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Original Article

MORINDA CITRIFOLIA HAS VERY WEAK ESTROGENIC ACTIVITY IN VIVO

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Morinda citrifolia (Noni) appears to restore normal menstrual cycle and alleviate menstrual symptoms; however, its estrogenic activity has not been tested *in vivo*. The present study tested whether the beneficial effects of *Morinda citrifolia* extract are mediated through estrogenic properties. Uterotrophic bioassay were done on tissue from prepubertal mice that were subcutaneaous treated with the substance for 3 days at various doses in water and alcohol vehicle and compared to mice treated for the same time with doses of 17-β estradiol. The frequency of vaginal opening and vaginal cornification in mice receiving *Morinda citrifolia* was not different from control mice. The relative wet and blotted uterine weight of mice receiving low doses but not higher doses of *Morinda citrifolia* was significantly higher than control group. The relative estrogenic potency of alcohol and water extracts of *Morinda citrifolia* was 1:1,000 and 1:10,000 respectively, indicating that the estrogenic activity in *Morinda citrifolia* is only seen at low doses, and even then it has very low potency in comparison to estradiol. This suggests that the beneficial effects of *Morinda citrifolia* are not closely linked to estrogen-mediated action.

Key words: estrogenic activity, in vivo bioassay, estradiol, estrone, *Morinda citrifolia* (Noni)

Morinda citrifolia has several common names, i.e., Noni or Nhau (in Thailand). Its fruit has been used as a food in tropical regions throughout the world (McClatchey W, 2002), and it appears to have many health benefits including alleviation of the menstrual symptoms (www.hawaiiannoni.com/noni/benefits). Scientific studies on *Morinda citrifolia* have recently increased and demonstrated several biological effects (Wang MY, et al., 2002). Further, *Morinda citrifolia* has a number of major biologically active components: 23 different phytochemicals as well as 5 vitamins and 3 minerals (Duke,1992). Estrogenic compounds can significantly alter the reproductive and endocrine system of both animals and humans (Herbst et al., 1981). For instance, vaginal bleeding is experienced in some post-menopausal Noni's

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user (unpublished data), and testimonials offered by Noni users at the International Noni Communication Council (INCC) indicated its usefulness in restoration of normal period in pre-menopausal women (www.incc.org).

Estrogens are steroid hormone with important functions in the regulation of specific sexual processes in the female. A variety of phytoestrogens have been identified which bind to the estrogen receptor, and these could induce estrogen actions (Davis et al., 1999). The flavone glycosides reported to be in Noni's fruits (Wang et al., 2002) is structurally classified as one type of phytoestrogen (Cos et al., 2003). While these phytoestrogens display very modest estrogenic binding to the estrogen alpha-receptor compared to endogenous or synthetic steroids, they may provide more selective actions on reproductive and non-reproductive estrogen targets.

Given the world-wide use of Noni and the purported risks of estrogen replacement therapy, (especially relative to breast cancer), there is a need to characterize the estrogenic characteristics of substances like Morinda citrifolia. Both aqueous and ethanol extracts of Noni should be tested since the water extract is the common form available to users but alcohol is a better solvent for extraction of steroids.

Although, in vitro assays can demonstrate interactions between components, they cannot replicate the myriad of pharmacokinetic and pharmacodynamic interactions that likely influence the estrogenic activity of a substance. A reliance on in vitro assays for predicting in vivo disrupter affects may generate false-negative as well as false-positive results. Therefore, in vivo assay must be conducted to test the in vitro-generated hypotheses (Joseph GV, 2003). In vivo studies indicate that 3-days uterotrophic assay in prepubertal rats or mice is an efficient method for determination of an estrogenic activity (Laws et al., 2000). The present study evaluates the estrogenic activity in Morinda citrifolia using mouse uterotrophic method.

Materials and Methods

Preparation of plant and estrogen standards

The fresh unripe fruits of Morinda citrifolia were collected from its natural habitat in the countryside of central part of Thailand. It was chopped into small pieces, dried at 60 ^{o}C and ground into powder. Two hundred grams of dry powder was boiled in 300 ml-distilled water for 30 min. The liquid obtained by filtration was freeze-dried to yield a solid residue of aqueous extract.

Eight hundred grams of dry powder was extracted by the process of maceration in an aspirator for 8 -10 hours during daytime using 95 % ethanol as menstruum. In the next morning, the ethanol was changed and another fresh volume of ethanol was added to the powder once again. All extract collected was concentrated under vacuum reduced pressure by the rotary evaporator (Büchi Laboratory Techniques Ltd., Flawil, Switzerland) to get thick syrupy mass and kept at 4^{o}C. The same procedure was repeated for 5 days using altogether 3000 ml of ethanol.

The per cent yield of water and alcohol extract was 3.02 % and 4.46 % of fresh fruit respectively. The working concentrations of the extracts were made in an appropriate media, sterile non-pyrogenic distilled water (Thai Otsuka Pharmaceutical Co. Ltd., Bangkok, Thailand) or corn oil (C63156, Fluka, NY, USA) before using in the experiment.

The stock standard solution of 17-βestradiol (E8875, Sigma) was prepared to contain 10 µg/ml in absolute ethanol (Merck, Darmstadt, Germany) and kept at 4 ^{o}C. On the day of the experiment, dosing solutions of standard were made and evaporated under nitrogen stream until dry and dissolved in corn oil before injecting into the animals.

Animals

Immature female mice (International Cancer Research strain, Japan), 21 days old were obtained from the National Laboratory Animal Center of Thailand. Their weight ranged 12-21 g at the beginning of the experiment. The animals were fed with animal pellets (C.P. Mice Feed, Bangkok, Thailand) and water ad libitum. They were housed in a temperature- controlled room (23-27 ^{o}C), humidity (50-80 %) and 12-h light/ 12-h dark conditions.

Acute toxicity test (LD50)

The intraperitoneal acute toxicity (LD50) test of Noni fruit's extracts was evaluated in group of 3-5 mice per dose (Lorke D, 1983; Nakanishi K et al., 1965). An aqueous and alcohol plant extract was dissolved in distilled water and gum acacia respectively to obtain the different doses per gram body weight before giving a single-dose intraperitoneal injection. The LD50 was calculated based on the number of death observed during 72 hours after injection (Lorke D, 1983).

Uterotrophic bioassay

A group of eight to ten immature mice was daily injected with a dose of the following standards;17-β estradiol (0.01, 0.02, 0.04, 0.08, 0.16 and 0.32 mg/g), water extract (50, 100, 200, 400, 800 and 1,600 mg/g) or alcohol extract (10, 15, 40, 87.5, 175, 375, 750, 1,500 and 3,000 mg/g) of Morinda citrifolia via subcutaneous route for 3 consecutive days. The control animals were injected in the same manner with distilled water or corn oil in corresponding with the test materials. Corn oil has been reported to be a suitable vehicle in the uterotrophic assay since it does not change the body weight, sex organ and accessory sex organ weights of animals (Yamasaki K, et al., 2001). Body weight of mice was recorded on the first and the fourth day of the experiment approximately 24 h after last injection before sacrifying the animals with ether. The uteri were removed and adhering fat was trimmed away. Care was taken not to lose the uterine luminal fluid. Both wet weight and dry uterine weight after fluid blotting were recorded on the Roller-Smith torsion balance (Federal Pacific Electric Co, NJ, USA). Vaginal opening and vaginal cytology were also examined as additional indicators of estrogenicity.

The experiment to obtain the standard curve of estrogens and plant extracts was performed two to three times to ensure the results. In order to find out the estrogenic potency of plant, single dose of plant extract that gave significantly highest uterotrophic response in comparison to the control group was selected. The relative potency of estrogenic effect on uterus in comparison to 0.01 μg (3.67 x 10 – 5 nM) estradiol was calculated.

Statistics

Distribution of data was tested by the Kolmogorov Smirnov test (SPSS program version 11.5). The significance of difference between control and treated groups of wet or dry uterine weight alone or relative uterine weight (ratio with body weight x 100) was determined using ANOVA or student's t-test and Kruskal-Wallis test or Mann-Whitney test whenever appropriate. The frequence of vaginal opening and cornification detected among the different groups were compared using Chi-square test. The significance was determined at p value < 0.05.

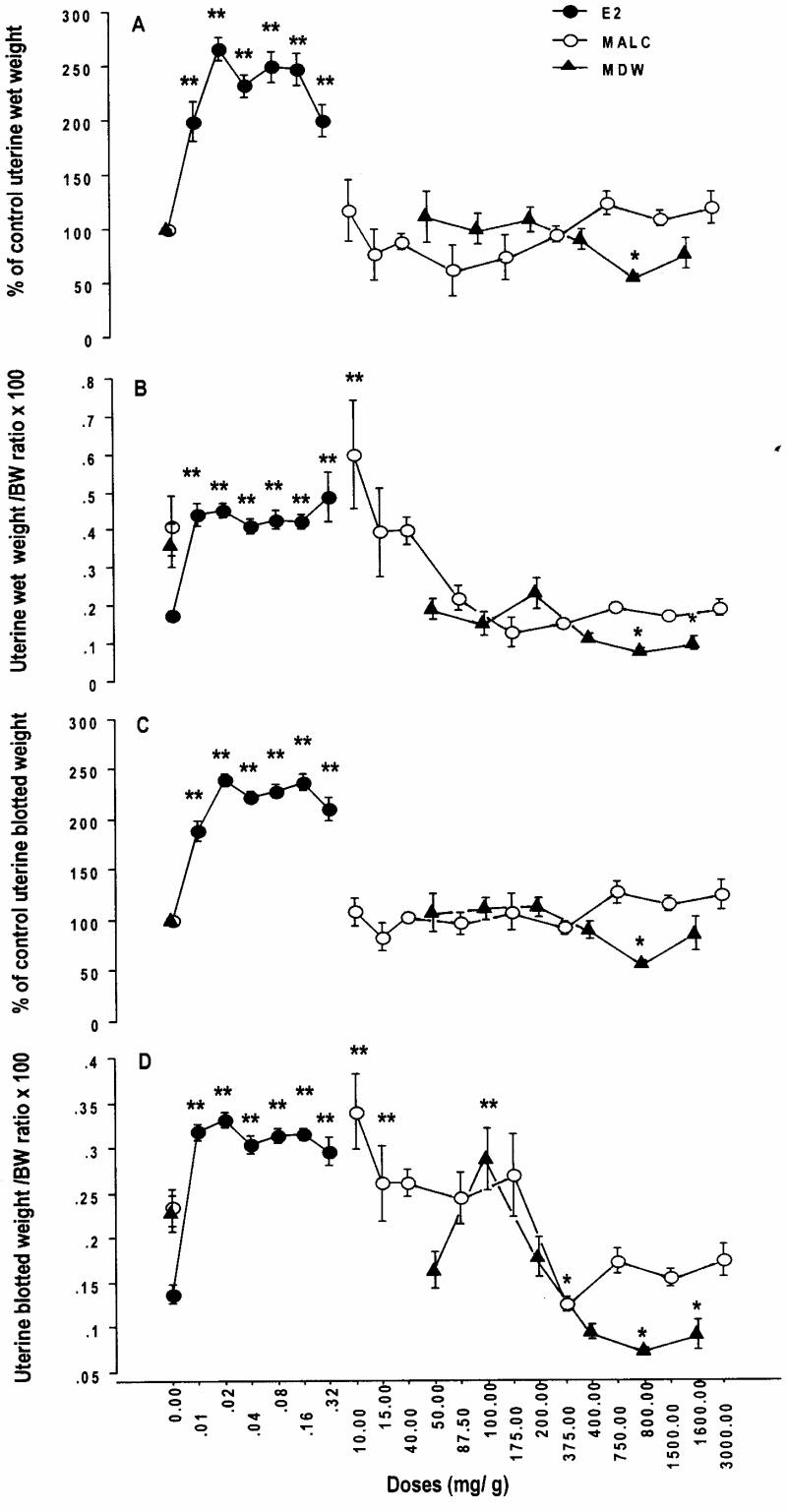

Figure 1. Results of uterotrophic assay in immature mice. Panels A and C show the dose-response curves for wet (A) and blotted (C) uterine weight as a percentage of that in control animals. Panels B and D demonstrate the relative wet (B) and blotted (D) uterine weight with corresponding body weight of each mouse.
Data are mean ± SEM.
(E2 = Estradiol, MALC and MDW = alcohol and water extract of Morinda citrifolia, ** $p < 0.0001$, * $p < 0.05$)

Results

The LD50 of aqueous and alcohol extracts of Morinda citrifolia.

The LD50 of aqueous and alcohol extracts of Morinda citrifolia by intraperitoneal injection was 7500 and 3500 mg/kg body weight respectively.

Uterotrophic response of Morinda citrifolia

Compared to the control (corn oil) treatment, estradiol at 0.01 μg/g or above caused significantly greater uterotrophy (Figure 1). In contrast, neither the water nor the alcohol administered Noni extract caused a significant change in uterine weight, compared to control (Figure 1). This uterotrophic response was similar whether measured by wet or blotted uterine weight.

The relative uterine weight (per body weight ratio x 100) at each dose of standard is also illustrated in Figure 1 panels B and D. Compared to the control and water extracted of Noni, the alcohol plant extract caused a significantly greater uterotrophy at a dose of 10 μg/g (both wet and blotted). Also a significantly greater uterotrophic response was seen in the water extracted group compared to control, but only at 100 mg/g. The percent increase in both groups (compared to control) was between 26 to 45 %. Higher doses of Morinda citrifolia depressed the uterotrophic response to below control levels.

Figure 2 illustrates the relative potency of plant extracts compared to the 17-β estradiol at doses producing the greatest uterotrophic response (see Figure 1). The calculated potency for water and alcohol extracts of Morinda citrifolia was approximately 1:10,000 and 1:1,000 times that of estradiol, respectively.

The vaginal opening observed in mice receiving estradiol was over 99 %, while it was only 20% in the control group (Table 1). The vaginal opening was not significantly greater in control mice compared to either group receiving Morinda citrifolia. An examination of cornified vaginal epithelium cells, as another index of estrogenic activity, indicated that only estrogen caused a significant increase in this index (Table 2).

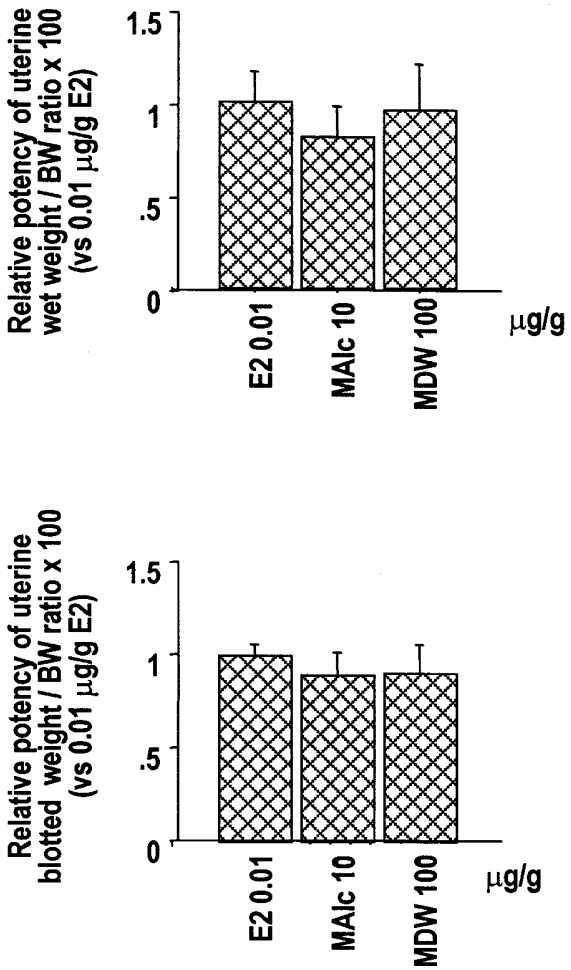

Figure 2. The effect (mean ± SEM) of 10 μg/g alcohol extract and 100 μg/g water extract of Morinda citrifolia (compared to 0.01 μg/g estradiol) on relative wet and blotted uterine weight (/ body weight ratio x 100).

Discussion

The investigation of acute toxicity is necessary as an initial step in the characterization of the biological effects of any substance. The LD50 of water and alcohol extracts of Morinda citrifolia's fruit (via single dose intraperitoneal injection) was 7500 and 3500 mg/kg body weight, respectively. These results agree with value > 1000 mg/kg reported by Nakanishi's group who tested for the LD50 of Morinda citrifolia in four-week old mice by the same method (Nakanishi et al., 1965). The difference in strain, age and preparation of plant extract may have contributed to the slightly different values.

Wet and dry uterine weight, the diameter of the vaginal opening and the cornification of the vaginal epithelium are reliable measures of estrogenicity (Diel et al, 2002; Laws et al., 2000; Jefferson et al., 2002). An early marker of estrogen action is uterine water imbibition due to enhanced microvascular permeability, which may be mediated by growth factors (Rockwell et al., 2002). A classical mouse uterotrophic assay is simply a measure of the increase in uterine wet/dry weight after exposure to a chemical (Marky et al., 2001). However, some scientists have suggested that mitotic activity is the best index of an estrogenic effect (Hertz, 1985; Marky, 2001). Thus, substances that directly stimulate mitotic activity in the female genital tract have estrogenic activity. The uterotrophic response of the extracts from Morinda citrifolia does not display these characteristics (either wet or blotted uterine weight). However, mean relative uterine weight in mice received very low dose of plant extracts was significantly higher than that of the control group. The body weight adjusted value of uterus ensures that the body weight of mice does not confound the results. The difference of relative wet and blotted uterine weight (panels B and D) indicates that the estrogenic activity of Morinda citrifolia was due to both water imbibition and the mitotic activity of uterine tissues.

Several mechanisms of action have been proposed to underlie the purported beneficial effects of phytoestrogens on menopausal symptoms. These include weak estrogenic action on the release of pituitary hormones (www.ifst.org/hottop34). Morinda citrifolia at very low doses displays estrogenic activity that is not observed at higher dose. Similarly, the biological responses to other phytoestrogens, (e.g., genistein and bisphenol A) occur at low doses but not at higher doses (Anderson et al., 1998; Rubin et al., 2001). These findings support Calabrese's concept of hormetic-like biphasic dose response model characterized by a low-dose stimulation and a high-dose inhibition (Calabrese et al., 2003). They have shown that stimulatory biological responses to low-doses are modest, usually, only

Table 1. An opening of vagina in groups of mice receiving estradiol and both types of Noni extracts.

	% Vaginal opening		**p value**
	Yes	**No**	
Corn oil	20.0	80.0	<0.0001
Estradiol	99.5	0.5	
Corn oil	70.6	29.4	0.92
Noni: alcohol extract	69.7	30.3	
Corn oil	60.0	40.0	0.08
Noni: aqueous extract	43.8	56.2	

Table 2. Cornified vainal epithelial cells as an index of estrogenic activity in groups of mice receiving estradiol and both types of Noni extracts.

	% Cornified cells positive	p value
Corn oil	0	<0.0001
Estradiol	21.0	
Corn oil	2.9	0.50
Noni :alcohol extract	1.4	
Corn oil	2.2	0.27
Noni :aqueous extract	2.2	

30 to 60% above control. Our result demonstrated a similar response. Previous results that induced changes in MCF-7 breast cancer cell line through exposure to estradiol, show that low level responses to estradiol act by hormone binding to the estrogen receptor, whereas, toxicological response at high level exposure take place independent of binding with the estrogen receptor. (Welshons et al., 2003) The authors concluded that the endocrine-dirupting chemicals with estrogenic activity should be tested at a much wider range of doses, including doses that are physiological relevant. Most phytoestrogens have estrogenic activity at ranges of 1:500 to 1:1,000, compared to 17-β estradiol (www.ifst.org /hottop34). The relative potency of Morinda citrifolia by alcohol extraction was 1:1,000 of estradiol activity which was ten times more than that obtained from water extraction. An opening of vagina and the cornification of vaginal epithelial cells were not observed in mice receiving Morinda citrifolia. According to EDSTAC final report, the sensitivity of vaginal cornification and vaginal opening for measurement of estrogenic activity in intact animals is less than that of uterine weight measures (www.epa.gov/scipoly/oscpendo/docs/edstac/app-kv14, 1998). Longer exposure periods may be required to detect changes in vaginal cytology during exposure to weak estrogenic activity (Laws et al., 2000).

In conclusion, the phytoestrogen(s) in Morinda citrifolia has very low potency for estrogenic action. Short-term, moderate consumption of Morinda citrifolia is unlikely to cause physical problem due to its estrogenic effect; however, long-term exposure to higher doses may cause either pharmaceutical benefits or a risks in humans. The chemical nature of phytoestrogen in Morinda citrifolia is still unknown and needs further investigations.

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