	Antimicrobial, antitumor
Resins	Anti-microbial, Anti-bacterial
Diterpenes	Anti-inflammatory, anti-angiogenic\
Xanthones	Anti-fungal, Anti-bacterial, Anti-cancer
Lignins	Antioxidant activity, Antimicrobial activity, Biocompatibility
Reducing Sugars	Anti-oxidant potential

In summary, gamma irradiation at 4 and 8 kGy induces dose-responsive phytochemical changes in *Pinus kesiya* ethanolic extracts, particularly promoting the emergence and intensification of alkaloids, cardiac glycosides, and saponins. The qualitative constancy of other groups may reflect limitations in detection methods rather than a true lack of chemical transformation. These findings suggest that gamma irradiation may serve as a tool to modulate the phytochemical profile and potential bioactivity of plant extracts.

Oxidation

The oxidative effects induced by gamma irradiation may provide a possible basis for the phytochemical transformations observed in this study. Gamma radiation generates hydroxyl radicals (-OH) through the radiolysis of water, which in turn initiate structural oxidation, bond cleavage, and rearrangements in plant compounds. These effects vary significantly across different phytochemical groups and are highly dose-dependent. Findings in this study, which showed an emergence or intensification of certain compounds—such as alkaloids, cardiac glycosides, and saponins—at 4 kGy and 8 kGy, are consistent with known oxidative behaviors under gamma exposure.

Alkaloids are among the most susceptible to oxidative transformation. The appearance and intensification of alkaloids in irradiated samples likely result from the oxidative cleavage of nitrogen-containing precursors. As described by Shim et al. (2022), gamma irradiation induces oxidative modifications at moderate doses by promoting oxygen incorporation into molecular structures, which may enhance solubility or reactivity. However, at higher doses, radiation leads to extensive bond cleavage, resulting in fragmentation of the core ring systems of bioactive compounds. While this study's applied doses (4–8 kGy) are significantly lower than those causing total degradation (50 kGy+), they may have facilitated molecular rearrangements that contributed to the increased detectability of alkaloids.

Steroids, which remained qualitatively stable across doses in the phytochemical analysis, are known to undergo oxidative degradation at standard sterilization doses (25 kGy). However, at lower doses like those used in this study, oxidative damage is minimal, supporting the observed qualitative constancy. According to Cauwenbergh et al. (2022), controlled doses such as 4–8 kGy help preserve molecular integrity while avoiding the formation of oxidative impurities.

The limited oxidative data on cardiac and anthraquinone glycosides prevents definitive conclusions, but their increased visibility at higher irradiation doses in this study may stem from glycosidic bond cleavage or increased solubility post-oxidation. This interpretation aligns with similar oxidative effects observed in structurally related compounds.

Flavonoids and phenolics demonstrated no qualitative changes across treatments, a result supported by existing literature. According to Patil et al. (2018), these compounds generally respond well to low-dose irradiation between 50 and 100 Gy, as it enhances antioxidant activity and preserves structural integrity, whereas degradation only becomes significant at much higher exposure levels—typically 150 and 200 Gy or 25 and 35 kGy—which far exceeds the doses applied in the current study. Thus, their apparent stability in the analysis likely reflects both their chemical resilience and the relatively mild oxidative environment produced at 4–8 kGy.

Tannins in this study showed no distinct qualitative variation, yet literature suggests they undergo slight enhancement at doses up to 7 kGy and degradation beyond 9 kGy (Hasanin et al., 2022). The use of doses just below the degradation threshold may have maintained their presence, although any oxidative modifications or potential roles in biological activity remain unclear and may not be detectable by qualitative tests.

Saponins, absent at the control group but present at irradiated doses, are known to resist direct oxidative breakdown. However, improved extraction or release from cell matrices due to mild oxidative membrane disruption—similar to the effects seen under E-beam irradiation—likely explains their increased detectability (Shi et al., 2022). This mechanistic explanation aligns with the enhanced foam formation observed post-irradiation.

For less abundant or more chemically inert compounds such as coumarins, phytosterols, quinones, and terpenoids, responses to gamma irradiation vary and are less well-characterized. While coumarins do undergo hydroxylation under -OH exposure (Náfrádi et al., 2019) the doses required for significant degradation often exceed the levels applied in this study. Likewise, terpenoids may exhibit enhanced bioactivity at lower doses and degradation at higher ones (Hadiati et al., 2021), but their apparent stability here suggests the irradiation protocol preserved these compounds, though further analysis is needed to determine any subtle structural or functional changes.

Resins and lignins, which are complex, high-molecular-weight polymers, require extreme irradiation levels (often >500 kGy) for significant degradation (Wu et al., 2018). Therefore, their stability in the samples is expected. The same applies to diterpenes and xanthones; while xanthones like mangiferin are known to degrade into hydroxylated derivatives under ROS attack (Jo et al., 2016), the low to moderate doses used may have induced limited structural modification. However, their specific functional outcomes were not directly evaluated in this study.

Formation of free radicals

In this study, the exposure of *Pinus kesiya* crude extracts to gamma radiation may have induced the formation of various reactive radicals across multiple phytochemical classes, which could have contributed to the observed pro-angiogenic

activity in the CAM assay. In phenolic compounds, flavonoids, and xanthones—compounds identified in all dose groups—gamma irradiation facilitates the generation of phenoxyl radicals by promoting hydrogen atom transfer. This process may enhance their intrinsic antioxidant and anti-inflammatory properties, particularly at the 4 kGy and 8 kGy doses used in the experiment (Platzer et al., 2022). These enhanced properties are biologically relevant, as oxidative stress modulation and inflammation is known to influence angiogenic activity.

In the case of alkaloids, which became detectable only at irradiated doses, nitrogen-centered radicals may have formed, which are reported to inhibit abnormal cellular proliferation and could potentially support controlled angiogenic responses (Mladenova et al., 2025). Cardiac glycosides—shown in this study to increase in intensity with radiation dose—may have formed carbon- and oxygen-centered radicals, subtly altering their glycosidic structures and possibly enhancing their angiogenic or cardiotonic bioactivity. Similarly, saponins, which appeared only after irradiation, react with water molecules to form additional radicals that can amplify their anti-inflammatory and pro-regenerative functions (Hasanin, et al., 2022).

Other phytochemicals found in *P. kesiya*, including steroids, coumarins, phytosterols, terpenoids, diterpenes, and reducing sugars, are also subject to radical formation under irradiation, particularly carbon-centered radicals. While such radicals can be cytotoxic at high concentrations, at controlled doses they may selectively enhance the biological behavior of these compounds, including antioxidant activity and possible modulation of angiogenesis-related pathways. Furthermore, anthraquinone glycosides and quinones in the extract likely underwent mild oxidation, forming semiquinone radicals that are associated with anti-inflammatory effects (Yazan et al., 2014). Lignins, also present in *P. kesiya*, contribute additional phenoxyl and semiquinone radicals when irradiated, further supporting antioxidant capacity.

Collectively, the potential gamma radiation-induced radical transformations may help explain the increased vessel formation observed in the CAM assay, particularly in the 4 kGy and 8 kGy groups. By potentially enhancing the biological activity of key phytochemicals, gamma irradiation may have contributed to the extract's vascular effects through modulation of angiogenesis-related responses. These findings suggest that gamma irradiation may alter the phytochemical composition and influence the angiogenic potential of *P. kesiya* extracts.

CAM Assay

Using one way ANOVA, the statistical analysis confirmed that gamma irradiation had a significant effect on the vascular response elicited by Pinus kesiya crude extracts, $F(2, 57) = 13.76$, $p < 0.001$. Post hoc comparisons using Tukey's HSD revealed significant differences between the control and both irradiated groups ($p < 0.001$), while the difference between 4 kGy and 8 kGy was not significant ($p = 0.862$). These results validate that irradiation enhanced angiogenic activity, particularly at 4 kGy, and that the response may have plateaued beyond this dose.

The significant difference between the control and 4 kGy group suggests that irradiation at 4 kGy effectively enhanced the pro angiogenic properties of the extract. This

may be attributed to improved extractability or reactivity of certain phytochemicals after moderate radiation exposure. Hammad et al. (2024) explained that moderate oxidative stress induced by irradiation activates NRF2 signaling, which promotes cellular regeneration and angiogenesis. Jabbari et al. (2019) also reported enhanced angiogenic and wound healing effects in HUVECs exposed to irradiated conditioned media, demonstrating that irradiated substances can trigger vascular responses. Zaib et al. (2018) further found that antioxidant and biological activity increased in medicinal plants subjected to low to moderate gamma doses, consistent with the vessel proliferation observed in the CAM assay after 4 kGy exposure.

The comparison between the control and 8 kGy groups also yielded a statistically significant difference, indicating that higher-dose irradiation still promoted vascularization relative to the non-irradiated extract. However, the increase in angiogenic activity at 8 kGy may not necessarily reflect a linear relationship. Sharma et al. (2020) and Pradeep et al. (2020) noted that gamma irradiation can modify phenolic and flavonoid structures to improve bioactivity, while Song et al. (2017) showed enhanced antioxidant performance in plant-based systems post-irradiation. These mechanisms may underlie the angiogenic response seen at 8 kGy. Furthermore, Marques et al. (2020) demonstrated that low-dose radiation could stimulate angiogenesis in zebrafish embryos, reinforcing the potential relevance of radiation-enhanced plant extracts in vascular models like the CAM assay.

Despite these findings, the absence of a significant difference between the 4 kGy and 8 kGy groups suggests that the biological response reaches a threshold, beyond which further radiation does not yield additional angiogenic benefit. This plateau effect is well-documented in literature. Kim et al. (2012) and Zaib et al. (2018) both observed that excessive irradiation can reduce efficacy due to oxidative degradation or molecular saturation. Kavita et al. (2025) also reported that the enhancement of antioxidant activity in irradiated onion skin extracts peaked at an optimal dose, supporting the idea that biological effects do not always increase with higher exposure. Jung (2015) added that phytochemical responses to radiation may not be visibly dose dependent, indicating that observed bioactivity could result from cumulative or synergistic effects rather than measurable changes in individual compounds.

Effects of the identified and other bioactive compounds in angiogenic activity

Alkaloids have been found to accelerate wound healing in full-thickness cutaneous wound models in rats by promoting angiogenesis and in regenerating tissues. They stimulate cellular proliferation, migration, and tube formation, and maximizes capillary growth, potentiating the effects of VEGF in an angiogenic cascade. It is likely that the presence of alkaloids in the irradiated crude extracts may have contributed to the observed increase in vessel formation in the duck embryos (Shi et al., 2022).

Coumarins exhibit hepatotoxicity, dermatological toxicity, and reproductive and developmental toxicity. Depending on presence and concentration, these either promote angiogenesis, or cause necrosis and apoptosis (Heghes et al., 2022). While this study did not isolate or quantify coumarins, their potential presence in the extract may be

associated with the embryo mortality observed across all dose groups, although this remains speculative and requires further investigation.

Diterpenes are susceptible to inducing angiogenesis during biotransformation, which occurs during the formation and growth of an embryo. During biotransformation, the cytotoxicity of diterpenes decreases, causing susceptibility to angiogenesis and carcinogenesis. Since the duck embryos used were 10 days old and undergoing biotransformation, this may explain why there is a number of vessel growth and development in all dose groups, even in the control (de Sousa et al., 2018).

Flavonoids have been demonstrated to have pharmacological effects such as upregulating the expression levels of morphogenetic proteins and promoting osteoblast proliferation in growing bones. These have the potential to promote vascularization and vessel generation, and to increase fibrous connective tissue formation in bone and vessels. This fact is exhibited in the duck embryos, where there were cited major and minor branchings of vascularization in all dose groups (Shen et al., 2022). Lignins improve blood flow by its ability to enhance the angiogenic capacity of the endothelial cells. It has a prominent proangiogenic effect. It can thus be suggested that the emergence and development of numerous mother vessels in all dose groups are due to the presence of lignins and flavonoids in the crude extract, alongside other proangiogenic compounds (Pan et al., 2023).

Polyphenols, when associated with anthocyanins, show an increased activity in fibroblast and keratinocyte migration. These allow polyphenols to amplify its ability to induce cell migration and to increase the production of the vascular endothelial growth factor (VEGF) which is an important cytokine involved in angiogenesis. It is likely that this synergistic effect allowed the growth of multiple vessels in the duck embryo (Van de Velde et al., 2019).

Phytosterols, which are richly concentrated in crude extracts, promote pro-inflammatory properties as well as the hyper-absorption and lack of elimination of triglycerides. This then leads to vessel occlusions. In the dose group control, 6 embryos were found non-viable, whilst others showed vessel growth, contributory to these may be afflicted by the phytosterols as duck embryos are rich in triglycerides (Souleymane Zio et al., 2024).

Quinones are sensitive and delicate to oxidation. These cause oxidative stress, which in turn produces oxidized cellular macromolecules associated with angiogenesis and carcinogenesis. With the dose group 8 kGy having the highest mean vessel count, oxidation of the crude extract with the 8 kGy irradiation increased, thereby increasing vessel formation and its extensive branching (Bolton & Dunlap, 2017).

Glucose, fructose, and sucrose are linked to carcinogenesis through upregulation of vascular endothelial growth factor (VEGF) and tumor cell proliferation. Since crude extracts contain significant amounts of reducing sugars, it is possible therefore that the vessel growth in all dose groups were feeding directly from the crude extract, thus why the vessels were directed towards the paper disc (Hasan et al., 2024). Resins contain statins and vascular endothelial growth factors (VEGF) which can be upregulated by sugar, hence its ability to stimulate both angiogenesis and

arteriogenesis. Pine resin, specifically, is reported to enhance angiogenesis in umbilical vein endothelial cells as well as a dose-dependent effect on angiogenesis. This is supported by the constant presence of vessel growth and networks in the three dose groups of the duck embryos (Muqri et al., 2020).

Saponins have a role in vascular changes in post-stroke ischemia as studied by Xiao et al (2024), as these promote vascular neovascularization as assessed by molecular docking methods. They revitalize the proteins HIF1-α (Hypoxia-inducible factor 1-alpha)/VEGFA (Vascular Endothelial Growth Factor A)/VEGFR2 (Vascular Endothelial Growth Factor Receptor 2) and their signaling pathways, hence promoting post-stroke ischemic region revascularization by facilitating endothelial cell and endothelial progenitor cell proliferation, resulting in restoration of oxygen and nutrient supply to tissue microvasculature. A connection is likely to exist with this fact and the number of vessel formation since duck embryos are rich in progenitor cells (Xiao et al., 2024). Xanthones are reported to dose-dependently affect cell proliferation and angiogenesis with an increase in protein expressions of vascular endothelial growth factor (VEGF) in umbilical vein endothelial cells. This is relevant to the use of duck embryos which have umbilical veins and rich in progenitor cells, and its presence of vessel growth in all dose groups (Gunter et al., 2020).

Steroids, and its types of corticosteroid hormones such as Glucocorticoids have direct effects on tumor-derived vasculature, cellular populations in a tumor microenvironment, cancer cell-derived factors, and in the upregulation or downregulation of angiogenesis in various subtypes of cancers. In Glioblastoma multiforme and bladder cancer, it decreases the effectiveness of radiotherapy and chemotherapy due to its inability to control angiogenesis. This suggests that the steroids of the crude extract allowed the continued growth and development of the vessels in all dose groups ("The Effect of Glucocorticoids on Angiogenesis in the Treatment of Solid Tumors," (Liu & Goodwin, 2020).

Tannins exhibit antinutritional properties by its engagement and formation of complexes with elements such as calcium, magnesium, and phosphorus, as well as with macromolecules like carbohydrates and proteins, rendering them to be toxic for utilization. These are classified as Class 1 Carcinogens due to their generation of necrosis, ability for mutagenesis, and inclination to increase microvasculature and vessel growth when partnered with other carcinogenesis-inducing elements like hypoxia, which was the state the duck embryos were under, thence allowing vessel formation (Sharma et al., 2019). Terpenoids have neurotoxic effects, particularly in early developmental stages of life, and serve as an antagonist to neurotransmitters which leads to sudden death due to overstimulation of the nervous system. In the control dose group, the death of 6 embryos can be correlated to the abundance of antinutritional properties of tannins and neurotoxicity of terpenoids both to the duck embryo and in the crude extract (Castilhos et al., 2017).

Anti-inflammatory compounds

Flavonoids, identified in all treatment groups of *P. kesiya* extracts, are well-documented for their anti-inflammatory properties and modulatory effects on angiogenesis. While not specifically

quantified in this study, flavonoids such as kaempferol have been shown in other contexts to exhibit angiogenic activity when interacting with vascular endothelial growth factor (VEGF) (Hu et al., 2020). This literature-based evidence supports the possibility that similar flavonoid compounds present in *P. kesiya* may contribute to the observed increase in neovascularization in the CAM assay, potentially via VEGF-mediated pathways, especially following gamma irradiation which may enhance bioavailability.

Biphasic effect

The Biphasic effect when it comes to angiogenesis may be due to synergistic interactions among the compounds used. Sample study of Liu et al. (2016) shows a result that MTS antioxidant activity showed the combination of C-3 + EGb exhibited synergistic effects especially at the ratio 5:1. Components of isorhamnetin and caffeic acid in C-3 and EGb displayed strong antioxidant activities on MTS and their combination also showed significant synergy in promoting H2O2 production. The combinations of C-3 + EGb and isorhamnetin + caffeic acid promoted CAT and SOD enzyme activities and the ratio 1:1 exhibited the strongest synergy while no obvious promotion on POD and GSH-PX enzyme activities was found. In this study, the 8 kGy group showed strong positive results for three phytochemicals—alkaloids, cardiac glycosides, and saponins—that have been previously reported to exhibit anti-angiogenic and antioxidant properties. Despite this, the 8 kGy group exhibited the highest vessel formation among all treatments, suggesting a possible biphasic or synergistic effect. These phytochemicals include Alkaloids which have antioxidant property, Cardiac Glycosides which has Anti-angiogenic property (Fu et al., 2019), and Saponins which has Anti-angiogenic and antioxidant properties (Majnooni et al., 2023). In contrast, the control where alkaloids and saponins are not detected and Cardiac Glycosides is weakly positive produces lower formation of blood vessels which supports the possibility of a biphasic effect and further discussion of its synergistic effect is done separately.

Effect of environmental conditions to phytochemicals causing angiogenesis

Angiogenesis is markedly influenced by environmental stressors such as hypoxia. In response to diminished oxygen level, cells frequently activate transcriptional pathways involving hypoxia-inducible factor 1-alpha (HIF-1α). This factor plays a central regulatory role by promoting the transcription of several genes associated with blood vessel formation (Basheeruddin & Qausain, 2024). The experiment uses cling wrap to cover the exposed membrane tightly, which may cause hypoxia. This can be a factor in which all the 3 distinct groups—control, 4 kGy, and 8 kGy—have vascularization, especially for control. The extract from *P. Kesiya* included a variety of phytochemicals. Terpenoids, alkaloids, steroids, tannins, xanthones, reducing sugar, and saponins were in order of decreasing phytochemical content from high to low. The terpenoid test revealed a very deep color, indicating that terpenoids were present in greater quantities than any other chemical.

(Pocasap et al., 2021). These compounds are rich in *P. kesiya* extracts, explaining why all dose groups exhibit vascularization in addition to hypoxia.

Tubulogenesis and neovascularization are stimulated by an extracellular acidic pH of 5.4 to 6.4, which is moderate or weak. The release of extracellular growth factors around the endothelial cells has a more indirect proangiogenic effect. Stronger alkaline pH (8.4 and 9.4) and extracellular acidic pH (4.4) inhibit neovascularization and tubulogenesis. The same things happen during tumor angiogenesis, when the metabolic processes of malignant cells produce hypoxia, an acidic environment, and a number of growth factors, including platelet-derived endothelial cell growth factor, basic fibroblast growth factor, and VEGF (Saghiri et al., 2020). Tropical pine species *Pinus kesiya* Royle ex Gordon and *P. dalatensis* Ferré coexist in the mountain ranges of south-central Vietnam at intermediate elevations, although their distribution zones are very different in size. From the Philippines to northeast India, *Pinus kesiya* is widely distributed. Its tolerance to harsh environmental circumstances, such as acidic, nutrient-poor soils, low water availability, and exposure to wildfire, accounts for its vast spread, even though it thrives on somewhat humid, well-drained soils with a sufficient supply of nutrients. Even at somewhat dry and acidic locations, this species is utilized for nature-oriented reforestation because of its excellent adaptability and comparatively quick growth, particularly in its early life stages. Although this study did not directly investigate the environmental conditions of the plant source, it is possible that these challenging growth conditions contributed to the phytochemical richness of the crude extract. The presence of these bioactive compounds may, in part, explain the observed angiogenic responses across all dose groups in the CAM assay.

Synergistic Effect

The increasing angiogenic response across dose groups - control, 4 kGy, and 8 kGy shows that certain phytochemicals may act synergistically to enhance blood vessel formation. Van de Velde et al. (2019) demonstrated that polyphenolic extracts from berries promoted fibroblast migration and tissue regeneration, thereby creating a microenvironment conducive to vascular growth. In crude extracts, phenolics can enhance the function of other compounds by modulating oxidative stress and inflammation, both of which are closely linked to angiogenic responses that result in several blood vessel formations in the 3 different dose groups.

Flavonoids, when co-present with phenolics, have been shown to increase vascular endothelial growth factor (VEGF) expression and nitric oxide (NO) production. These signaling molecules are central to the angiogenic cascade. Zhao et al. (2016) reported that quercetin, a common flavonoid, activates redox-sensitive transcription factors that upregulate pro-angiogenic genes. In crude extracts, flavonoids can work synergistically with phenolics to amplify these effects.

The role of glycosides in angiogenesis is increasingly recognized, particularly their ability to modulate membrane-bound enzyme activity and intracellular signaling. Yang et al. (2014) demonstrated that by influencing autophagy and apoptosis, low concentrations of the cardiac glycoside convallatoxin enhanced vascular modulation. When included in a multi-compound extract, glycosides may

help stabilize the angiogenic process and support endothelial responsiveness. This may help explain the observed presence of both minor and major vessel formations across all dose groups in the CAM assay.

Saponins contribute to angiogenesis by enhancing the bioavailability of co-existing compounds. They increase cell membrane permeability, allowing other phytochemicals, such as flavonoids and steroids, to more effectively penetrate an act on endothelial cells. Zhao et al. (2016) found that saponins from Polygonum hydropiper L. enhanced endothelial cell function and antioxidant activity, facilitating capillary development. With the significant number of progenitor cells in duck embryos, there is probably a relationship between this fact and the quantity of vessels that form.

Steroids, particularly phytosterols, may modulate immune responses and stabilize vascular structures, providing additional support for angiogenesis. Valerio et al. (2017) suggested that phytosterols influence lipid signaling and reduce endothelial inflammation, thereby improving angiogenic outcomes in a synergistic context when present with other bioactives.

The pro-angiogenic effect of crude plant extracts in the CAM assay can be explained by the multi-targeted, synergistic interactions among these bioactive compounds. Their combined activity enhances angiogenic signaling more effectively than any individual compound, highlighting the therapeutic potential of whole-plant extracts in regenerative medicine and vascular therapy.

Mechanical Stress

Mechanical stress is known to influence endothelial progenitor cell (EPC) behavior by regulating VEGF receptor endocytosis, a critical process in promoting angiogenesis (Bai et al., 2016). While mechanical stress can initially suppress VEGF expression, it may subsequently restore angiogenic activity by enhancing VEGF signaling. Mechanical forces such as extracellular matrix (ECM) stiffness, cyclic stretch, and shear stress modulate endothelial function through distinct mechanotransduction pathways. For instance, ECM stiffness activates YAP signaling to upregulate angiogenic genes, while cyclic stretch and shear stress influence endothelial behavior via calcium signaling and chromatin remodeling (Flournoy et al., 2022). Although mechanical stress was not directly manipulated in this study, it is plausible that natural mechanical forces—such as membrane tension during embryonic development—may have been present and interacted with the bioactive compounds in the irradiated extracts. This hypothetical interaction could have contributed to the observed increase in neovascularization, suggesting that the mechanical microenvironment of the CAM may enhance phytochemical-induced angiogenic responses.

Effect of the distilled water for dampening the CAM

The application of distilled water improves the adhesion of the paper discs by dampening the exposed membrane. The initial pH of distilled water is neutral, approximately 7. It does, however, have a tendency to absorb carbon dioxide when exposed to air, which lowers its pH and frequently lowers it to around 5.8. This happens because the water absorbs carbon

dioxide and keeps doing so until it balances out with the atmosphere. Hydronium ions, which are the same as free hydrogen ions, are released into solution when carbon dioxide and water react to form carbonic acid. Although distilled water was used primarily to facilitate disc adhesion, its slightly acidic pH may have influenced the local microenvironment in a manner that indirectly supported angiogenesis.

Effect of the ethanol as solvent

Ethanol was used as a solvent in the extraction of the different secondary metabolites from the pine needles of *Pinus kesiya*. The solvent was not recovered after the extraction process and was incorporated in the crude extract. However, based on the result of this study it appears that the CAM test was unaffected by the presence of ethanol in the crude extract used. Ethanol is a highly volatile organic compound, hence, it is possible that evaporation has occurred in the succeeding steps after extraction. It is also possible that further evaporation occurred during the conduct of the CAM assay. The cling wrap that was used to cover the egg window is made of Low Density Polyethylene which is a common component of plastic wrap. This is an extremely versatile material with low water vapour permeability but with high permeability to gas (Versaperm, n.d) such as ethanol vapors. In addition, ethanol may not have affected the results since a minimal amount of extract was placed on the paper disks. A 6mm filter paper disk can be impregnated with approximately 30 microliters of the extract solution (Yoshida et al., 2022).

CONCLUSION

This study demonstrated that gamma irradiation induces dose-dependent modifications in the phytochemical profile and angiogenic activity of Pinus kesiya ethanolic crude extracts. Exposure to 4 kGy and 8 kGy significantly enhanced the detectability of key bioactive compounds—namely alkaloids, cardiac glycosides, and saponins—which are associated with pro-angiogenic mechanisms such as endothelial proliferation, VEGF pathway activation, and tissue regeneration. In contrast, compounds like phenolics, flavonoids, steroids, and anthraquinone glycosides remained qualitatively stable across all treatments, potentially due to their inherent structural resilience or the limitations of qualitative detection methods.

The vascular responses observed in the duck chorioallantoic membrane (CAM) assay further supported these biochemical changes. Mean vessel counts increased with radiation dose—from 1.70 in the control group to 3.75 at 4 kGy and 4.0 at 8 kGy—indicating that gamma-irradiated extracts exert a clear pro-angiogenic effect. This enhancement is likely driven by both the presence of specific phytochemicals and the synergistic interactions among them, potentially amplified by radiation-induced oxidative processes.

The more pronounced angiogenic activity observed in the irradiated groups affirms the capacity of gamma irradiation to serve as a modulatory tool that enhances the biological efficacy of crude plant extracts. Notably, the lack of a significant difference between 4 kGy and 8 kGy suggests a plateau in effect, highlighting the importance of dose optimization to balance enhancement with compound integrity. Altogether, these findings emphasize the therapeutic potential

of irradiated *Pinus kesiya* extracts, with implications for their future use in regenerative medicine, vascular modulation, and the development of plant-based pro-angiogenic agents.

LIMITATIONS AND RECOMMENDATIONS

While this study demonstrated a dose-dependent trend in both the phytochemical expression and angiogenic activity of *Pinus kesiya* extracts, the findings are primarily based on qualitative assessments and descriptive observations. Although statistical analysis was applied through a one-way ANOVA to validate differences in mean vessel counts, the evaluation was limited to a single dimension of angiogenic response. Other important variables, such as vessel branching complexity, density patterns, and directional orientation, were not quantified. Moreover, the phytochemical screening relied on qualitative methods that do not measure actual concentrations of compounds, restricting the depth of interpretation regarding dose-dependent chemical changes. The use of crude extracts without further fractionation or compound isolation also limits the ability to identify which specific phytochemicals are responsible for the observed effects.

To strengthen the validity and specificity of future investigations, researchers are encouraged to incorporate more advanced statistical tools such as Two-Way ANOVA or MANOVA, which can simultaneously assess multiple variables related to angiogenesis. In addition, future studies should perform quantitative analysis of vascular patterns, including branching and density, and apply molecular assays such as VEGF or CD31 expression to confirm angiogenic activity at the protein level. Quantitative phytochemical techniques, such as HPLC or LC-MS, should be employed to measure and compare compound concentrations across dose groups. Furthermore, isolation and characterization of individual bioactive constituents from the crude extracts are recommended to determine their specific roles in modulating angiogenesis. These improvements will enhance the mechanistic understanding of radiation-induced phytochemical transformation and its implications for therapeutic development.

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