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Osteogenic Potential in *Piper sarmentosum* Roxb.: A Systematic Review of *in vitro* and *in vivo* Evidence

(Potensi Osteogenik *Piper sarmentosum* Roxb.: Tinjauan Sistematik Pembuktian secara *in vitro* dan *in vivo*)

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ABSTRACT

Piper sarmentosum Roxb. (Ps) has various therapeutic properties, however, the *in vitro* and *in vivo* effects on bone health, suitable dosage, and involvement of key metabolites remain unclear. This systematic review aims to evaluate Ps role in bone formation, dosage considerations, and active metabolites, highlighting the potential in future bone health therapy. The search was conducted in PubMed (Medline), Scopus, and Web of Science databases from 2014-2025. Related original articles published in English were included, while duplicates, secondary, and unrelated sources were excluded. Six articles from 1,523 met the inclusion criteria, and two additional eligible articles were manually selected from the reference lists, bringing the total to eight articles. Four studies used *in vivo* glucocorticoid-induced osteoporotic Sprague-Dawley rat models, while one employed an *in vivo* zebrafish model. The *in vitro* study involved two human Peripheral Blood Stem Cells and one MC3T3-E1 cell-line. The articles showed osteoprotective effects, particularly in glucocorticoid-induced models. Leaf extracts improved bone microstructure and osteoblast activity both *in vitro* and *in vivo*, evidenced by increased *OPG* mRNA expression, reduced *RANKL*/*OPG* mRNA expression ratio, enhanced mineralization, upregulation of osteoblast gene markers, and antioxidant activity with asarone and gamma-asarone as potential metabolites. Ps extract promotes osteoblast differentiation and bone formation *in vitro* and *in vivo*. *In vivo* effective dosage is 125 mg/kg/day in rats and 100-400 µg/mL in zebrafish, while 1-50 µg/mL for *in vitro*. Metabolites such as asarone and gamma-asarone need further investigation to clarify their role in bone health.

Keywords: Bone formation; metabolites; osteoblast differentiation; osteogenic; *Piper sarmentosum* Roxb.

ABSTRAK

Piper sarmentosum Roxb. (Ps) mempunyai pelbagai sifat terapeutik, namun kesannya terhadap kesihatan tulang secara *in vitro* dan *in vivo*, dos optimum serta metabolit utama masih belum jelas. Penyelidikan ini bertujuan menilai peranan Ps dalam pembentukan tulang, dos yang sesuai dan metabolit aktif yang berpotensi untuk rawatan kesihatan tulang pada masa hadapan. Pencarian pangkalan data PubMed (Medline), Scopus dan Web of Science dilakukan pada 2014-2025. Artikel asal berkaitan diterbitkan dalam Bahasa Inggeris telah dikenal pasti, manakala artikel bertindan, sumber sekunder dan tidak berkaitan diasingkan. Enam daripada 1,523 artikel memenuhi kriteria kemasukan, manakala dua artikel tambahan dipilih secara manual daripada senarai rujukan, memberikan keseluruhan lapan artikel. Secara *in vivo*, empat artikel menggunakan tikus Sprague-Dawley osteoporosis teraruh glukokortikoid, manakala satu kajian melibatkan ikan zebra. Kajian *in vitro* pula melibatkan dua artikel sel stem darah periferi dan satu artikel titisan sel MC3T3-E1. Kesemua artikel menunjukkan kesan pelindung tulang terutamanya dalam model *in vivo* teraruh glukortikoid. Ekstrak daun telah menambahbaik struktur mikro-tulang dan aktiviti osteoblas secara *in vitro* dan *in vivo* melalui peningkatan pengekspresan mRNA *OPG*, penurunan nisbah pengekspresan mRNA *RANKL*/*OPG*, peningkatan mineralisasi dan pengawalaturan penanda gen osteoblas dan aktiviti antioksidan serta kehadiran asaron dan asaron-gamma sebagai metabolit berpotensi untuk rawatan kesihatan tulang. Ekstrak Ps mengaruh pembezaan osteoblas dan pembentukan tulang secara *in vitro* dan *in vivo*. Dos berkesan bagi *in vivo*

adalah 125 mg/kg/hari (tikus) dan 100-400 µg/mL (ikan zebra), manakala 1-50 µg/mL pula untuk kajian *in vitro*. Metabolit seperti asaron dan asaron-gamma memerlukan kajian lanjutan bagi mengenal pasti peranannya dalam kesihatan tulang.

Kata kunci: Metabolit; osteogenik; pembentukan tulang; pembezaan osteoblas; *Piper sarmentosum* Roxb.

INTRODUCTION

Throughout history, medicinal plants have been essential to the maintenance of human health, with their therapeutic effects used to improve and preserve health sustainability (Akinyemi, Oyewole & Jimoh 2018). Therapeutic plants have been utilized either on their own or in combination with commercial drugs as complementary remedies for a variety of health conditions (Petrovska 2012). The natural origins of plant-based remedies, their usage simplicity, easy accessibility, and relatively lower potential for side effects are reasons people are attracted to their use (Shah 2022).

Piper sarmentosum Roxb. (Ps), is a natural plant that has attracted researchers over the decades. Ps, locally known as 'kaduk', belongs to the genus Piper and family Piperaceae (Sun et al. 2020). It is widely dispersed throughout Southeast Asia and the southeast coast of China, particularly indigenous to Malaysia, Northeast India, and Indonesia (Ugusman et al. 2011). Morphologically, it has a creeping or climbing growth habit with glossy green leaves with a heart-shaped base, a small, pointed apex, and small spiky white flowers (Mathew, Mohandas & Nair 2004; Rahman, Sijam & Omar 2016; Sun et al. 2020). It is consumed by local Asians in the form of 'ulam', i.e., a traditional salad from fresh leaves which is eaten raw or soaked in hot water (Abidin et al. 2021). The plant's different parts, such as leaves and roots, are used therapeutically to relieve headaches, diabetes, cough, hypertension, muscle and joint weakness, and bone pain (Salehi et al. 2019; Subramaniam et al. 2003). Several studies have reported its benefits, including antibacterial, antihypertensive, anticarcinogenic, antidepressive, antiatherosclerosis, antihyperglycemic, antioxidant, and anti-inflammatory properties (Ariffin et al. 2009; Azlina et al. 2009; Sanusi et al. 2017; Sun et al. 2020). The plant contains several potential antioxidants and bioactive metabolites such as flavonoids, alkaloids, vitamin E, carotenoids, xanthophylls, propenyl phenols, and carboxylic acids (Asri et al. 2020; Sun et al. 2020; Zainudin, Zakaria & Nordin 2015). The antioxidants and natural bioactive metabolites from the plant can act as bone-related therapy and enhance osteogenesis (Abidin et al. 2023a).

Osteogenic differentiation is a crucial process in which stem cells develop into osteogenic progenitors that can be developed into osteoblast cells, i.e., the processes that are responsible for bone repair and formation. This multistage process includes proliferation, matrix maturation, and mineralization are important to maintain bone integrity and function (Valenti, Dalle Carbonare & Mottes 2016). Disruption in the osteogenic differentiation process can lead to bone diseases such as osteoporosis, i.e., characterized by bone density reduction and fracture susceptibility. Thus, maintaining mineral homeostasis, repairing microfracture and skeletal remodeling are important to maintain bone health (Yazid et al. 2010).

Several techniques can analyze the progress of osteoblast development and offer comprehensive understanding of the underlying mechanisms of bone formation and differentiation. The enzymatic alkaline phosphatase (ALP) activity analysis is a main biochemical marker of osteoblast differentiation and activity (Trivedi et al. 2020). The von Kossa staining, on the other hand, can visualize bone matrix mineralization (Blair et al. 2017). The molecular analysis of genes and protein expressions of osteoblast-specific markers provides further insight at a molecular level into the differentiation process and functional state of osteoblast cells (Ramli et al. 2023; Wang et al. 2015). Other methods that correlate with bone formation, osteoblast proliferation, and differentiation such as histomorphometry, can detect osteoblasts by assessing parameters such as osteoblast surface and number, osteoid surface and volume, bone formation rate, mineralized surface, and mineral apposition rate (Asri et al. 2020; Dempster et al. 2013).

No systematic review has examined Ps as an osteogenesis and bone cell differentiation agent. The existing literature provides data on its effects on osteoblast differentiation with various dosages for *in vitro* and *in vivo* studies. This systematic review addressed these gaps in addition to analyzing Ps's metabolite composition and evaluating dosage considerations. This highlights the potential for developing plant-based supplements for bone health therapies.

METHODS

SEARCH STRATEGY

This systematic review was conducted in accordance with the guideline set in Preferred Reporting Items for Systematic Reviews and Meta Analyses (PRISMA) standards (Page et al. 2021). The search was performed until 2025 through PubMed (Medline), Scopus, and Web of Science (WoS) databases covering a decade of studies (2014-2025). The keyword combinations used in all three database searches are detailed in Table 1.

SELECTION CRITERIA

Original articles published between 2014 and 2025 in English were included. Duplicate publications, review articles, non-English articles, book chapters, books, conference papers, editorials, meeting abstracts, notes,

letters, data reports, and bibliographies were excluded. Both *in vitro* and *in vivo* study models demonstrating the potential of Ps in cellular osteogenic differentiation or bone development were included. This encompassed any primary cells, cell lines, or animal models. Studies on the plant's metabolite analysis were also included. Any articles that studied Ps effects on other biological activities without addressing bone formation or differentiation were excluded. Lastly, any articles that were unrelated to the search topic were excluded. The inclusion and exclusion criteria are shown in Table 2.

SCREENING PROCESS & DATA EXTRACTION

Three independent reviewers (R.M.A.A., I.Z.Z.A. and S.H.Z.A.) conducted the database search and screened the titles, abstracts, and full texts of the article to ensure they met the specified selection criteria. Any reviewer disagreement was resolved through consensus in collaboration with the co-authors (Z.Y. and M.D.Y.). All authors performed subsequent data extraction and further discussion. Initially, duplicate articles were removed. Subsequently, articles were screened based on inclusion and exclusion criteria. Articles unrelated to the search scope or not meeting the inclusion criteria were excluded. Data extraction was tabulated accordingly in the following order: 1) References, 2) Extract (preparation approach) and model of study, 3) Dosage concentration, 4) Methodology, and 5) Summary of findings. The main authors published four of the studies; three studies identified through the search strategy and one from the reference lists of two included articles. This gave a comprehensive review and valuable insights into the current knowledge. All studies, including the author's own, were evaluated using consistent and rigorous criteria to ensure quality and objectivity.

BIAS ASSESSMENT OF *in vitro* AND *in vivo* STUDIES

The included articles' quality check and bias assessments were conducted independently by (R.M.A.A) and further reviewed by (I.Z.Z.A and S.H.Z.A) using an adapted version of the 'Modified CONSORT' checklist for *in vitro* studies (Ariffin et al. 2022), and the Animal Research: Reporting of *In Vivo* Experiments; 'ARRIVE 2.0 (Essential 10)' checklist for *in vivo* studies (García-González et al. 2021; Percie du Sert et al. 2020). Any disagreement between the reviewers was addressed and resolved.

TABLE 1. The keywords combination for database search

Search strategy
('osteogenesis' OR 'osteogenic' OR 'bone formation' OR 'bone fracture' OR 'bone healing' OR 'osteoporosis' OR 'osteoblast' OR 'osteoblastogenesis' OR 'bone cell' OR 'preosteoblast' OR 'bone') OR ('differentiation' OR 'cell differentiation' OR 'cellular differentiation' OR 'osteoblast differentiation' OR 'osteogenic differentiation' OR 'preosteoblast differentiation' OR 'bone cell differentiation' OR 'cell regeneration' OR 'osteoblast mineralisation' OR 'osteoblast mineralization') AND ('*Piper sarmentosum*') OR ('*Piper sarmentosum* extract') OR ('kadok') OR ('kaduk') OR ('*Piper sarmentosum* roxb')

TABLE 2. Summary of journal articles' inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Primary sources (Original articles)	Secondary sources (Reviews, book chapters, books, proceeding/conference papers, editorials, meeting abstracts, notes, letters, data reports, and bibliography)
Published in the last decade (2014-2025)	Languages other than English
Open access articles	Redundant articles
In vitro and *in vivo* experiments	Non-related articles; covering scopes other than measuring bone formation or osteoblast differentiation activity of Ps
Articles related to Ps effect on bone formation or cell differentiation (Namely osteoblasts)	Evaluating other biological activities of Ps extracts

In the *in vitro* studies, the key domains assessed of the modified CONSORT included (1) a structured summary of the abstract, (2) the scientific background, (3) objectives and/or hypothesis, (4) detailed description of interventions, (5) statistical methods, (6) results, (7) limitations, (8) sources of funding, and (9) protocol availability. The domains of this tool are evaluated using (+), (-), (?), and (/) which stand for present, absent, unclear, and not mentioned, respectively. Meanwhile, for the *in vivo* studies, the 'ARRIVE 2.0 (Essential 10)' checklist includes assessing the following main domains: study design, sample size, inclusion and exclusion criteria, randomization, blinding, outcome measures, statistical methods, experimental animals, experimental procedures and results, which are designed to ensure comprehensive and transparent reporting of animal research. The domains of 'ARRIVE 2.0' checklist evaluation are simplified as (+), (-), and (/) which stand for low risk, high risk and unclear risk, respectively.

RESULTS AND DISCUSSION

OVERVIEW OF SEARCH RESULTS: A SCARCITY OF STUDIES AND OPPORTUNITIES TO EXPLORE Ps OSTEOGENIC POTENTIAL

The initial search of Ps effect on bone formation and cell differentiation through electronic databases resulted in a total of 1,523 studies with 63 from PubMed (Medline), 988 from Scopus, and 472 from Web of Science (WoS). Each source was evaluated based on specified inclusion and exclusion criteria. Initially, 81 duplicated articles and 348 articles from various sources including reviews, book chapters, books, proceeding/conference papers, editorials, meeting abstracts, notes, letters, data reports, and bibliography, as well as non-English language articles were removed. The remaining 1,094 articles were individually screened by title, excluding irrelevant studies unrelated to role in bone formation or osteoblast differentiation. Articles focusing on other biological activities of Ps or not meeting the inclusion criteria were also excluded, resulting in six articles eligible for inclusion. As an additional source of data collection, the reference lists of the eligible articles were also screened to identify any potential articles. This step helps to expand the search coverage and account for any potential limitations such as database coverage or indexing. As a result, two additional articles (Abidin et al. 2021; Fadziyah et al. 2016) were manually selected from the reference lists that did not appear during the search of the three databases (PubMed (Medline), Scopus and WoS) although they met the inclusion criteria of assessing Ps effect on osteoblast differentiation, bringing the total up to 8 studies selected for qualitative synthesis. The article selection process using the PRISMA 2020 flow diagram is shown in Figure 1.

Out of the eight studies, five studies involved *in vivo* models, i.e., four studies with rat models (Asri et al. 2020, 2016; Fadziyah et al. 2016; Ramli et al. 2023) and one study

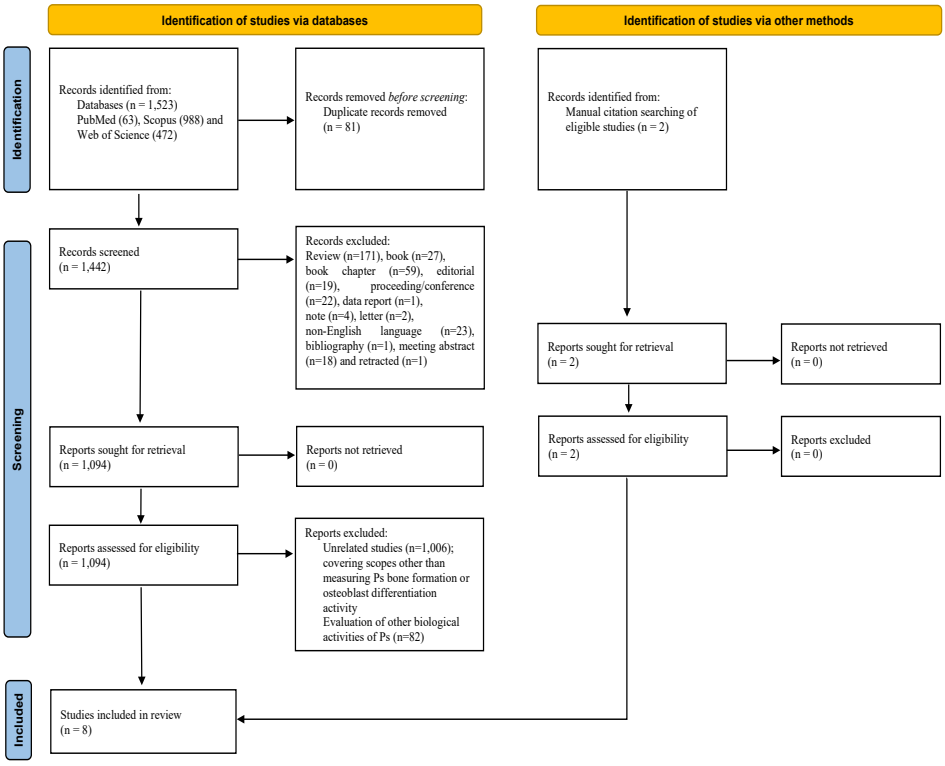

FIGURE 1. The process of article selection (PRISMA 2020 flow diagram)

with a zebrafish model (Abidin et al. 2020). Meanwhile, the remaining three studies involved the *in vitro* models (Abidin et al. 2023a, 2023b, 2021) including MC3T3-E1 cell line (Abidin et al. 2021), and human Peripheral Blood Stem Cells (hPBSCs) models (Abidin et al. 2023a, 2023b). The summary of data extraction from the selected articles was tabulated into four categories, i.e., Extract (preparation approach) and model of studies, dosage concentration, methodology, and summary of findings, as shown in Table 3. The two added articles (Abidin et al. 2021; Fadziyah et al. 2016) that were extracted from the reference list and not been cited in any three databases (PubMed (Medline), Scopus and WoS) have also been indicated in Table 3 to provide comprehensive and valuable information for the osteogenic potential of Ps extract.

Generally, the selected articles have featured an abstract, scientific background, or introduction followed by clear study objectives. The interventions and methods were clearly explained, along with the results and statistical methods used. Nonetheless, the limitations were not explicitly stated by the authors. On the other hand, the *in vivo* studies had inadequate detailed reporting when it comes to the randomization process, blinding, and sample size calculation.

BIAS ASSESSMENT ANALYSIS

The 'Modified CONSORT' checklist was used to assess the quality and risk of bias of the *in vitro* studies from selected articles as shown in Table 4. The key domains presented in all three studies include a structured summary of the abstract, scientific background, objectives, and/or hypothesis, description of interventions, statistical methods, results, and funding sources. However, the studies did not explicitly mention limitations and information on protocol availability. The overall risk of bias was finalized based on the total number of (+ or present) assessed, whereby six to seven = low risk, four to five = moderate risk, and one to

TABLE 3. Evaluation of *Piper sarmentosum*: Extract types, models, dosage, methodology, and outcomes

Study	Extract type (preparation method) and model of study	Dosage (Type of test)	Methodology	Summary of Findings
Asri et al. (2016)	Aqueous leaf extract (boiled aqueous extraction) Rats* (*in vivo*)	125 mg/kg/day	*ELISA:* 11-dehydrocorticosterone, corticosterone levels, femur corticosterone, *11β-HSD1* protein expression	• ↑ 11β-HSD1 dehydrogenase activity • ↓ Femur corticosterone • ↑ Body weight in treated rats • Potential osteoporosis treatment
Fadziyah et al. (2016) #	Aqueous leaf extract (boiled aqueous extraction) Rats* (*in vivo*)	125 mg/kg/day	*mRNA analysis: OPG*, *OCN*, *RANKL*, *11β-HSD1*	• ↑ *OPG*, ↓ *11β HSD1* • ↓ *RANKL*/*OPG* ratio • *OCN*: no significant increase • Potential osteoporosis treatment
Abidin et al. (2020)	Aqueous leaf extract (boiled aqueous extraction) and GC-MS analysis for metabolite screening Zebrafish (*Danio rerio*) embryo and adults (*in vivo*)	0-60 µg/mL (embryo toxicity), 0-400 µg/mL (caudal fin regeneration)	Embryo toxicity, caudal fin regeneration, DPPH assay, GC-MS analysis	• 60 µg/mL: lethal; 50 µg/mL: non-viability; 40 µg/mL: slowed development • 100-400 µg/mL: ↑ caudal fin regeneration • Antioxidant activity IC_{50}: 50.56 mg/mL

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Asri et al. (2020)	Aqueous leaf extract (boiled aqueous extraction) Rats* (*in vivo*)	125 mg/kg/day	*Bone Histomorphometry:* Dynamic: bone formation rate, mineralization rate, mineralized surface (single/double labeling) Static: osteoid volume and surface and osteoblast surface Microstructure: bone volume, trabecular thickness, number and separation	• Prevention of bone loss and improved bone microstructure • Bone formation parameters: significant increase • Potential osteoporosis treatment
Abidin et al. (2021) ##	Aqueous leaf extract (boiled aqueous extraction) MC3T3 -E1 cell line (*in vitro*)	1-4 mg/mL (cell differentiation)	*Osteoblast differentiation:* von Kossa staining, ALP activity, gene expression (*ALP*, *Dkk2*, *Col I*)	• ↑ ALP activity • ↑ Osteoblast markers • MC3T3-E1 cells mineralization
Abidin et al. (2023a)	Ethanolic leaf extract (Soxhlet extraction) and GC-MS analysis for metabolite screening hPBSCs** (*in vitro*)	1-50 µg/mL (cell differentiation)	*Osteoblast differentiation:* von Kossa staining, ALP activity, gene expression (*ALP*, *RUNX2*, *OPN*, *OCN*)	• ↑ ALP activity • Mineralization & ↑ osteoblast markers • GC-MS: 54 metabolites identified; 21 with reported biological activity (potential contribution in osteogenic differentiation)
Abidin et al. (2023b)	Ethyl acetate leaf extract (Soxhlet extraction) hPBSCs** (*in vitro*)	1-50 µg/mL (cell differentiation), 1-900 µg/mL (cytotoxicity)	*Cytotoxicity assay, osteoblast differentiation:* von Kossa staining, *ALP* gene expression	• Dose-dependent cytotoxicity • ↑ MC3T3-E1 Proliferation & mineralization • ↑ *ALP* gene expression
Ramli et al. (2023)	Aqueous leaf extract (boiled aqueous extraction) Rats* (*in vivo*)	125 mg/kg/day	*Immunohisto-gold labelling: OCN* protein expression in femoral bone	• ↑ *OCN* protein expression • Potential osteoporosis treatment

*Rats : Glucocorticoid-induced osteoporotic Sprague Dawley, **hPBSCs : human Peripheral Blood Stem Cells, # : article extracted from Asri et al. (2016) & Ramli et al. (2023), ## : article extracted from Abidin et al. (2023a) & Abidin et al. (2023b)

three = high risk (Benli, Al-Haj Husain & Ozcan 2023). As a result, all assessed articles were categorized as overall low risk (Table 4).

The quality check and bias assessment results for the *in vivo* studies (Abidin et al. 2020; Asri et al. 2020, 2016; Fadziyah et al. 2016; Ramli et al. 2023) conducted using 'ARRIVE 2.0 (Essential 10)' checklist is presented in Table 5. The results of the quality check showed overall methodological adherence with all studies demonstrating a low risk of bias in the study design, inclusion and exclusion criteria, outcome measures, statistical methods, and reporting of experimental procedures. However, although the studies have reported the sample size (number of animals) tested, the studies did not provide the justification for the selected sample size and lacked reporting on randomization methods leading to an unclear risk of bias in these areas (Table 5). Furthermore, the lack of reporting on blinding in all studies increases the risk of potential bias in this domain.

While the studies were well-structured and reported essential methodological aspects, the quality check identified areas for improvement in the included *in vivo* studies, as the clear reporting on sample size justification, randomization and blinding would strengthen the robustness and reliability of future research in this area, finally establishing the evidence base for the effect of Ps on bone formation and differentiation.

METABOLITE COMPOSITION OF Ps*:* EXTRACTION METHODS AND YIELDS

Two articles have reported on metabolite screening of Ps extracts from the eight selected articles. Abidin et al. (2020) carried out the metabolite screening on the aqueous

extract, while Abidin et al. (2023a) on the ethanolic extract (Table 3). The key metabolites in both aqueous and ethanolic extracts of Ps are displayed in Figure 2.

The metabolite screening in the two studies was conducted using Gas Chromatography-Mass Spectrometry (GC-MS), after the boiled aqueous extraction for Ps aqueous leaf extract and Soxhlet extraction for its ethanolic leaf extract (Table 3). The ethanolic extract screening showed 54 metabolites, of which only 21 were notable for their biological activities (Abidin et al. 2023a). The key metabolites in the extract were 2,4-di-tert-butylphenol; 2,5-bis (1,1-dimethylethyl); Gamma-asarone; Benzene 1,2,3-trimethoxy-5-(2-propenyl) and Asarone with peak area percentages of 32.24%, 32.24%, 10.97%, 10.97% and 10.31% respectively (Figure 2).

Meanwhile, the analysis identified 13 metabolites in the aqueous extract, seven of which were reported to have antioxidant activities. Following 3-(4-methoxyphenyl) propionic acid (10.90%), Gamma-asarone (9.89%), and n-Hexadecanoic acid (9.34%), the two most prevalent metabolites found to have the biggest peak percentages were Hydrocinnamic acid (16.49%) and Asarone (16.49%) (Abidin et al. 2020) (Figure 2). Both Asarone and Gamma-asarone have been identified to possess cell differentiation abilities, i.e., the common metabolite between the aqueous and ethanolic extract from these two studies (Abidin et al. 2023a, 2020).

INFLUENCES OF EXTRACTION METHODS AND DOSAGES ON OSTEOGENIC EFFECTS

Three different extraction approaches were reported, i.e., aqueous, ethanol, and ethyl acetate extractions (Table 3). The aqueous extraction involved boiling dried and grounded leaves in distilled water, followed by a freeze-drying approach to obtain a powdered form. On the other hand, both ethanolic and ethyl acetate extractions involved Soxhlet extraction of dried grounded leaves with

TABLE 4. Quality check and risk of bias assessment results by the 'Modified CONSORT' checklist for *in vitro* studies

Domain/Journal	1	2	3	4	5	6	7	8	9	Overall risk of bias
Abidin et al. (2021)	+	+	+	+	+	+	-	+	/	Low
Abidin et al. (2023a)	+	+	+	+	+	+	-	+	/	Low
Abidin et al. (2023b)	+	+	+	+	+	+	-	+	/	Low

(1) = structured summary of the abstract, (2) = scientific background, (3) = objectives, and/or hypothesis, (4) = detailed description of interventions, (5) = statistical methods, (6) = results, (7) = limitations, (8) = sources of funding, (9) = protocol availability, (+) = present, (-) = absent and (/) = not mentioned

TABLE 5. Quality check and risk of bias assessment results by the 'ARRIVE 2.0 (Essential 10)' checklist for animal (*in vivo*) studies

Criteria	Abidin et al. (2020)	Asri et al. (2016)	Ramli et al. (2023)	Asri et al. (2020)	Fadziyah et al. (2016)
Study design	+	+	+	+	+
Sample size	/	/	/	/	/
Inclusion & exclusion criteria	+	+	+	+	+
Randomization	/	/	/	/	/
Blinding	-	-	-	-	-
Outcome measures	+	+	+	+	+
Statistical methods	+	+	+	+	+
Experimental animals	+	+	+	+	+
Experimental procedures	+	+	+	+	+
Results	+	+	+	+	+

(+) = Low risk, (-) = High risk, (/) = Unclear risk

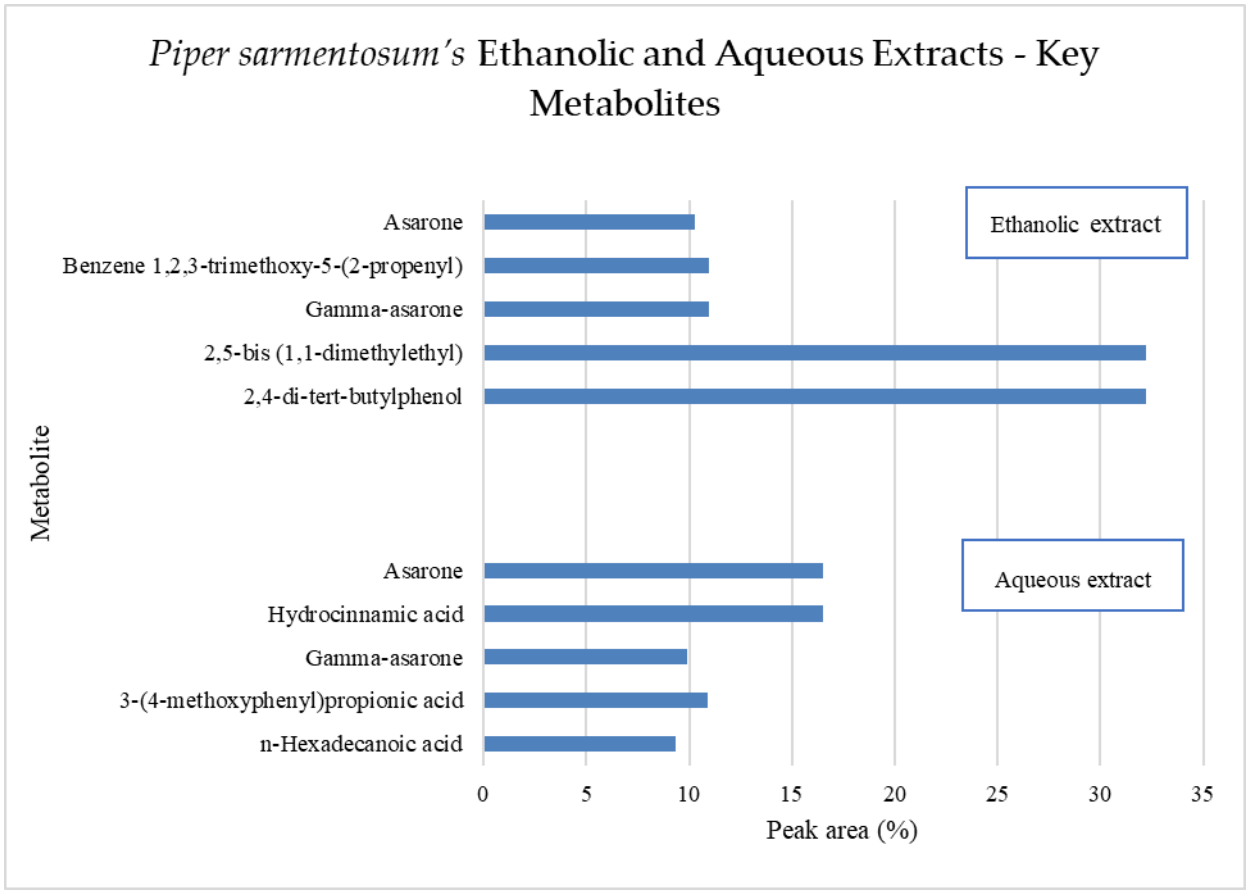

FIGURE 2. The key metabolites of *Piper sarmentosum's* extracts and their peak area (%)

the respective solvent (ethanol or ethyl acetate), followed by rotatory evaporation to remove the solvent.

It was reported that aqueous and ethanolic extractions showed higher levels of Asarone (Abidin et al. 2023a, 2020). Asarone consists of alpha-asarone, beta-asarone, and gamma-asarone isomers which are phenylpropenes; alpha-asarone has shown potential link to osteoblast differentiation (Tian et al. 2022). Although asarone appears as a key metabolite for both aqueous and ethanolic extracts, the specific mechanism of action on osteoblast differentiation remains unclear. On the other hand, metabolites in Ps may act alone or synergically to enhance osteoblast differentiation and bone healing (Abidin et al. 2023a). Therefore, further investigation is essential to elucidate the mechanisms underlying their biological effects and to clarify their contribution and osteoporosis-protective properties.

The *in vivo* studies utilized a dosage of 125 mg/kg/day for Ps given orally to the osteoporotic rat models, making it the current standard dosage of Ps for *in vivo* models (Table 3). The comparison of this dosage with other natural plants' dosages studied for osteoprotective effects might be useful due to the limited *in vivo* studies on Ps. For instance, *Labisia pumila* at 100 mg/kg/day (Effendy et al. 2015) a blend of *Ulmus davidiana* and *Cornus officinalis* at 100 or 200 mg/kg/day (Kim et al. 2022) and *Eleutherococcus senticosus* at 100 mg/kg/day (Lim et al. 2013) were reported to enhance bone strength and possess anti-osteoporotic effects. These findings suggest that the dosage of Ps is within a reasonable range compared to other natural plants with osteoprotective properties. Additionally, the choice of this dosage of Ps in osteogenic studies is supported by earlier evidence demonstrating its osteogenic benefits in fracture healing of animal models (Estai et al. 2011). In the current review, four *in vivo* studies have tested this dosage of Ps (125 mg/kg/day) on glucocorticoid-induced osteoporotic rats (Table 3). Three studies used 32 rats (Asri et al. 2020; Fadziyah et al. 2016; Ramli et al. 2023), while one used 24 rats (Asri et al. 2016).

Meanwhile, Ps concentration range of 0-60 µg/mL and 0-400 µg/mL were utilized on the zebrafish *in vivo* model for embryotoxicity and caudal fin regeneration assessments, respectively (Table 3). The *in vitro* studies utilized concentrations ranging between 1-50 µg/mL (Abidin et al. 2023a, 2023b) and 1-4 mg/mL (Abidin et al. 2021) to evaluate Ps osteoblast differentiation potential and 1-900 µg/mL to evaluate the cytotoxic effects (Abidin et al. 2023b) (Table 3). This study showed the cell

viability decrease was in a dose-dependent manner in the cytotoxicity assay, with IC_{50} values after 48 h and 72 h treatment of 153 µg/mL and 84 µg/mL, respectively, hence the 1-50 µg/mL seems to be an appropriate dosage for *in vitro* osteoblast differentiation.

REGULATION OF 11-HYDROXYSTEROID DEHYDROGENASE TYPE 1 ENZYME (11β-HSD1) ACTIVITY BY Ps IN SPRAGUE-DAWLEY RATS

The 11-Hydroxysteroid Dehydrogenase Type 1 Enzyme (11β-HSD1) enzyme in bone cells converts inactive cortisone to active cortisol, regulating glucocorticoid levels within the bone. This regulation is essential because when active glucocorticoids like cortisol reach high levels to trigger osteoblast differentiation, the body naturally maintains low activation of glucocorticoids through 11β-HSD1. This is important to ensure that osteoblast differentiation and local glucocorticoid levels are regulated effectively and harmoniously (Eijken et al. 2005). Hence, the proper functioning of 11β-HSD1 helps maintain bone health, while its dysregulation can lead to bone loss and osteoporosis (Cooper et al. 2002; Eijken et al. 2005). Fadziyah et al. (2016) showed that Ps aqueous extract significantly reduced the mRNA expression of 11β-HSD1 in glucocorticoid-induced osteoporotic rats. This reduction indicates lower enzyme activity, leading to decreased conversion of inactive cortisone to active cortisol hence decreasing levels of bone-inhibiting cortisol will favor bone formation (Fadziyah et al. 2016; Ramli et al. 2012). Meanwhile, Asri et al. (2016) reported that Ps extract significantly reduced corticosterone levels in the femur of Sprague-Dawley rats. This suggests that the extract enhances the dehydrogenase activity of 11β-HSD1, promoting the conversion of active corticosterone to its inactive form, thereby protecting against bone loss. These two studies collectively demonstrated that Ps could regulate 11β-HSD1 activity, either by decreasing its expression or enhancing its dehydrogenase function, which also has a probable major role in regulating osteoblast proliferation and differentiation (Eijken et al. 2005), thus showing promise as complementary treatment in glucocorticoid-induced osteoporotic rat models.

EFFECTS OF Ps ON TRABECULAR MICROARCHITECTURE

Bone histomorphometry analysis examines bone growth and remodeling at both cellular and structural levels. Visual observation of structural changes particularly in osteoporosis patients is essential during fracture healing as it helps assess the impact of treatments on bone and its healing process. Asri et al. (2020) reported that Ps extract significantly enhanced improvements in bone health by increasing bone volume, trabecular thickness, and trabecular number to indicate stronger and denser bone structures. In addition, the trabecular separation was also reduced to enhance bone integrity. Furthermore, the extract stimulated osteoblast activity through increased osteoid and osteoblast surface reflecting increased osteoblast differentiation that contributed to bone formation. The elevation of double-labelled trabecular surface, mineralizing surface, and bone formation rate, further contributed to bone formation. Overall, these observations suggest Ps extracts not only prevent bone loss but also promote new bone formation thus acting as a potential treatment for osteoporosis induced by long-term glucocorticoid therapy (Asri et al. 2020).

BONE TISSUE REGENERATION POTENTIAL IN ZEBRAFISH MODELS

The Ps aqueous effect on zebrafish (*Danio rerio*) involved the embryos and adult fish. Based only on one study, the research evaluates embryonic toxicity, tissue regeneration on the fish caudal fin, antioxidant activity, and metabolites' profile (Abidin et al. 2020). The embryos were treated with the extract at a range of 0-60 µg/mL to assess the toxicity, survival rates, heartbeat, and morphological changes for 24, 48, and 72 h. The results show that 60 µg/mL was lethal which led to embryo death, followed by slowed development at 40-50 µg/mL. On the heartbeat analysis, the heartbeat reduced significantly at 40 µg/mL where higher concentrations stopped the heartbeats entirely. A high concentration of Ps caused a significant abnormal development and was finally lethal through embryo morphological assessment. Meanwhile, for tissue regeneration analysis, the adult zebrafish with the caudal fin were amputated and treated for 10 days at different concentrations of the extract (0-400 µg/mL) (Table 3). A fin's shape is mainly supported by a segmented, calcified ray, which is a hollow cylinder at the tip, formed and lined by osteoblasts on both the inside and outside (Lebedeva et al. 2020). All the tested concentrations (100, 200, 300 and 400 µg/mL) showed a significant increase in tissue regeneration when compared to untreated samples, demonstrating that Ps extract enhanced the caudal fin regeneration of adult zebrafish, where the highest concentration (400 µg/mL) of the extract generated the fastest and highest regeneration rates (Abidin et al. 2020).

The study has also examined the antioxidant activity of the extract as compared to ascorbic acid, which is a common standard of antioxidant using 2,2-diphenyl-1-picryhydrazyl (DPPH) assay demonstrated significant free radical scavenging activity with the IC_{50} value of 50.56 mg/mL. This indicates that in contrast to embryonic toxicity at higher concentrations, the extract shows a strong regenerative potential for caudal fin regeneration of adult zebrafish. This could be due to antioxidant activity, highlighting the potential of Ps extract for therapeutic application in bone tissue regeneration.

in vitro OSTEOGENIC EFFECTS ON MC3T3-E1 AND hPBSCs

The *in vitro* studies have also shown the effect of Ps aqueous, ethyl acetate, and ethanolic extracts on other osteoblast gene expressions (Table 3). The extracts were

able to upregulate osteoblast-specific genes in MC3T3-E1 cell line and hPBSCs including *ALP* (Abidin et al. 2023a, 2023b, 2021), *RUNX2*, *OPN*, *OCN* (Abidin et al. 2023a), *Col I* and *Dkk2* (Abidin et al. 2021). All these findings suggest the ability of Ps to affect and regulate the molecular level of osteoblast cells, which are indicators of osteoblast cell differentiation.

The *in vitro* models have also shown the aqueous and ethanolic extracts' abilities to increase the alkaline phosphatase (ALP) specific enzyme activity, which is the most well-known biochemical indicator of osteoblast activity (Sabokbar et al. 1994). Ps aqueous extract (2 mg/mL) and ethanolic extract (50 µg/mL) exhibited the highest ALP enzyme activity in MC3T3-E1 and hPBSCs models, respectively (Abidin et al. 2023a, 2021). Moreover, the *in vitro* models showed that aqueous, ethyl acetate and ethanolic extracts caused the differentiation of the MC3T3-E1 cell line and hPBSCs, respectively, into osteoblasts. Alongside the gene expression and enzymatic analysis previously mentioned, this was also proven by the later stage of bone differentiation, mineralization, detected by MC3T3-E1 and hPBSCs mineralized cells with dark brown calcium nodules using von Kossa staining, at doses between 1-50 µg/mL, for both ethanolic and ethyl acetate extracts (Abidin et al. 2023a, 2023b), as well as 1-4 mg/mL for aqueous extract (Abidin et al. 2021). Ps aqueous extract at the concentration of 2 mg/mL, ethyl acetate extract at 1 µg/mL, and ethanolic extract at 35 µg/mL and 50 µg/mL, exhibited the highest mineralisation rates in these studies (Abidin et al. 2023a, 2023b, 2021).

MOLECULAR MECHANISMS OF BONE REMODELING: *RANKL*/*OPG* AND *OCN*

The receptor activator of nuclear factor kappa-B ligand (*RANKL*) is a signaling molecule that plays a crucial role in bone metabolism that is produced by osteoblast cells to help regulate the bone remodeling process, by activating the subsequent osteoclast activity (Giuliani, Colla & Rizzoli 2004). Fadziyah et al. (2016) reported that Ps leaf aqueous extract showed no significant changes in the *RANKL* mRNA expression when compared to the adrenalectomized (Adrx) control group treated with dexamethasone (DEX) (Table 3). Meanwhile, the mRNA expression of osteoprotegerin (*OPG*) was increased (Fadziyah et al. 2016). Moreover, the ratio of *RANKL* to *OPG* mRNA expression decreased by 1.2-fold in the Ps-treated group, indicating reduced osteoclast activity, hence promoting a favorable environment for bone formation, as a higher ratio favors bone resorption (Jura-Półtorak et al. 2021).

OPG, secreted by osteoblasts, binds to *RANKL*, which together maintains bone remodeling by inhibiting osteoclast formation to maintain the balance between bone resorption and formation (Raje, Bhatta & Terpos 2019). One of the key metabolites which is common between the aqueous and ethanolic extract in Ps extract was alpha-asarone. Alpha-asarone may contribute to this mechanism, as it was shown to affect the regulation of *RANKL* such as causing the suppression of the key pathways activated by *RANKL* (Protein Kinase B, p38 Mitogen-Activated Protein Kinase, and Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells) (Tian et al. 2022). This suppression reduces osteoclast activity, which shifts the balance of bone remodeling toward the bone formation, thereby helping to preserve bone health and creating a favorable environment for osteoblast activity.

The aqueous extract also showed no significant increase in the osteocalcin (*OCN*) mRNA expression in the Ps-treated group (Fadziyah et al. 2016). In contrast, another *in vitro* study on hPBSCs showed that Ps ethanolic extract was able to upregulate *OCN* expression throughout the 14-day treatment period, especially at 50 µg/mL (Abidin et al. 2023a). Fadziyah et al. (2016) also suggested that increasing the dosage of Ps extract and prolonging the study duration would be necessary for more comprehensive and conclusive results. Meanwhile, the study done by Ramli et al. (2023) showed that Ps aqueous extract mediated an increase in *OCN* protein expression, as demonstrated by the immunohisto-gold labelling.

CONCLUSION

This review demonstrates the therapeutic potential of various Ps extracts in enhancing osteogenesis and bone health. Using *in vitro* and *in vivo* models, highlighted increased osteoblast activity and differentiation, bone mineralization, expression of key osteogenic markers (*ALP*, *OCN*, *RUNX2*, *OPN*, *Col I*, *Dkk2*, *OPG,* and reduced *RANKL*/*OPG* ratio), enzyme activities (increased ALP and 11β-HSD1 dehydrogenase activities), also improved bone microstructure and regeneration. Aqueous leaf extracts, prepared through boiled extraction, were consistently effective in enhancing bone formation and reducing markers linked to osteoporosis in glucocorticoid-treated models. Cytotoxicity assays showed dose-dependent responses with high doses causing cytotoxicity or reduced viability. Dosage considerations for bone formation study are 125 mg/kg/day and 100-400 µg/mL for *in vivo* involved rats and zebrafish, respectively, on the other hand, the *in vitro* study of hPBSCs is 1-50 µg/mL. Metabolite analysis using GC-MS identified bioactive metabolites potentially responsible for osteogenesis such as asarone, gamma-asarone, and other metabolites that should be investigated further. These observations suggest Ps extract especially aqueous and ethanolic extracts as potential candidates for managing and improving bone health.

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We declare no conflicts of interest. First author (I.Z.Z.A.) declares that four of the studies analyzed in this review were authored by herself. These studies were included based on their relevance to the research topic and the limited availability of studies in this field. We have taken steps to mitigate any potential bias by adhering to standardized evaluation criteria for all included studies.

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