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Review Article

Systematic Review: Anti-Osteoporosis Potential Activities Of Phytoestrogen Compounds In *Chrysophyllum cainito* L., *Elaeis guineensis* Jacq., *Lannea acida* Rich., *Marsilea crenata* Presl., and *Medicago sativa* L.

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ABSTRACT

Phytoestrogens are compounds from plants that have a structure and function similar to estrogen (17β-estradiol). Phytoestrogens can be found in *Chrysophyllum cainito* L., *Elaeis guineensis* Jacq., *Lannea acida* Rich., *Marsilea crenata* Presl., and *Medicago sativa* L. This systematic review aimed to prove that all of these five plants have phytoestrogens which were observed with several instruments, and to assess activities and bone forming mechanisms from these five plants in the female mice (*Mus musculus*) and female rats (*Rattus norvegicus*). This systematic review was done by identifying articles in several databases (Google Scholar, PubMed, and Science Direct). The process of selecting the articles used the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines to create a flowchart with inclusion and exclusion study criteria. Meta-synthesis was done to analyze, identify, and interpret all of the data in the articles systematically. 31 articles in total were obtained from the selection process, with 27 articles containing a discussion about chemical compound content and 4 articles describing research results for in vivo testing of the plants that were reviewed. The results showed that these five plants have phytoestrogens and by in vivo testing have the activity in increasing trabecular bone density in the experimental animals.

INTRODUCTION

Osteoporosis is a condition where the bones become thinner, fragile, porous, and easily broken because of decreasing bone density which happens over a long time. Osteoporosis in postmenopausal women happens because of an imbalance between the bone erosion process and bone formation due to estrogen deficiency (Wahjudi and Putriana, 2014; Afni and Hanafi, 2019; Sugiritama and Adiputra, 2019). Today, the osteoporosis prevalence in the world is more than 200 million people. Recent research conducted by the International Osteoporosis Foundation in 2015 revealed that 1 out of 4 women in Indonesia with the age range 50-80 years old have osteoporosis risk 4 times higher than men (Kemenkes, 2015; Sozen *et al*., 2017). Estrogen deficiency is one of the essential factors that cause an imbalance in the bone remodeling process which involves increasing bone resorption that induces osteoporosis. First-line therapy in osteoporosis is hormone replacement therapy (HRT), but the long-term application may evoke severe side effects for the body, like emboli cancer, breast cancer, and stroke. Accordingly, using HRT as osteoporosis therapy has been limited and discontinued (Chen *et al*., 2015; Agarwal *et al*., 2018).

Phytoestrogens are compounds from plants that have a structure like estrogen or may replace estrogen's function. Structurally, phytoestrogen is divided into two groups, which

are flavonoid and non-flavonoid. Not only is the phytoestrogen compound group easy to obtain and has no side effects, but it also has the efficacy to raise bone density (Schröder *et al*., 2016; Ma'arif *et al*., 2019). Plants that contain phytoestrogen compounds are *Chrysophyllum cainito* (*C. caimito*) L., *Elaeis guineensis* (*E. guineensis*) Jacq., *Lannea acida* (*L. acida*) Rich., *Marsilea crenata* (*M. crenata*) Presl., and *Medicago sativa* (*M. sativa*) L. (Figure 1).

Plants such as *C. cainito*, *E. guineensis*, *L. acida*, and *M. sativa*, empirically have been utilized as rheumatic medication and plant-like *M. crenata* has been applied as an anti-osteoporosis treatment, when rheumatic symptoms and osteoporosis occur due to estrogen deficiency disorder in postmenopausal women (Muhaisen, 2013; Shailajan and Gurjar, 2014; Widjayant, 2016; Febrina and Febriyanti, 2017; Rafińska *et al*., 2017).

C. cainito is a plant that is often found in tropical areas. Research of the methanol extract from *C. cainito* leaves indicated it has the potential to reduce inflammation reactions in the joints because of the anti-inflammatory effects from triterpenoid compounds (Meira *et al*., 2014). Furthermore, research of methanol extract in *E. guineensis* leaves identified 28 derivates of flavonoids that have been observed with liquid chromatograph mass spectrometry (LC-MS) instrument (Tahir *et al*., 2012). Additionally, an *in vivo* study identified the 96% ethanol extract from *L. acida* bark which was induced in MCF-7 cells has the potential to raise the marker from bone formation, which is alkaline phosphatase (ALP) (Oumarou *et al*., 2017).

Various research has been conducted on *M. crenata* leaves that have potential as anti-osteoporosis. One i*n silico* study showed the compounds in *M. crenata* leaves have a high affinity to ER-β, while, an *in vitro* study of MC3T3-E1 preosteoblast cells found the n-hexane extract and its fraction can increase proliferation process and differentiation in MC3T3-E1 preosteoblast cells (Ma'arif *et al*., 2018). Moreover, research of the methanol extract from *M. sativa* leaves observed with ultra-high performance liquid chromatography (U-HPLC) instrument, found phytoestrogen compounds from flavonoid group which have the potential as anti-osteoporosis agents (Chiriac *et al*., 2020).

These five plants had been studied *in vivo* to find out their estrogenic effect. This systematic review aimed to study the phytoestrogen compounds from these five plants that have been observed with numerous instruments, and to study the mechanism of bone formation in increasing trabecular bone density in the experimental animals. Therefore, this systematic review may be considered as a primary contribution and source of scientific information for further research related to the development of these five plants as anti-osteoporosis agents.

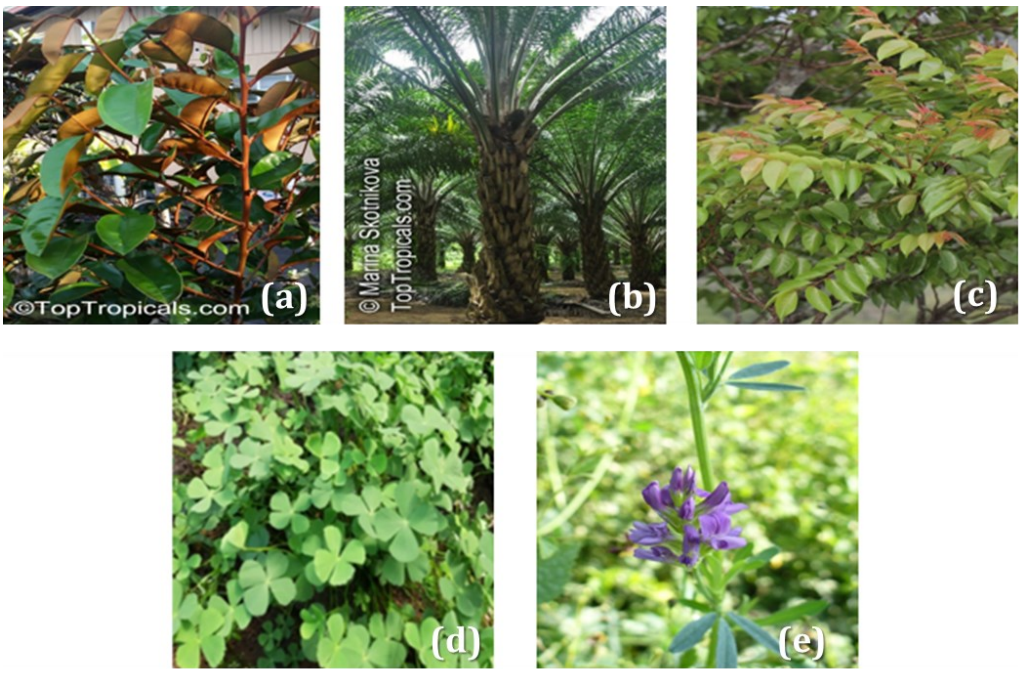


Figure 1. (a). Kenitu leaves (*C. cainito*) (b). Kelapa sawit leaves (*E. guineensis*) (c). Faruhi bark (*L. acida*) (d). Semanggi leaves (*M. crenata*) (e). Alfalfa leaves (*M. sativa*) (Source: Agil *et al*., 2021; Plantsoftheworldonline.org; TopTropicals.com)

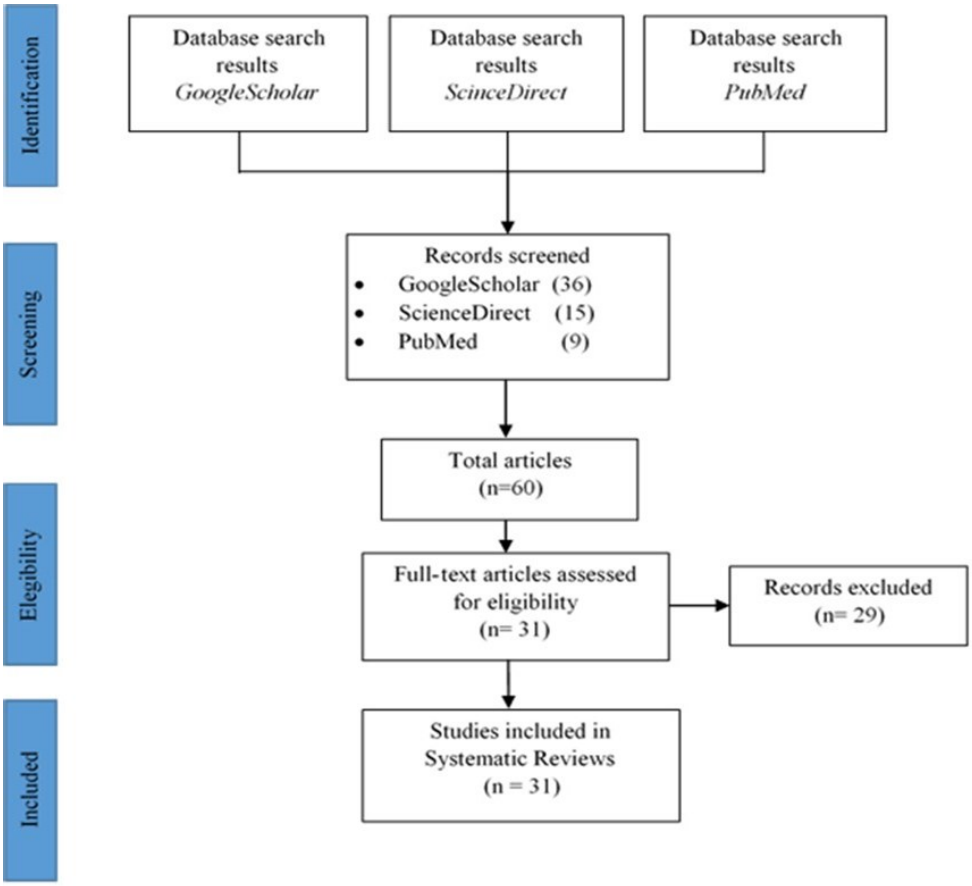


Figure 2. Flow diagram of the study selection process following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines

METHODS

Materials

Criteria of collecting data

Inclusion criteria used to select the articles were: (i) Studies that referred to *in vivo* research models that focused on osteoporosis; (ii) Studies that used plants which contain phytoestrogens; and (iii) Studies that reported the benefits of phytoestrogens in bone formation.

The exclusion criteria applied to each article were: (i) Article in another language besides English and Indonesian; (ii) Research article that published before the last ten years; and (iii) article that was not in full text or could not be fully accessed.

Collecting strategy and article selection

Article searching used the electronic databases of Google Scholar, ScienceDirect, and PubMed in January 2021. The keywords used were "Chemical content, leaves or bark, *Chrysophyllum cainito* or *Elaeis guineensis* or *Lannea acida* or *Marsilea crenata* or *Medicago sativa,* phytoestrogen and *in vivo* antiosteoporotic". After that step, by screening the title and abstract from the articles that were considered compatible with the research, article screening was done based on the inclusion and exclusion criteria that were developed for this research. Furthermore, the article was selected to be a discussion analysis objective or primary article. Any article that was considered relevant could be used as a supported analysis in this systematic review.

Methods

This review literature with a systematic review used the Meta-Synthesis methods with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Selcuk, 2019; Snyder, 2019).

Data extraction

Collected data from all of the studies were organized and analyzed using the PRISMA guidelines and Meta-Synthesis methods. Moreover, data that met the inclusion criteria were arranged in a table and the articles were analyzed to match the aim of this systematic review.

Table 1. Investigation results for phytoestrogens articles from plants C. cainito, E. guineensis, L. acida, M. crenata, and M. sativa

No.	First Author, Year of Publication	Material / Extract	Phytochemical Components (Phytoestrogens)	Observation Method
1	(Ningsih *et al*., 2016)	70% ethanol extract of *C. cainito* leaves	Flavonoids	UV-Vis
2	(Arrijal *et al*., 2018)	Ethyl acetate extract of *C. cainito* leaves	Flavonoids	Color test
3	(Ma'arif *et al*., 2019)	Methanol extract of *C. cainito* leaves	Myricetin, dibutyl phthalate	UPLC-QToF-MS/MS
4	(Arana-Argáez *et al*., 2017; Sayed *et al*., 2019)	Methanol extract of *C. cainito* leaves	Flavonoids, Terpenoids	GC-MS
5	(Soundararajan and Sreenivasan, 2012)	Methanol extract of *E. guineensis* leaves	Flavonoids	FTIR analysis
6	(Vargas *et al*., 2016; Zain *et al.,* 2020)	95% ethanol extract of *E. guineensis* leaves	Flavonoids	UHPLC-MS/MS
7	(Tahir *et al*., 2012)	Methanol extract of *E. guineensis* leaves	Apigenin, Luteolin	LC–MS
8	(Yin *et al*., 2013; Ajayi *et al,* 2016)	Methanol extract of *E. guineensis* leaves	Flavonoids, Terpenoids	Color test
9	(Soundararajan and Sreenivasan, 2012)	Methanol extract of *E. guineensis* leaves	Flavonoids	FTIR analisis
10	(Vargas *et al*., 2016; Zain *et al.,* 2020)	95% ethanol extract of *E. guineensis* leaves	Flavonoids	UHPLC-MS/MS
11	(Tahir *et al*., 2012)	Methanol extract of *E. guineensis* leaves	Apigenin, Luteolin	LC–MS
12	(Yin *et al*., 2013; Ajayi *et al,* 2016)	Methanol extract of *E. guineensis* leaves	Flavonoids, Terpenoids	Color test
13	(Muhaisen, 2013)	Acetone extract of *L. acida* bark	Flavonoids	UV-Vis
14	(Ogunsina, 2020; Olatunji *et al*., 2020; Olusola *et al*., 2020)	Methanol extract of *L. acida* bark	Flavonoids	Color test
15	(Ma'arif *et al*., 2019)	96% ethanol extract of *M. crenata* leaves	Prochlorperazine, 12-Aminododecanoic acid, 1-methyl-2-[(4-methylpiperazin-1-yl)methyl]benzimidaol-5-amine.	UPLC-QToF-MS/MS
16	(Ma'arif *et al.,* 2016)	*n*-hexane extract of *M. crenata* leaves	Monoterpenoid, diterpenoid, and palmitic acid	GC-MS
17	(Ma'arif *et al*., 2019)	*n*-hexane extract of *M. crenata* leaves	Triterpenoid	UV-Vis
18	(Puspitasari *et al*., 2015)	*n*-hexane fraction of *M. crenata* leaves	Terpenoids	^{1}H-NMR
19	(Nurjanah *et al*., 2012; Hardoko *et al*., 2019)	Methanol extract of *M. crenata* leaves	Flavonoids	Color test
20	(Susilowati *et al*., 2014; Widyowati *et al*, 2014)	70% ethanol extract and 96% ethanol extract of *M. sativa* leaves	Flavonoids	TLC
21	(Rodrigues *et al.,*2014)	Methanol extract of *M. sativa* leaves	Flavonoids	HPLC
22	(Krakowska *et al.,* 2017)	70% ethanol extract of *M. sativa* leaves	Flavonoids	HPLC-MC
23	(Doss *et al*., 2011)	Ethanol extract of *M. sativa* leaves	Flavonoids	Color test

Notes: UV-Vis (Ultraviolet-Visible),UPLC-QToF-MS/MS (Ultra-High Performance Liquid Chromatography with Quadrupole Time-of-Flight Mass Spectrometry), ^{1}H-NMR (Proton Nuclear Magnetic Resonance), GC-MS (Gas Chromatography Mass Spectrometry), FTIR (Spektrofotometer Fourier Transform Infra Red),UHPLC-MS/MS (Ultra-High Performance Liquid Chromatography with Mass Spectrometry), LC–MS (Liquid Chromatography-Mass Spectrometry), TLC (Thin Layer Chromatography), HPLC (High Performance Liquid Chromatography).

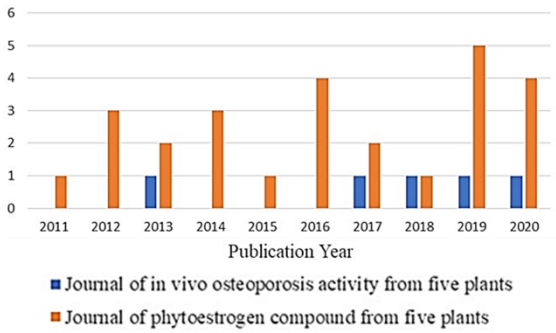


Figure 3. Total of article publications in the 2011-2020 period

RESULTS AND DISCUSSION

Article selection

Articles in this systematic review study were selected using the PRISMA guidelines to create a flowchart diagram as shown in Figure 2. There were 31 articles obtained from the database searching. Among them, 16 articles were from Google Scholar, 6 articles were from ScienceDirect, and 3 articles were from PubMed. After identifying the titles, abstracts, and article discussions that were relevant to the research, there were 31 articles that met the inclusion criteria, and 29 articles that did not meet the inclusion criteria or exclusion criteria. From the 31 articles analyzed, 26 articles discussed phytochemical component analysis that was observed with various instruments, and 5 articles used the *in vivo* research model; all of which were published within the last 10 years (Figure 3). The articles were discussed with the Meta-Synthesis method which involves analyzing, identifying, and interpreting article data that were presented systematically.

Article characteristics

The primary characteristics of the articles that were included in this review discussion are summarized in Tables 1 and 2**,** with a total of 31 articles or primary analyzed data. There are 5 articles involving *in vivo* research models from the 5 plants that have potential as anti-osteoporosis agents. Also, the articles that focus on phytoestrogen content in *C. cainito* leaves are 5 articles, 4 articles on *E*. guineensis leaves, 4 articles on *L. acida* bark, 3 articles on *M. crenata* leaves and 4 articles on *M. sativa* leaves.

Phytoestrogen as anti-osteoporosis agents

Phytoestrogens are chemical compounds from plants that have a similar structure as estrogen or can replace the function of estrogen. Structurally, phytoestrogens are divided into two main groups based on their chemical structure. There are flavonoid compounds including isoflavones (genistein, daidzein, glycitein, biochanin A, and formonoetin), coumestans (equol), prenyflavonoids, and non-flavonoid compounds including lignans (pinoresinol, 4-methoxy pinoresinol, and eudesmin), and terpenoids (α-amyrin, and β-amyrin) (Virk-Baker *et al*., 2010; *Zhou et al*., 2014; Kiyama, 2017; Křížová *et al*., 2019) (Table 3). A chemical compound will have similar pharmacological properties as phytoestrogen if it has similar pharmacophore distances, and has a similarity of the type of bound amino acids (Ma'arif *et al*., 2019).

Estrogen biological effect in bone formation begins when estrogen (as a ligand) diffuses into cells then binds with estrogen receptor (ER). The receptor will be activated and form an estrogen-receptor complex that is able to penetrate the nucleus (translocation). This estrogen-receptor complex will bind to a particular part in the DNA, which is called estrogen-response-element (ERE). This process is followed by complex genetic transcription, which causes an estrogenic effect in bones which includes forming osteoblast cells and inhibiting the overproduction of osteoclast cells (Sihombing *et al.*, 2012). Since phytoestrogens have a similar structure or activity with the estrogen hormone in the body, phytoestrogens potentially can replace the function of estrogen to induce the estrogenic effect in bone formation.

Phytoestrogen compound from plants C. *cainito, E. guineensis, L. acida, M. crenata,* and *M. sativa*

C. cainito leaves chemically contains phytoestrogen from the flavonoid compound group and terpenoids that was observed with color test, ultra-violet (UV)-Vis Instrument, UPLC-QToF-MS/MS, and gas-chromatography and mass spectrometry (GC-MS) (Table 1). Myricetin and dibutyl phthalate are compounds that are found in methanol extract of *C. cainito* leaves, where the compounds are derivates from flavonoids (Mayanti *et al*., 2017; Ma'arif *et al.*, 2019; Yahmin *et al.*, 2019). Remarkably, the GC-MS instrument identified *C. cainito* leaves have 7 derivate compounds of flavonoids: 3//galloyl myricitrin, rutin, quercitrin, myrecetrin, myricetin, and quercetin. Also, there are 3 terpenoid compounds, are α–amyrin, β–amyrin, and lupeol (Arana-Argáez *et al*., 2017; Sayed *et al*., 2019). The UV-Vis instrument identified *C. cainito* leaves have flavonoid total content of 14.039 ± 0.030 mg gallic acid equivalent (GAE)/g extract (Ningsih *et al.*, 2016). Additionally, the qualitative chemical test which is the flavonoid

Table 2. Search results for articles containing in vivo analysis from plants *C. cainito*, *E. guineensis*, *L. acida, M. crenata,* and *M. sativa*

No	First Author, Year of Publication	Material	Animal Model	Osteoporosis Model	Observed section	Analysis Results (Histomorfo-metri)	Bone Remodelling Mechanism
1	(Utaminingtyas *et al*., 2018)	70% ethanol extract of *C. cainito* leaves	Female Mice (*M. musculus*)	Induction with the drug dexamethasone	Trabecular Vertebra	Has activity in increasing trabecular bone vertebrae of female mice (*M. musculus*) with an optimum dose of 400 mg / kgB /day.	↑Osteoblastogenesis ↓Osteoclastogenesis
2	(Bakhsh *et al*., 2013)	50% ethanol extract of *E. guineensis* leaves	Female Rat (*R. norvegicus*) *Sprague Dawley*	OVX	Trabecular Femur	At a dose of 300 mg/ kgBW/day from the leaves extract of *E. guineensis*, it increased the trabecular bone density of female rats (*R. norvegicus*).	↑ ALP activity ↑ Calcium deposition ↑Ekspression mRNA of Runx2 ↑ Protein osteocalcin ↓ Cytokines IL1, IL6, dan IL7 ↓ TNF production ↓ Ekspression COX-2 of RANKL
3	(Oumarou *et al*., 2017)	95% ethanol extract of *L. acida* bark	Female Rat (*R. norvegicus*) *Albino Wistar*	OVX	Trabecular Femur	Trabecular bone density of female rats (*R. norvegicus*) increased due to the induction of *L. acida* leaves ethanol extract at a dose of 200 mg/kgBW/day.	↑ ERβ activition ↑ Production of osteoblasts cell
4	(Agil *et al*., 2019)	n-hexane extract of M. crenata leaves	Female Rat (*R. norvegicus*)	Induction with the drug dexamethasone	Trabecular Vertebra	In the n-hexane fraction of *M. crenata* has activity in increasing the trabecular bone density of female rats (*R. norvegicus*), at a dose of 3.08 mg/20gBW	↑ Production of osteoblasts cell ↓ Production of osteoclasts cell
5	(Jdidi *et al*., 2020)	Ethanol extract of *M. sativa* leaves	Female Mice (*M. musculus*)	OVX	Trabecular Femur	There was an increase in the trabecular bone density of the female mice (*M. musculus*) which was induced by the ethanol extract of *M. sativa* leaves at a dose of 0.75 g/kgBW/ day.	↑ Production of osteoblasts cell ↓ Lipid and protein oxidation biomarkers

test showed a changing of orange color indicating there are flavonoid compound groups derived from *C. cainito* leaves (Arrijal *et al.,* 2018).

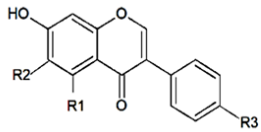
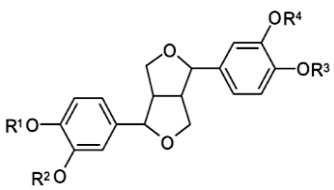
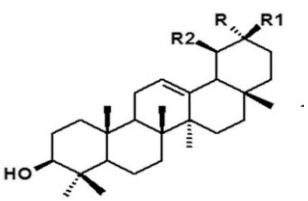
The compounds from *E. guineensis* leaves contain phytoestrogens from the flavonoid compound group, including flavone, apigenin, and luteolin which were observed with the color test, Fourier-transform infrared spectroscopy (FTIR) instrument, UPHPLC-MS/MS, and LC-MS (Table 1). Flavonoid compounds from *E.*

guineensis leaves were found in the FTIR instrument analysis that showed stretching vibrations from flavonoids and other minor flavone groups with wave range 100-1700 cm^{-1} (Soundararajan and Sreenivasan, 2012). Apigenin and luteolin derivate compounds from *E. guineensis* had been identified with the UPHLC-MS/MS instrument (Vargas *et al*., 2016; Zain *et al.,* 2020). Moreover, the LC-MS instrument identified that *E. guineensis* leaves have 28 flavonoid derivate compounds (Tahir *et al*., 2012)**.** In qualitative phytochemical screening analysis, the yellow color from the Shinoda test shows that there are the flavonoid compound group and terpenoid compounds in *E. guineensis* leaves (Yin *et al*., 2013; Ajayi *et al*, 2016).

The compounds in *L. acida* bark are flavonoid compounds that have been observed with the UV-Vis instrument and by chemical qualitative methods (Table 1). There were 4 flavonoid compounds found with the UV-Vis instrument, 6,7-(2",2"-dimethyl chromeno)-8-γ,γ-dimethyl allyl flavanone, 3',4'dihydroxy-7,8 (2",2"- dimethyl chromeno)-6-γ,γ dimethyl allyl flavanol, 7-methyltectorigenin, and Irisolidone (Muhaisen, 2013). Additionally, the flavonoid qualitative analysis test showed a yellow color meaning there is a flavonoid compound group (Ogunsina, 2020*;* Olatunji *et al*., 2020; Olusola *et al*., 2020).

The content of *M. crenata* leaves was observed with UPLC-QToF-MS/MS, GC-MS, and UV-Vis instrument which identified several phytoestrogens (Table 1). The UPLC-QToF-MS/MS instrument found Prochlorperazine,12-Aminododecanoic acid, and1-methyl-2- (4-methyl piperazine-1-yl) methyl benzimidazole-5-amine components, that are potentially active as phytoestrogen compounds due to their similar affinity that has been predicted in silico. Monoterpenoid, diterpenoid, and palmitic acid compounds are compounds that had been found with the GC-MS instrument, whereas monoterpenoid and terpenoid are terpenoid groups. Palmitic acid is a fatty acid group that has a mechanism to increase osteogenesis (Ma'arif *et al.,* 2016). In the UV-Vis instrument, there is a triterpenoid component that was found in *M. crenata* with maximum absorption in λ 232 nm and 242 nm wavelength. Identification *M. crenata* with spectroscopy ^{1}H-NMR showed terpenoid compounds, due to the result of analysis contains signals on δH 0.8 to 1.3 ppm, which indicated that these are isolated terpenoid compounds because it has a long-chain altakane group (Puspitasari *et al*., 2015). Moreover, the flavonoid qualitative test of *M. crenata* leaves shows yellow color, which is a characteristic of the flavonoid compound group (Nurjanah *et al.*, 2012; Hardoko *et al*., 2019).

Table 3. Structure of phytoestrogens such as flavonoid and non-flavonoid (Virk-Baker et al., 2010; Zhou et al., 2014 Křížová et al., 2019)

Flavonoid	Structure	R1	R2	R3	R4
Genistein (isoflavones)		OH	H	OH	OH
Daidzein (isoflavones)		H	H	OH	OCH_3
Glycitein (isoflavones)		H	OCH_3	OH	OCH_3
Formonoetin (isoflavones)		H	H	OCH_3	OH
Biochanin A (isoflavones)		OH	H	OCH_3	OH
Equol (coumestans)		H	H	OH	OH
Non-Flavonoid	**Structure**	**R1**	**R2**	**R3**	**R4**
Pinoresinol (lignans)		H	CH_3	H	CH_3
4-methoxy pinoresinol (lignans)		H	CH_3	CH_3	CH_3
Eudesmin (lignans)		CH_3	CH_3	CH_3	CH_3
α-amyrin (terpenoids)		H	CH_3		
β-amyrin (terpenoids)		CH_3	H		

Phytoestrogen compounds from *M. sativa* leaves are a flavonoid compound group that was observed with the thin-layer chromatography (TLC), HPLC, and HPLC-MC instruments (Table 1). The content of *M. sativa* that has been observed with TLC densitometry shows a spot yellow color, which is a characteristic of the flavonoid compound group (Susilowati *et al.*, 2014; Widyowati *et al*., 2014). The HPLC instrument identified that the average content of isoflavone is about 2.3 (mg/kg/db) (Rodrigues *et al.,* 2014). Also, the HPLC-MC instrument showed that there are derivate compounds from flavonoids, such as apigenin, luteolin, and quercetin (Krakowska *et al.,* 2017). The qualitative phytochemical analysis screening in *M. sativa* leaves also showed there is a flavonoid compound group (Doss *et al.,* 2011).

Activities of bone formation from plants *C. cainito, E. guineensis, L. acida,* and *M. sativa* for in vivo

Table 2 explains that *in vivo* research results from plants C*. cainito, E. guineensis, L. acida, M. crenata,* and *M. sativa* have anti-osteoporosis potential because of their phytoestrogen content. There are two kinds of osteoporosis models that are used in these five plants, which are ovariectomy (OVX) and induced by medicine. OVX is an ovary removal method in a female experimental animal, so the ovary cannot produce estrogen (Yuliawati *et al*., 2019; Yousefzadeh *et al*., 2020). This model had been applied to *in vivo* study of *E. guineensis* leaves, *M. sativa* leaves, and *L. acida* bark. Furthermore, the osteoporosis model by inducing experimental animals with medicine was used in the research conducting *in vivo* study of *C. cainito* leaves and *M. crenata* leaves, where the experimental animal is given the medicine that is proved to have a side effect of osteoporosis; one of which is dexamethasone. Dexamethasone is a synthetic substance from the corticosteroid group that has high glucocorticoid content which may cause side effects, and this higher glucocorticoid content will cause inhibition of bone formation from long-term use (Agil *et al.*, 2019).

Moreover, in observations of bone density in experimental animals from the *in vivo* study, histomorphometry has been used. Cells or tissue measurement methods to study the changing shape and activity of the cells are done through volume, thickness, length, and wide measurement with an optic microscope, for example, using the *Carl Zeiss teaching, Olympus cellsens* (Yanti *et al.,* 2019). Histomorphometry observation in bone density was done by observing the quantity or sign marker of bone formation. This method was performed by i*n vivo* study of *E. guineensis* leaves (in U/L), increasing quantity of bone inorganic matrix in *L. acida* bark, and *M. sativa* leaves (in mg/g or mmol/L). Also, it is used to measure growth by directly counting on bone specimen thickness value for an *in vivo* study of *C. cainito* and *M. crenata* leaves (in µm).

Giving ethanol extract in *C. cainito* leaves with 400 mg dose to mice (*M. musculus*) has activity in increasing the vertebrate trabecular bone density which has the average bone specimen thickness of 626.96 µm. This research suggests increases in bone density occurs because phytoestrogen compounds in the *C. cainito* leaves potentially replace the estrogen function in binding with ER. As a result, its binding causes decreasing in osteoclastogenesis and bone resorption. Also, it causes an increase in osteoblastogenesis and bone formation (Utaminingtyas *et al*., 2018).

Giving *E. guineensis* leaves extract with 300 mg/kg BW/day dose to female rats (*R. norvegicus*) revealed increasing femur bone density with alkaline phosphatase (ALP) (U/L) 272.33± 3.80 because the phytoestrogen compounds from this plant are able to raise ALP activity, increase calcium deposition, increase mRNA expression from runx2, and increase osteocalcin protein in increment bone formation. Moreover, the phytoestrogen compounds from *E. guineensis* potentially suppress the inflammation cytokines: interleukin (IL)-1, 6, and 7) protein production and osteoclast activity thereby inhibiting tumor necrosis factor (TNF) production, and cyclooxygenase expression (COX-2) in RANKL expression (Bakhsh *et al*., 2013).

Giving the extract of *L. acida* bark with 200 mg/kg BW/day doses to female rats (*R. norvegicus*) showed increasing in femur bone formation in the experimental animals which was characterized by increasing of inorganic bone matrix quantities: specifically, *Calcium* (Ca) 0.043 ± 0.005 mmol/L, and *Phosphorus* (P) 4.30 ± 0.482 mmol/L. In this study, phytoestrogen content from *L. acida* potentially increased the inorganic bone matrix. Osteoblast cells are the cells that form the bone matrix. Consequently, a phytoestrogen in *L. acida* affects bone formation through ERβ localized activity on osteoblast cells (Oumarou *et al*., 2017).

The n-hexane extract from *M. crenata* leaves with 3.08 mg/20g BW dose which was given to female rats (*R. norvegicus*) showed an

increasement of vertebrate bone density with the result of average bone thickness ± standard deviation (SD): 8.0 ± 0.3 µm. This occurs due to the content of phytoestrogen in *M. crenata* leaves. Phytoestrogen compounds bind ER in the main cells, thereby decreasing osteoclastogenesis and bone resorption, while also increasing osteoblastogenesis and bone formation (Agil *et al*., 2019).

In addition, giving ethanol extract of *M. sativa* leaves with 0.75 g/kg BW/day dose also had activity on femur bone density in the female mice (*M. musculus*), which was indicated by increasing inorganic bone matrix quantities (mg/g): specifically, *Calcium* (Ca) 21.942 ± 0.133, *Phosphorus* (P) 7.530 ± 0.056, and *Magnesium* (Mg) 0.423 ± 0.010. This article explained that increasing bone matrix density occurs because there is a growth of the inorganic matrix, which is an indicator of higher bone replacement. Accordingly, the phytoestrogen content from *M. sativa* leaves may lower oxidation lipid and protein biomarkers due to high levels of oxidant production that disturb the balance of normal redox and shift the cells into oxidative stress condition (Jdidi *et al*., 2020).

CONCLUSIONS

Anti-osteoporosis activity from plants *C. cainito*, *E. guineensis*, *L. acida*, *M. crenata*, and *M. sativa* can affect trabecular bone density in the experimental animals by different mechanisms, but in general, it occurs because there is an increase in bone formation and a decrease in bone resorption due to the phytoestrogen content from each plant.

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