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REVIEW

Medical applications of phytoestrogens from the Thai herb *Pueraria mirifica*

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Abstract ***Pueraria mirifica*** **Airy Shaw et Suvatabandhu is a medicinal plant endemic to Thailand. It has been used in Thai folklore medicine for its rejuvenating qualities in aged women and men for nearly one hundred years. Indeed, it has been claimed that *P. mirifica* contains active phytoestrogens (plant substances with estrogen-like activity). Using high performance liquid chromatography, at least 17 phytoestrogens, mainly isoflavones, have been isolated. Thus, fairly considerable scientific researches, both *in vitro* in cell lines and *in vivo* in various species of animals including humans, have been conducted to date to address its estrogenic activity on the reproductive organs, bones, cardiovascular diseases and other climacteric related symptoms. The antioxidative capacity and antiproliferative effect on tumor cell lines have also been assessed. In general, *P. mirifica* could be applicable for preventing, or as a therapeutic for, the symptoms related to estrogen deficiency in menopausal women as well as in andropausal men. However, the optimal doses for each desirable effect and the balance to avoid undesired side effects need to be calculated before use.**

Keywords white Kwao Krua; miroestrol; estrogenic activity; reproduction; osteoporosis; breast cancer; Alzheimer

What is *Pueraria mirifica*?

Pueraria mirifica Airy Shaw et Suvatabandhu is a leguminous plant that is endemic to Thailand [1,2], and belongs to the Family Leguminosae, subfamily Papilionoideae or the soy, bean and pea subfamily. This plant is widely distributed in deciduous forests throughout Thailand at altitudes of 300–800 m, and is commonly and abundantly found in the north, west and north-east [3,4]. It has also been claimed that this plant is found in Myanmar, but this has not been confirmed [4]. *P. mirifica* is locally called Kwao Krua, Kwao Krua Kwao, Kwao Krua Kao, white Kwao Krua or Guao Krua [4], of which white Kwao Krua is the common name that is popularly used in the scientific literature (e.g. Refs. 5–7).

P. mirifica is a woody perennial climber (Fig. 1a) with tuberous roots that look like a chain of round-shaped bulbs of various sizes connected to the next one via a small root (Fig. 1c), and which occur throughout the entire length of the root. Its tuberous root contains whitish and round starch granules (diameter of 3–5 μm) [8] (Fig. 2a–d). The shape and size of the tuberous roots are diverse and depend on the environment in which they are grown and on their genetics [3,9,10]. A single tuberous root weighs between 60–100 kg. The flowers are bluish purple in color with a long inflorescence (maximum 30 cm), somewhat similar to a bean flower (Fig. 1b). The plant flowers from February to March and produces seedpods in April [4]. The seedpods of *P. mirifica* are 7 mm wide and 3 cm in length with hair and when mature are brown, flat and contain up to 3–5 seeds [1].

The use of *P. mirifica* was first mentioned in a pamphlet written by Suntara in 1931 as a source of rejuvenation and good health [11]. A year later, Kerr, the director of the botanical section of the *Journal of the Siam Society*, widely drew the attention of the scientific community to the rejuvenating qualities of this plant [12]. Later, Sukhavachana reported the estrogenic activity of the extract from its tuberous root in ovariectomized rats, in terms of stimulating a vaginal cornification and increasing uterine weight [13]. However, in those reports, the plant was mistakenly identified as *Butea superba* Roxb (currently named as red Kwao Krua in Thai), no doubt because of their superficial resemblance [14]. Later, in 1952, white Kwao Krua was recognized as a new species and reclassified as *Pueraria mirifica* (Latin, *mirificus* = amazing or wonderful) by Kashemsanta and Suvatabandhu with the help of Airy Shaw [1,15].

In Thailand there are indeed two plants species known as

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Fig. 1 *P. mirifica* (a) cultivated in north-eastern Thailand, and its leaves, flowers (b) and tuberous roots (c) (with permission from N. Urasopon).

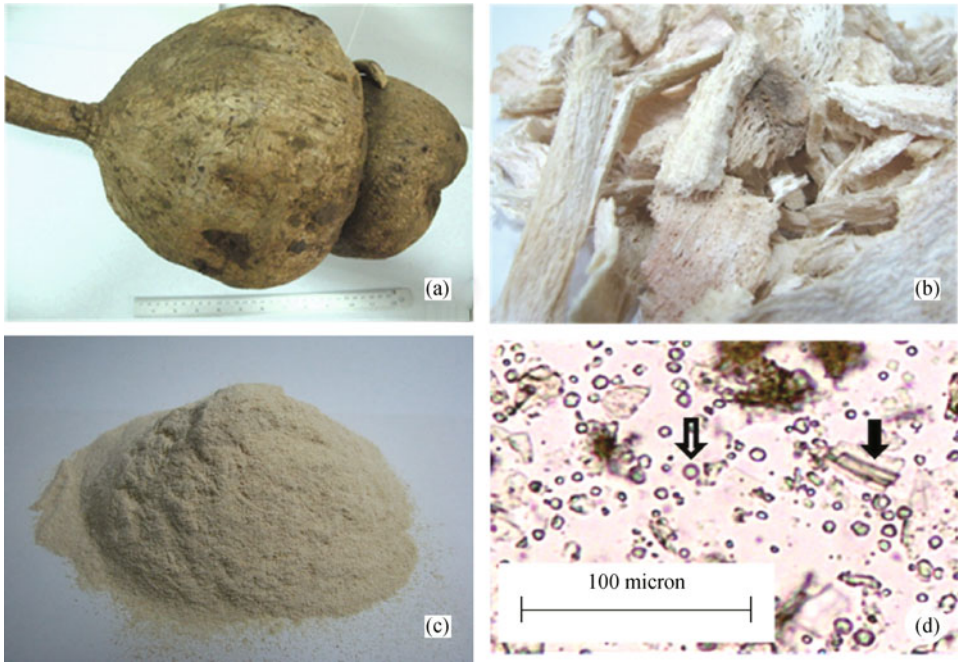

Fig. 2 The structure of *P. mirifica* tubers showing (a) the roots and tuber system, (b) texture of the cut and dried roots, (c) 100 mesh sized dried powder and (d) the number and shape of fibers and starch granules under the light microscope. The arrows indicate (⇑) starch granules and (↑) fibers.

white Kwao Krua: *P. candollei* var. *candollei* (syn. *P. candollei*) and *P. candollei* var. *mirifica* (syn. *P. mirifica*). These two species have similar botanical characteristics (Fig. 3) and both have been used in Thai traditional medicine [4]. Indeed both species contain comparable amounts of phytoestrogens [16].

Ethnobotany of *P. mirifica*

The bioactive compounds in *P. mirifica* are found in the tuberous root, in accordance with the observation that it is the roots in particular that have been used by local communities in Thailand for over a hundred years, and specifically for its rejuvenating qualities in menopausal women and andropausal men. The usage of *P. mirifica* in folklore medicine suggests that it contains some active substances alike female hormones [17]. The ordinary dosage of *P. mirifica* for women and men is about a peppercorn-sized piece [11], equivalent to about 250 mg/50 kg BW or 5 mg/kg BW, taken once a day at night. Suntara [11] stated that *P. mirifica* can serve as the "fountain of youth" for aged men and women, with use as an anti-wrinkle agent for wrinkled skin, to darken white hair and increase hair growth, alleviate cataracts in the eyes, help with memory loss, increase energy and vigor, provide more reflexive bodily movements, increase blood circulation and appetite and alleviate sleep disorders. Thai people also used it to recover black hair, promote an appetite and increase their longevity [18]. Recently, it has been popularly used to alleviate the symptoms of the aging process and estrogen deficiency, such as sagging breasts, wrinkled skin, bone loss and gray hairs. At present, *P. mirifica* has been included into the cosmetic, dietary supplement and pharmaceutical products.

P. mirifica has long been traditionally prepared in the form of pills for oral intake. To this end the dried *P. mirifica* roots are finely ground, sometimes mixed with other medicinal plants, packed in capsules and marketed as a dietary supplement. However, in some commercial brands the *P. mirifica* capsules are prepared from the extracts of the root rather than the crude ground root powder. Indeed, the use of *P. mirifica* root extracts was introduced several years ago since, with modern technology, the extract from dried *P. mirifica* roots could be standardized for bioactivity and prepared in the form of either a powder or a solution and so offers consistency between batches. In addition, the liquid extract solution is used in the cosmetic industry, such as in the preparation of *P. mirifica* breast cream, eye gel and skin moisturizer, while the solid extract powder is used as a dietary supplement similar to the traditional crude ground tuber powder, but with a greater degree of consistency in the bioactivity between batches as mentioned. Various forms of *P. mirifica* products are sold on the markets, such as cream, serum, spray, soap, cookies, gel and gum.

Chemical constituents of *P. mirifica*

Kerr [12] was the first to create international awareness of the rejuvenating qualities of this plant, which subsequently led to

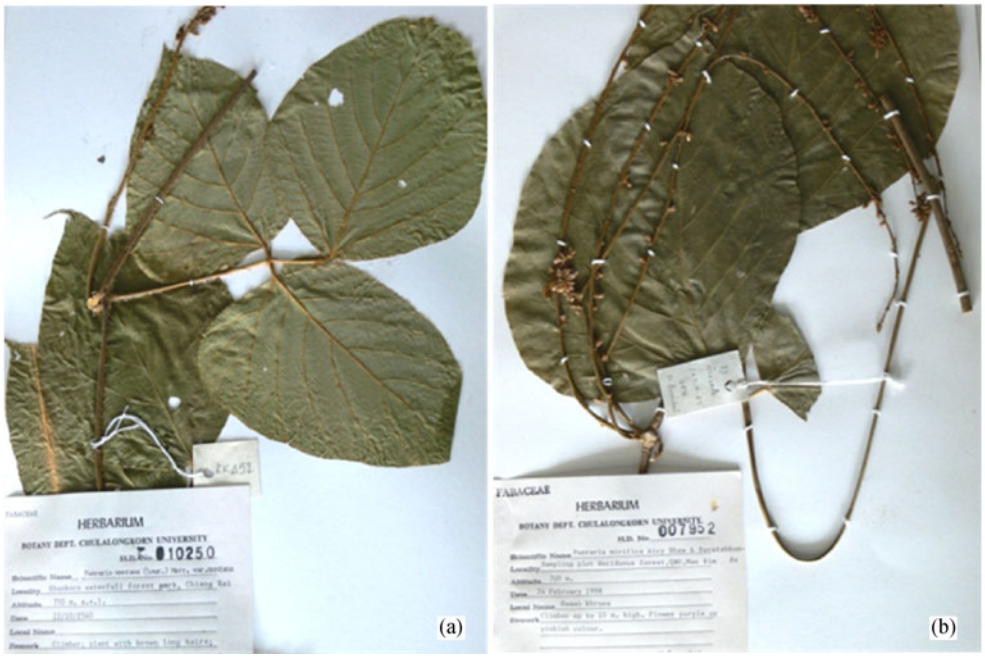

Fig. 3 Voucher specimens, numbers (a) BCU010250 and (b) BCU011045, of *P. mirifica* (Airy Shaw & Suvatabhandhu) kept at the Professor Kasin Suvatabhandhu Herbarium, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand.

the isolation of a potent phytoestrogen, i.e. a non-steroidal plant compound exhibiting estrogenic-like activity. The first phytoestrogen isolated from *P. mirifica* was miroestrol [17,19–21], which has the highest estrogenic potency among all the subsequentially isolated phytoestrogens from *P. mirifica* [22,23]. Compared to 17β-estradiol, miroestrol exhibited a 4-fold lower bioactivity level [24]. The estrogenic activity is found in the roots, leaves and stems of the plant, but the estrogenic activity in the latter two plant parts is minimal compared to that in the roots [21], and so the chemical analysis of the leaves and stems has not been performed. The tuberous roots of *P. mirifica* were analyzed by high performance liquid chromatography (HPLC) with estrogenic bioactivity screening of the fractions, from which at least 17 chemical substances with estrogenic activities were found. These can be categorized into three groups as (i) ten isoflavonoids, comprised of daidzein, daidzin, genistin, genistein, kwakhurin, kwakhurin hydrate, tuberosin, puerarin, mirificin and puemiricarpene; (ii) four coumestrans, comprised of coumestrol, mirificoumestan, mirificoumestan glycol and mirificoumestan hydrate, and (iii) three chromenes, comprised of miroestrol, deoxymiroestrol and isomiroestrol [9,16,17,22,25–30] (Fig. 4a–c). These are all phytoestrogens which have structures similar to that of 17β-estradiol (Fig. 4d) [17,31,32]. Puerarin is found only in *Pueraria* plants [30]. While miroestrol [16] and kwakhurin [1,33] are only found in *P. mirifica*, and so they can be used as index of species identification and also qualification.

It was recently reported that miroestrol and isomiroestrol are in fact artifacts, as they are derived from the oxidation of deoxymiroestrol during the extraction and isolation procedure [22], and thus it is deoxymiroestrol that is *P. mirifica* specific. The estrogenic activity of deoxymiroestrol extracted from *P. mirifica* was found to be 10-fold higher than that for miroestrol and isomiroestrol, as assayed by the proliferative effect on the MCF-7 human breast cancer cell line [22].

Chansakaow *et al*. [22] noted that 100 g of dry powdered *P. mirifica* tubers contains 46.1 mg of daidzein and 2–3 mg of miroestrol and deoxymiroestrol, although there is likely to be cultivar and cultivation dependent variation in those levels. Malaivijitnond *et al*. [34] analyzed the isoflavone content in *P. mirifica* cultivar Wichai III using HPLC and found that this cultivar contains a significant amount of isoflavones (187.1 mg total isoflavones/100 g dry powdered tubers). Generally, puerarin accounted for about half of the total isoflavone content with a slightly comparable amount of genistin and daidzin [29,30]. The content of puerarin in *P. mirifica*, as analyzed by HPLC, ranged from 53.2–870.5 μg/g *P. mirifica* ground tuber powder [9,29,30]. Indeed, puerarin, genistin and daidzin are isoflavones in glycoside forms and could be partially hydrolyzed by cleaving a C-glycosyl bond to the aglycoside forms of daidzein, genistein and daidzein, respectively, by intestinal microflora [35–37]. This is important since these aglycoside forms possess a considerably stronger estrogenic activity than the original glycoside forms [1,28]. The potential alteration of bioactivity through metabolic conversion following oral administration by either gut microbes prior to absorption or in the liver following absorption serves to remind all of the need for caution in evaluating bioactive compounds solely by the use of *in vitro* tissue or cell line culture.

Cherdshewasart *et al*. [29] examined the levels of five

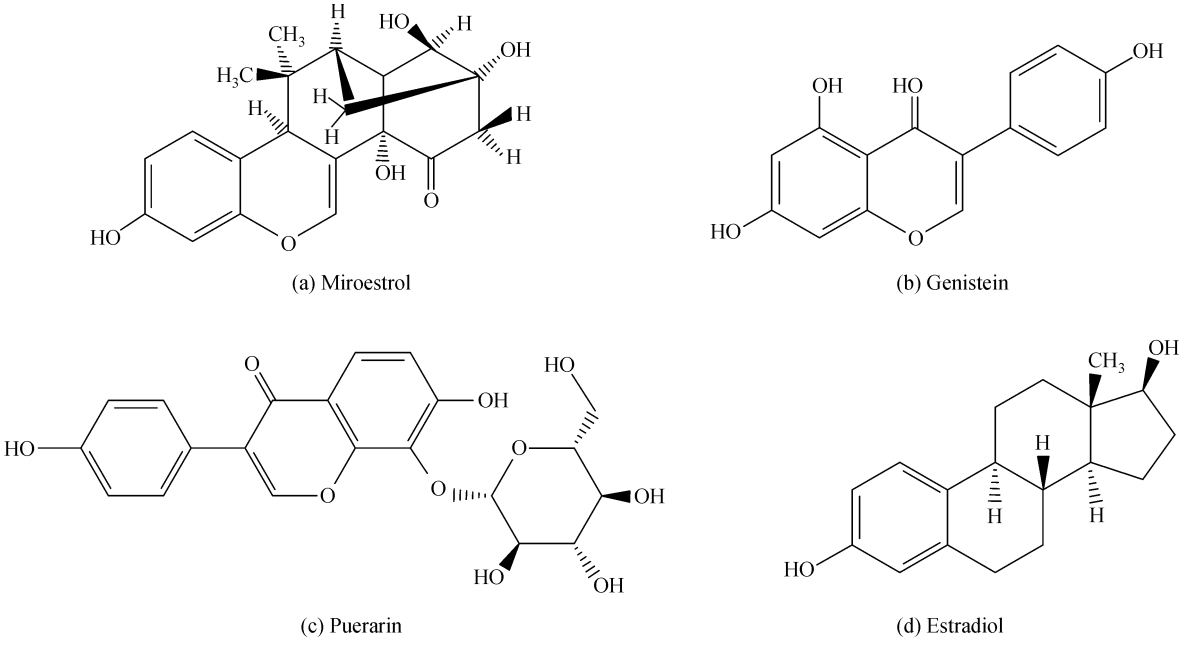

Fig. 4 Chemical structures of (a) miroestrol, (b) genistein, (c) puerarin and (d) 17β-estradiol.

isoflavonoids in the roots of three-year-old *P. mirifica* plants collected from 28 of the 76 provinces in Thailand. They found a high (up to 10-fold) variation of isoflavonoid contents between locations, ranging from 18.1 to 198.3 mg/100 g dry tuber powder, with a higher variation between provinces than between districts within each province. However, within the same population, the isoflavonoid contents were almost homogeneous. They concluded that a major factor for the difference was genotype, although environmental effects and plant age may cause some effects [9,29]. The major isoflavones found in their study were puerarin and genistin. Confirming the conclusion of Cherdshewasart *et al*. [29], the isoflavonoid levels in tuberous roots of the same cultivar (*P. mirifica* cultivar Wichai-III), cultivated in the same location, did not differ much over the different seasons, ranging from 123.2 to 157.3 mg/100 g dry tuber powder [30]. However, while the same cultivar grown in the same location (intra-cultivar variation) still showed variations in the isoflavone content, it was lower than the variation between different cultivars (inter-cultivar variation) [30].

The isoflavonoid contents in the tuberous roots of *P. mirifica* were also dependent on the age of the plant. One-year-old plants, with smaller sized and fewer differentiated tubers, showed a greater variation in isoflavonoid levels than those that were at least three years old with their large and fully differentiated tubers. Indeed, fully differentiated tubers from plants within the same population did not exhibit much variation in their isoflavonoid contents [9]. Thus, the degree of chemovariation in the same plant cultivar was reduced as the tuber became more differentiated [9,29]. As a consequence, plants that are at least three years old were recommended for tuberous root collection [29,38].

The chemovariation in *P. mirifica* plants was also found to depend on the harvesting season. Plants collected in the summer had the higher isoflavonoid content than those harvested in the winter and rainy seasons, respectively. However, the distribution profile of puerarin and daidzin was such that they can be used as chemical markers to evaluate the influence of both the cultivation conditions and the cultivar genetics on the tuberous storage of isoflavonoids in *P. mirifica* [9]. When the amount of phytoestrogens in each part of the tuberous root of *P. mirifica* was compared, the tuber cortex contained higher levels of miroestrol, deoxymiroestrol and isoflavonoids than the other parts [16].

Toxicity study

Based on the toxicity tests in various species of animals, it is likely that *P. mirifica* does not exhibit significant toxicity effects. Acute toxicity studies in mice fed with *P. mirifica* powdered tubers revealed an orally administered LD_{50} value of greater than 2–16 g/kg BW [39,40]. These doses are far higher (> 400-fold) than the equivalent of those consumed by Thai people (250 mg/(50 kg BW·d) or 5 mg/(kg BW·d)), and is likely to be higher than that accounted for by the differences in metabolism between mice and humans. A subchronic toxicity study in female rats treated orally with *P. mirifica* powdered root suspension at doses of 10 100 and 1 000 mg/(kg BW·d) for 90 consecutive days revealed that *P. mirifica* at doses of 10 and 100 mg/(kg BW·d) caused neither any abnormalities of hematological and biochemical parameters nor any dose-related histopathological changes in the internal organs [39]. Likewise, at 2 and 40 mg/(kg·d) no chronic toxicity was observed when tested for six months in female rats including the absence of any abnormalities in the internal organ weights, liver and kidney functions and blood biochemical values [41]. However, adverse hematological effects in a six-month treatment were evident when the dose was increased to 250 mg/kg BW [42], and at 300–800 mg/kg BW the formation of micronuclei in polychromatic erythrocytes or a mild genotoxic effect in male rats was reported [43,44]. Based on these studies a safe dosage of *P. mirifica* as a dietary supplement for humans was suggested at 1–2 mg/(kg·d) or about 50–100 mg/d. Postmenopausal women administered *P. mirifica* (in a capsule) at a 20–100 mg/d for six months, or at 100–200 mg/d for 12 months, showed no significant changes in their hematologic, hepatic and renal functions [45–47]. Moreover, *P. mirifica* did not cause any significant irritation when topically applied in rabbits and guinea-pigs [40].

The results of Ames tests revealed that the *P. mirifica* tuber extract had no detectable mutagenic effect toward the TA98 and TA100 bacterial strains even when subjected to metabolic activation by the *in vitro* preincubation with a rat liver S9 microsome-cytosol mixture, used as an *in vitro* model for liver metabolic modifications. This result was supported by the negative results in the *rec* assay. In contrast, an anti-mutagenic activity of *P. mirifica* tuber extracts was revealed by both the Ames test and *rec* assay [44].

Bioassay of estrogenic activity of *P. mirifica in vitro* and *in vivo*

The estrogenic activity of *P. mirifica* was claimed several years (i.e. Kerr, 1932 [12] and Sukhavachana, 1941 [13]) before the identification of the plant species was certain in 1952. Miroestrol was the first non-steroidal estrogenic substance isolated from the plant [19]. The *in vitro* assay that is popularly used for determination of estrogenic activity, including for *P. mirifica*, is the *in vitro* MCF-7 cell line proliferation assay [22,28,38,48,49]. MCF-7 is an estrogen-responsive human breast cancer derived cell line that has widely been used to detect the estrogenic or anti-estrogenic action of chemicals [50]. In addition, the use of other types of estrogen responsive cell lines, such as HeLa cells [51], and transformed reporter cells, such as yeast cells [52], the HepG2

cell transient transfection assay [53] and the CV-1 monkey kidney cells transient transfection assay [23], are also sometimes utilized.

When the estrogenic bioactivity of *P. mirifica* root extracts derived from ethanol, dichloromethane or water extraction were examined by the MCF-7 proliferation assay, the dichloromethane extract exhibited the most potent estrogenic activity [49]. The proliferation effect of the ethanol extract of *P. mirifica* on MCF-7 required an average of 1 μg/ml, with a cytotoxicity or anti-proliferative effect appearing at 100–1 000 μg/ml [48,54] and with an IC_{50} value of greater than 1 000 μg/ml [54]. When a high concentration of this *P. mirifica* extract (1 000 μg/ml) was coincubated in the same assay with 17β-estradiol, a significant agonistic effect was observed [48]. Thus, the phytoestrogens from *P. mirifica* could effectively only outcompete 17β-estradiol binding to the ERα of MCF-7 cells at a high dose [48], suggesting a relatively high K_d. Comparing the estrogenic potency between *P. mirifica* and *P. lobata*, another type of *Pueraria* species that predominantly originated in China, Japan and Korea, using the *in vitro* MCF-7 proliferation assay, *P. mirifica* was found to have a stronger estrogenic activity [48].

The estrogenic activity of the eight isoflavonoids isolated from *P. mirifica*, in terms of the MCF-7 proliferation assay, showed considerable variation in their bioactivity levels, with genistein and coumestrol displaying a 10-fold stronger activity than daidzein and kwakhurin, while puemiricarpene, tuberosin, daidzin and puerarin exhibited a lower estrogenic activity [28]. Puerarin, a major constituent of the isoflavone content in *P. mirifica* (86.5 ± 5.4 mg/100 g dry root powder) [30], produced a significant proliferative activity in MCF-7 cells at high concentrations (10^{-6} M), whereas 17β-estradiol showed a similar effect at much lower doses (10^{-11} M). In comparison, puerarin exhibited a proliferative effect that was 10^2–10^5 times lower than that of 17β-estradiol [54]. However, puerarin did not show any cytotoxicity at high concentrations (10^{-6} M) [54]. Miroestrol is the main bioactive component in the *P. mirifica* root extract, and as already mentioned it was recently reported that it is an artifact of the isolation process results from the oxidation of the more active deoxymiroestrol [22]. Indeed, the strong estrogenic activity of deoxymiroestrol is almost equivalent to that of 17β-estradiol, in terms of the induction of expression of the aryl hydrocarbon receptor (AhR) and ER related genes [55] and the regulation of the testicular gene related sex hormone synthesis pathway [56]. The *in vitro* yeast estrogen screen (YES), consisting of human estrogen receptors α (hERα) and β (hERβ) and human transcriptional intermediary factor 2 (hTRF2) or human steroid receptor coactivator 1 (hSRC1), respectively, together with the β-galactosidase expression cassette, has been established as an assay system for screening for hERα and hERβ binding. In agreement with its phytoestrogen contents, and that the phytoestrogens exhibit a higher binding affinity to hERβ than to hERα, *P. mirifica* extracts showed a higher relative estrogenic potency with hERβ than with hERα [52]. Using the CV-1 monkey kidney cells transient transfection assay to compare the activation of the estrogen-responsive element (ERE) gene in the presence of ERα and ERβ, 17β-estradiol was found to preferentially enhance ERα-mediated activation, miroestrol and coumestrol enhanced both ERα- and ERβ-mediated transactivation, and daidzein and genistein preferentially modulated ERβ-mediated transactivation [23].

Although, the *in vitro* assay for estrogenic activity in *P. mirifica* plants is rapid and convenient, and quite applicable for commercial scale assays, it has some disadvantages. For example, using the *in vitro* MCF-7 proliferation assay to determine estrogenic activity levels may not represent the estrogenic response in animals including humans due to the lack of metabolic activation by gut microbes or the liver, as already mentioned, and the differences in ER types and expression levels between MCF-7 cells and those in the different tissues of the target organism. Furthermore, the evaluation of the estrogenic activity of phytoestrogen-rich plants using different estrogen responsive cell lines yielded significantly different results for each extract between the different cell lines [52,54], highlighting the assay-dependent variations.

To avoid these pitfalls, *in vivo* methods are often used. The most popularly used *in vivo* methods to assess the estrogenic activity of *P. mirifica* plants are uterotropic and vaginal cornification assays [3,5,30,34,38,49,57]. In addition, ovariectomy causes a deficiency in the endogenous estrogen levels and induces a high sensitivity of the animals to exogenous estrogen-like substances. The estrogenic activity of *P. mirifica* extracts was first tested by the uterotropic method in ovariectomized rats [13]. Gavaging 0.1 and 0.2 g/(ml·d) of *P. mirifica* tuber extract for seven days induced vaginal opening and also cornification and increased the uterine weight and thickness in immature ovariectomized rats [57]. The estrogenic activity of the dichloromethane extracts of *P. mirifica* exhibited the highest estrogenic activity (compared to the ethanol and water extracts), as determined by uterotropic assay in immature female rats [49], as already mentioned. However, the increase in uterine weight caused by the administration of the *P. mirifca* extract was associated with an increase in the water content, whereas the increase in the uterine weight after 17β-estradiol treatment was related to an increase in muscle mass [49]. Oral administration of a *P. mirifica* tuber powder suspension at 50–1000 mg/(kg BW·d) to ovariectomized female rats stimulated the cornification of vaginal epithelium cells within 2–3 days, depending upon the dose [5,30,34]. In comparison with the uterotropic assay, the vaginal cornification assay is more convenient and more sensitive. Furthermore, changes in the epithelium cells can be followed (as a readout) during the treatment and post-treatment periods, because there is no need to euthanize the animals [5]. In addition to the uterotropic and vaginal cornification assays, the *in vivo* estrogenic activity of *P. mirifica* can also be determined by changes in the body weight of rats, whereas the increased body weight of

ovariectomized rats was found to be attenuated by *P. mirifica* treatment, as well as by estradiol and puerarin treatment [58,59].

In agreement with the chemovariation found in *P. mirifica* plants that originated from various locations (28 out of the 76 provinces) of Thailand [29], the estrogenic activity, as assessed by both *in vitro* and *in vivo* assays, of the same *P. mirifica* extracts also varied [3,38]. Although testing the estrogenic activity of phytoestrogen substances with the *in vitro* MCF-7 cell proliferation assay is quicker, more convenient and allows a large number of samples to be conducted in a single run, MCF-7 cells have two potential drawbacks, as mentioned before, namely that they (i) contain only the ERα, and so cannot assay for ERβ binding and responses, which may be relevant in key tissues of the animal, and (ii) this direct *in vitro* assay does not allow for any metabolic activation, either by gut microbial flora or the animal tissues (such as liver microsomes), yet these metabolites may have different activities or potencies and so this assay may be prone to artifactual results [54]. Thus, some isoflavones need to be modified by intestinal microflora to gain their bioactivity, while phytoestrogens have a stronger binding affinity to ERβ than to ERα [60,61]. Thus, for these reasons, as well as for inter-species variations in the ERα and ERβ expression and responses in different tissues or liver metabolism, the MCF-7 assay may not represent the actual estrogenic response elicited in any given animal including humans. This pitfall can be solved in part by using the *in vivo* vaginal cornification assay, where both ERα and ERβ are present in the vaginal epithelium cells [35–37]. Overall, this may explain why the estrogenic response of MCF-7 cells did not correlate well with the estrogenic response of the vaginal epithelium cells [54].

Estrogenic activity of *P. mirifica* on the reproductive organs

Regarding the fact that phytoestrogens can be found in a variety of daily human diet items, including soy and soy products, it is very difficult to experimentally control the dietary intake of phytoestrogens in humans. Moreover, the longitudinal study of hormonal changes in humans is also difficult. Accordingly, laboratory animals, such as mice, rats and monkeys, have been chosen and used as alternative models for the *in vivo* study of the effects of *P. mirifica* on animals including on the reproductive organs [5,7,34,62–64].

Daily feeding of *P. mirifica* root powder as an aqueous suspension induced a vaginal cornification in ovariectomized rats in a dose- and cultivar-dependent manner [3,5,30,38], where higher doses produced an earlier and longer duration of cornification of the vaginal epithelium [3,5]. Of the 25 cultivars evaluated, the tuberous roots collected from *P. mirifica* in the Kanchanaburi Province, at the central-west of Thailand (Kanchanaburi cultivar) exhibited the strongest estrogenic activity in the vaginal cornification assay, and those collected from Uttraradith and Petchaburi Province (Uttraradith and Petchaburi cultivars, respectively), at the north and central-west part of Thailand, had the weakest activity [3]. Moreover, the estrogenic activity of *P. mirifica*, in terms of the stimulation of vaginal proliferation in ovariectomized rats, was also dependent upon the season that the tubers were harvested. Those harvested in the winter had a stronger and longer estrogenic potency than those harvested in the summer and rainy seasons, respectively [38]. The results for the vaginal cornification assay are in agreement with those from the uterotropic assay [3,5,38]. The estrogenic potency of *P. lobata* tubers was found to be lower than that of *P. mirifica* [5]. Overall, as mentioned above the estrogenic activity of *P. mirifica* varied upon their cultivar (assumed plant genetics), cultivated locations (likely environment as in cultivation conditions) and seasons.

The estrogenic activity of *P. mirifica* on the reproductive system was confirmed by the suppression of pituitary gonadotropin and inhibin (INH) secretion in both female and male mice and rats, and in female monkeys [6,7,34,62–66]. Although *P. mirifica* can influence the reproductive function in both sexes, the response in females is greater and more sensitive than in males [34,62,63]. The reduction in the serum follicle stimulating hormone (FSH) levels was observed only in female rats, along with the increase in the uterus weight and vaginal cornification, while the increase in epididymis weight only occurred in male rats after treatment at the higher dose of 1 000 mg/(kg·d) of *P. mirifica* for 14 days [34]. Indeed, administration of *P. mirifica* at doses of 10–1 000 mg/(kg·d) for 90 days increased the uterus weights of female rats [58], but without alteration to the seminal vesicle and prostate gland of male rats [67]. This implies that the *P. mirifica* tuber treatment dose for males should be higher than that for females if the same results are needed. Due to the suppression of the secretion of luteinizing hormone (LH), FSH and INH in female cynomolgus monkeys, experimental animals which have a broadly similar hormonal pattern and reproductive system to those of humans, *P. mirifica* could become an alternative choice for a contraceptive drug in reproductive women. Indeed, *P. mirifica* has been reported to suppress folliculogenesis and ovulation [6,64].

Similar to the stimulation of vaginal cornification in female rats, the suppressive effects of *P. mirifica* on serum gonadotropin levels in female cynomolgus monkeys were also dependent upon the doses administered [6,68]. The highest treatment dose administered (1 000 mg/(kg·d) for 90 days) in cyclic female monkeys completely suppressed menstruation during the treatment period, and induced lowered serum levels of estradiol and progesterone [6]. As mentioned above, after the cessation of *P. mirifica* treatment the recovery of serum gonadotropin levels was also dependent upon the administered doses [6,7]. Indeed, there is a direct correlation between the dosage given and the subsequent recovery time [7]. The rebounded increases in LH

and FSH levels after cessation of treatment with *P. mirifica* were also unexpectedly observed in aged female monkeys. Decreased urinary FSH but not LH levels were also observed in cyclic females as well as in aged monkeys [65,66]. Interestingly, in aged female cynomolgus monkeys, *P. mirifica* could also induce reddening of the sex skin, the nearly hairless large region of skin around the perineal/anogenital area that becomes reddened or swollen when female macaques are in estrus or in estrogen peak [68,69]. It has been reported that the sex skin of female monkeys possesses ERs [70], and so it assumed that the phytoestrogens in *P. mirifica* bind to the ERs leading to the reddening of the sex skin. *P. mirifica* has also been reported to alleviate menopausal symptoms, such as hot flush, frustration, sleep disorder and skin dryness, in women [50].

Together with its estrogenic activity, *P. mirifica* is considered as a potential endocrine disruptor. The consumption of high doses of *P. mirifica* led to infertility and reproductive disorders in mice [62,63], rats [34,71] and Japanese quails [72]. Administration of relatively high doses of *P. mirifica*, that is at 100 or 200 mg/(kg·time), three times a day for 14 days, decreased the number of sperm in the epididymis and the percentage of sperm motility in male rats [71], while when administered at 100 mg/(kg·d) for eight weeks it reduced the epididymis and seminal vesicle weights and suppressed the epididymal sperm motility and viability in sexually intact male mice [62]. However, no alterations in the mating efficiency in male mice, or in their ability to cause pregnancy in their female partners, were observed. Such a reduction could be attributed to a direct effect of *P. mirifica* on the epididymis function and so cause alterations in spermatogenesis of male mice. Similar to that found in cyclic female monkeys, oral administration of 100 mg/(kg·d) of *P. mirifica* for eight weeks induced prolonged estrous cycles in mature female mice with a decreased number of growing follicles in the ovary and reduced serum LH and FSH levels. This led to a decrease in the mating efficiency in female mice, which is in contrast to that seen in male mice [63]. However, there were neither malformations nor changes in the reproductive organ weights of the pups parented by the *P. mirifica* treated male and female mice and rats [62,63,71]. Oral treatment with *P. mirifica* at 50 or 100 mg/(rat·d) to female rats during the midterm of pregnancy (days 1–3 or days 1–10), interrupted the pregnancy and had a toxic effect on the fetuses [73]. Taken together, it can be concluded that *P. mirifica* has an estrogenic effect on the reproductive systems of both males and females.

P. mirifica and osteoporosis

Osteoporosis is a condition that is characterized by a low bone density and microarchitectural deterioration of the bone tissue, resulting in an enhanced bone fragility and an increased susceptibility to bone fracture, especially of the hip, spine and wrist [74]. Osteoporosis has recently emerged as a significant public health problem, and is becoming increasingly prevalent with the aging of the world's population. The incidence of osteoporosis is significantly higher in women than in men. Osteoporosis is a silent problem as the bone loss is typically asymptomatic and goes unnoticed until well developed. Indeed, many patients are unaware of this problem until they experience a fracture. Nearly all fractures of osteoporotic patients require hospitalization and cause considerable morbidity, disability and mortality. In Thailand, the increase in the life span of the Thai people has proceeded to an increased incidence of osteoporosis and bone fractures. This is to some extent expected, and the prevalence of osteoporosis in Thai women occurs rapidly after the age of 55 years and reaches a level of more than 50% after the age of 70 years [75]. The worst consequence of the bone fracture is not simple fatality but that the post-fracture patient needs to live dependently, with an impaired quality of life, for many years. The primary goal of osteoporosis treatment is to prevent a fracture, by slowing down the rate and decreasing the total amount of bone loss, maintaining bone strength and minimizing or eliminating factors that induce fractures.

Estrogen plays an important role in bone homeostasis [76], where it regulates bone homeostasis directly via acting on the bone cells, and indirectly on the immune system and oxidative stress [77]. The loss of serum estrogen levels during menopause has been reported to cause a decrease in the bone mineral density (BMD) and bone mineral content (BMC) [78,79]. Thus, estrogen deficiency is a major pathogenic factor in the development of osteoporosis in postmenopausal women. Although estrogen replacement therapy (ERT) has potential benefits for the prevention of the onset of, or as a cure for osteoporosis, the long-term exposure to high estrogen levels is considered to be the main risk factor for the development of breast cancer [80], endometrial cancer [81,82], hypertriglyceridemia and angiogenesis [83]. Considering these problems, many researchers have tried to develop alternative drugs for bone loss therapy that have no or only minimal side effects on the other organs.

Since the estrogenic activity of *P. mirifica* tuber powder or extracts on the reproductive organs has been found to be beneficial as mentioned above, the effects of such treatment on the bone tissue were evaluated to ascertain its potential use for curing bone loss. The effect of *Pueraria* plant extracts on the prevention of bone loss was first reported in *P. lobata* [84,85]. Subsequent research revealed that *P. mirifica* crude powder could prevent bone loss, both in terms of the BMD and BMC, in trabecular and cortical bones of the 4th lumbar vertebra, tibia and femur in ovariectomized female and orchidectomized male rats (Fig. 5) in a dose-dependent manner [58,67]. Interestingly, the effect in males appeared comparable to that in female rats. This may be due to the absence of any significant differences in distribution and expression levels of ERβ between male and female osteoblasts, the key bone forming cells [86]. Additionally,

although phytoestrogens can bind both estrogen receptors (ERα and ERβ), they have a higher binding affinity (smaller K_d) to ERβ [60,61]. Confirming these effects in sex hormone deficient rats, *P. mirifica* intake was observed to decrease serum parathyroid hormone and calcium levels in aged menopausal monkeys [87]. The mechanism of action of *P. mirifca* on bone cells is not been clearly understood. However, it seems to reduce the bone turnover rate, because serum levels of bone-specific alkaline phosphatase or alkaline phosphatase (a marker of bone formation) were reduced in *P. mirifica* treated post-menopausal women [23,47], while phytoestrogens have been reported to have anabolic effect on bone metabolism [88,89]. In bone cell culture, genistein induced a significant increase in the calcium content and alkaline phosphatase activity [88]. *In vitro* exposure (in cell culture) to genistein and daidzein caused a significant increase in the proliferation of the mouse osteoblastic cell line MC3T3-E1, as well as an increased alkaline phosphatase activity and DNA content [90]. Isoflavones can also inhibit osteoclast cell, the bone resorbing cells, formation from bone marrow cells and can stimulate apoptosis of these cells [91].

Thus, taking the above into consideration, *P. mirifica* tubers seem to have some potential for the treatment of osteoporosis in menopausal women and andropausal men. However, there are a few reports indicating that the oral intake of phytoestrogen-rich herbs at the required osteoporotic preventive doses actually induced a uterothophic effect [84].

In Thailand, the diagnosis of the onset of osteoporosis is usually ignored. Most of the people are unaware of it until the disease is well advanced and so early treatment is rarely performed. However, patients with a low bone mass or already having an osteoporotic fracture should in particular be considered for treatment. Although current antiresorptive agents can reduce bone resorption, they do not increase the bone mass. Various anabolic agents can induce new bone formation, but their side effects and cost are the main drawbacks to be considered. Therefore, an agent that can induce bone formation with fewer side effects and that is cheap should be sought out. The indigenous Thai herb *P. mirifica* is certainly one of the choices worthy of further evaluation and/or trials.

Other climacteric related symptoms and *P. mirifica*

As a woman ages, a number of both psychological and physiologic changes take place. The average age of women to enter the perimenopausal period is at 48–50 years when the reproductive function is declined. Psychological changes range from anxiety, tension and nervousness to chronic depression, whereas the physiologic changes range from hot flushes, fatigue and insomnia to more severe conditions such as bone loss, balding and degeneration of the reproductive organs. Consequently, post-menopausal women often need something to replace the loss of natural estrogen. ERT is currently extensively used to alleviate both the psychological and physiologic changes. However, the estrogen substitute used can be quite expensive and cause various undesirable side effects, such as breast cancer [80], endometrial cancer [81,82], hypertriglyceridemia and thromboembolism [83]. An effective alternative approach that is cheap and has no side effects, especially on cancers, should be considered.

A clinical trial conducted to evaluate the beneficial effect of *P. mirifica* tuber extracts as an oral supplement on menopausal symptoms has recently been performed [45,46,92]. The intake of *P. mirifica* at 200 mg/d for four months followed by a dose of 200 mg/2 days for eight months clearly improved the signs and symptoms related to menopause, such as hot flushes, frustration, sleep disorder, skin dryness, high blood cholesterol levels, oligomenorrhea and amenorrhea [45]. Patients receiving 50 mg/d for six months showed an alleviation of climacteric symptoms equivalent to that obtained from the use of conjugate equine estrogen with/without medroxyprogesterone acetate [46,92]. No changes in the blood cells, liver and kidney functions, as well as other physiologic status indicators were observed [45,46,92]. Pre- and post-menopausal women who received *P. mirifica* tuber preparations at either 50 or 100 mg/d for six months showed a decrease in the modified Greene climacteric scale from 35.6 to 15.1 and from 32.6 to 13.6 in those two groups, respectively [93]. Blood cholesterol levels were also decreased in postmenopausal women given oral supplements of *P. mirifica* [45], which is in agreement with the previous study in male and female rats [39]. Healthy postmenopausal volunteers that were orally administered with 100 mg/d of *P. mirifica* for two months showed an increased level of high-density lipoprotein (HDL) cholesterol and apolipoprotein (apo) A-1, decreased LDL cholesterol and apo B levels, and a decrease in the LDL cholesterol: HDL cholesterol and apo B: apo A-1 ratios compared to the control group [23]. Thus, *P. mirifica* might be applicable for the treatment of dyslipidemia, atherosclerosis or cardiovascular disease, which are very common in the aging population. Indeed, there is some evidence to support that *P. mirifica* consumption contributes to lower risks of cardiovascular disease. The administration of 100 mg/kg of *P. mirifica* extract a day for 90 days to OVX rabbits improved their endothelial function through the NO-dependent endothelial derived relaxing factor pathway, increased the response to 17β-estradiol and decreased the sensitivity to norepinephrine [94].

Other than hot flushes, frustration, sleep disorder and skin dryness, two other physiologic changes that can occur during the climacteric period are vaginal dryness and urethral atrophy. This not only causes problems to the menopausal women, but it also impacts on a couple-life by dyspareunia during sexual intercourse. Consistent with the previous reports indicating that *P. mirifca* induced vaginal proliferation in female rats [3,5,30,34,38,57], consumption of 20, 30 or 50 mg/d of *P. mirifica* for 24 weeks ameliorated vaginal dryness symptoms and dyspareunia, increased the vaginal maturation

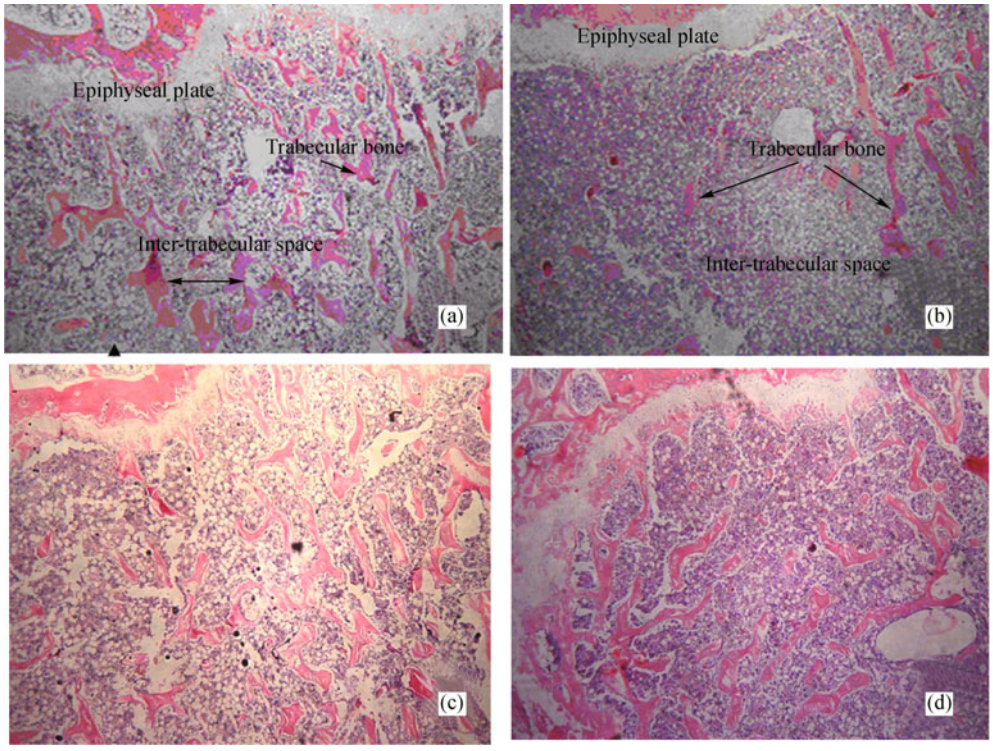

Fig. 5 Histological section (stained with H & E) in the longitudinal and medio-lateral plane of the proximal tibia at the epiphyseal growth plate and metaphyseal area of (a) sham control rats and (b–d) orchidectomized rats treated for 90 days with (b) 0 mg/(kg BW·d) of *P. mirifica*, (c) 0.1 mg/(kg BW·d) of 17-alpha-ethinylestradiol and (d) 1 000 mg/(kg BW·d) of *P. mirifica* (with permission from N. Urasopon).

index, and decreased the vaginal pH to acidity (pH $<$ 6) in postmenopausal women [95]. OVX rats forced-fed with *P. mirifica* for 28 days revealed an induced proliferation of urethral mucosa and an increased leak point pressure of urinary bladder [96].

The loss of sex steroid hormones accompanying the menopause and andropause has emerged as a significant risk factor for neurodegenerative diseases, such as Alzheimer and Parkinson diseases. Numerous studies have revealed the beneficial effects of estrogen on improving memory function. Estrogen modulates neuroprotection and cognitive function through hippocampus plasticity, an important part of the brain that is involved in memory and cognition. It can reduce the deposition of amyloid β peptide (Aβ), enhance neurotransmitter release and activity, protect against oxidative damage, increase expression of the presynaptic vesicle protein, synaptophysin, and exhibit neurotrophic effects [97–99]. Although estrogen is a prerequisite for neurogenerative treatment, alternative approaches based upon the use of natural products have been searched. *P. mirifica* extract has been shown to exhibit an *in vitro* neuroprotective effect in the glutamate-induced cell death of HT22 mouse hippocampal neurons [100]. Here incubating rat hippocampal neurons with 30 and 60 μg/ml of *P. mirifica* extract for six days increased the synaptophysin immunoreactivity and synaptophysin levels, reflecting an increase in the synapse formation [101]. Thus, *P. mirifica* may have potential for use in neurodegeneration protection. However, detailed studies are still needed to be conducted to gain insight into the mechanisms.

Antioxidative and antitumor effects of *P. mirifica*

ERT courses are currently administered to the elderly, and especially to aged menopausal women. However, as already discussed above, the long-term exposure to synthetic estrogen is a risk factor for the development of breast cancer [80], and endometrial cancer [81,82]. Thus, despite the many reported potential benefits of treatment with *P. mirifica* tuber extracts, it remains important to clarify if *P. mirifica* also induces the development of breast and endometrial cancers. Consistent with its antioxidative and antiproliferative effects, pretreatment of a high dose (1 000 mg/(kg·d)) of *P. mirifica* to female rats for four weeks before the induction of a breast tumor by a single injection of the 7,12-DMBA carcinogen actually resulted in decreased levels of tumor development and multiplicity. The proposed mechanism of action was through the suppression of ER expression, especially for ERα [102].

It is generally believed that antioxidants can aid in cancer prevention or have an anticancer property. Accordingly, the

potential antioxidant activity of *P. mirifica* has been evaluated. However, compared to other medicinal plants found in Thailand, such as *Stevia rebaudiana* Bertoni, *Curcuma longa* Linn., *Andrographis paniculata* (Burm.f.) Nees. and *Cassia alata* Linn., the *P. mirifica* extract showed the lowest antioxidant activity [103]. Indeed, *P. mirifica* tuber extracts also showed a weak antioxidant activity compared to that seen with α-tocopherol and *P. lobata* from China, when assessed by 1,1-dephenyl-2-picrylhydrazyl (DPPH) assay [104]. Puerarin exhibited an antioxidant activity as strong as that of α-tocopherol, which is of interest since of the *P. mirifica* plants collected from the 28 provinces throughout Thailand, only the puerarin phytoestrogen level was found to significantly correlate with the antioxidant activity of the plants, while a higher amount of daidzin, daizein and genistein is correlated with a lower antioxidant activity [29]. Based on the ranking of antioxidant activity of these 28 *P. mirifica* cultivars, the tuberous root extract that showed the lowest estrogenic activity, as determined by the vaginal cytology assay, i.e. Petchaburi and Nong Bua Lam Phu cultivars, in contrast, showed the highest antioxidant activity, and *vice versa* [38]. A similar trend was also found between the antioxidant activity and MCF-7 antiproliferative effect [48]. Thus, the puerarin level is not a prerequisite for the estrogenic and antiproliferative activities of *P. mirifica*, but it is for the antioxidant activity. This was confirmed by our current study determining the estrogenic activity of puerarin by the vaginal cornification assay [59]. The antioxidant activity of *P. mirifica* plant extracts was found to vary according to the time (or seasons) harvested and the cultivars [38].

Oxidative stress is viewed as one of the major causes leading to neurodegeneration. Antioxidants are being looked upon as persuasive therapeutic agents against neuronal loss, as they have the capability to neutralize free radicals. It is, therefore, of relevance that the *P. mirifica* tuber extract at 10 and 50 μg/ml exerted a sufficient *in vitro* antioxidative capacity as to protect HT22 cells against glutamate toxicity [100]. Glutamate-induced toxicity causes a reduction-oxidation imbalance, followed by glutathione deficiency and neuronal cell death. Consistent with the low antioxidative activity reported by Cherdshewasart and Sutjit [104], genistein or daidzein at concentration of 2.5 μg/ml did not exhibit any significant neuroprotective activity in HT22 cells.

Conclusions

P. mirifica has a long history of traditional folklore use in Thai society, and is currently prepared in the form of pills for oral intake of dietary supplements and in solution as a component of cosmetic products, such as breast cream, eye gel and skin moisturizer. Although their recorded folklore medicinal use dates from nearly 100 years ago, the main interest in *P. mirifica* consumption has developed and become pronounced within the past 10 years due to the rapid increasing awareness of the potential benefits to human health. This has led to scientific research into its bioactive chemical constituents, and especially those associated with its estrogenic, antiproliferative and antioxidative activities. Their effects have been clearly defined. With regard to the safety and efficacy of the use of *P. mirifica*, toxicity tests have been performed in various animal species including in humans with no detected toxicity. However, a precautionary concern is that *P. mirifica* plants harvested from different locations, ages or seasons, or even from different roots, can express different results when tested at different doses. This may suggest a complex interaction of several bioactive components as well as varying component levels within each cultivar and cultivation condition. Therefore, users need to consider and equilibrate between the desirable effects on specific target organs and any undesirable side effects on other systems before use.

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