

PHYTOCHEMICAL AND BIOLOGICAL STUDIES OF BUTEA FRONDOSA ROXB. LEAVES GROWING IN EGYPT

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SUMMARY: This study aimed to the discovery of new biologically active natural products with hormone-like activity from Butea frondosa Roxb., Family Leguminosae growing in Egypt. The investigation of estrogen-like activity showed that both the successive non-polar and methanolic extracts showed a significant estrogen-like activity in immature female rats. GLC analysis the non-polar bioactive revealed the presence of eicosane (22.5%) and β -amyrin (20.5%) as the major components in the unsaponifiable matter, while palmitic (24.9%) and linoleic (36.8%) acids were the main saturated and unsaturated fatty acids. The successive bioactive methanolic extract was subjected to chromatographic separation to yield five flavonoids identified as vicenin II, vitexin, chrysoeriol 7-o- β -D-glucuronic acid, 6,8-di-C-rhamnosyl apigenin and luteolin.

Key words: butea frondosa, estrogenic like activity, bioactive compounds

INTRODUCTION

Phytoestrogens are a group of compounds contained in plants that have structural and functional similarity to 17 β -estradiol and possess an array of pharmacological effects including anticancer properties (1). Phytoestrogens can interact with estrogen receptors (ER) and exhibit estrogenic/antiestrogenic activities.

There are two types of estrogen receptors in humans, hERR and hER β , with distinct tissue distributions throughout the body. Phytoestrogens exhibit weak estrogenic activity on the order of 10^{-2} - 10^{-3} that of 17 β -estradiol depending on the receptor subtype, but may be present in plasma at concentrations 100-fold higher than endogenous estrogens (2). The ability of phytoestrogens to display both estrogenic and anitestrogenic properties has led to the search for new plant sources and methods to induce phytoestrogens.

The genus *Butea* (Earl of Bute) belong to the Family Leguminosae (Fabaceae). It includes three or four species of trees or woody vines native to India and China with deep scarlet papilionaceous flowers in racemes and pinnate leaves. The species *Butea frondosa* Roxb. (Common name: flame of the forest, dahk, palas, bas-

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Table 1: Biochemical parameters of different experimental groups of the estrogenic test.

Plasma parameters	Group (Mean ± SE)					
	Control	Estradiol	Non-polar extract	Chloroform extract	MeOH extract	50% MeOH extract
Glucose (mg/dl)	58.3 ± 1.054	58.3 ± 0.882	58 ± 0.683	58.8 ± 0.833	59.5 ± 1.309	59.2 ± 0.792
Total Cholesterol (mg/dl)	73.5 ± 1.648	75.7 ± 1.646	76 ± 0.447	74.7 ± 1.706	74 ± 1.414	74.5 ± 1.088
Total protein (g/dl)	6.45 ± 0.118	6.15 ± 0.076	6.3 ± 0.085	6.2 ± 0.067	6.2 ± 0.112	6.2 ± 0.076
Creatinine (mg/dl)	0.612 ± 0.011	0.635 ± 0.015	0.602 ± 0.009	0.612 ± 0.005	0.613 ± 0.005	0.608 ± 0.008
Urea (mg/dl)	28 ± 0.856	34.2* ± 1.166	29.5 ± 0.764	28.3 ± 0.882	29.2 ± 0.307	27.2 ± 0.703
AST (U/l)	134.8 ± 1.681	147*** ± 0.966	139.8* ± 0.792	136.8 ± 1.077	137.8 ± 1.681	136.7 ± 0.882
ALT (U/l)	56.7 ± 1.891	63.2** ± 1.352	59.8 ± 1.939	57.3 ± 1.744	59 ± 1.983	56.8 ± 1.796
17-β Estradiol (pg/ml)	29.3 ± 0.882	56.8*** ± 1.869	39*** ± 0.856	31 ± 0.894	41.5*** ± 1.147	30 ± 0.577
% Change	-	94	33	6	42	2

Values significantly differ from control: *: $p < 0.05$, **: $p < 0.010$, ***: $p < 0.001$.

tard, teak, Bengal Kino), cultivated in Egypt, is a medium sized ornamental leafy tree native to east India and Burma yielding gum or lac, with three roundish pubescent beneath leaflets, the lateral ones are unsymmetrical. The flowers are two inch long, orange-crimson, very showy, and the stamens are nine together and one free. The tree reaches a height of 50 feet. Its inspissated juice is known as Bengal or Palas Kino, or *Butea* gum which has astringent properties resembling true Kino. The trees also yield

a stick-lac. The coarse fibrous material obtained from the inner bark is used for caulking the seams of boats; dried flowers yield a yellow or orange dye. The plant is used by Hindus and Buddhists in religious ceremonies, the wood is burnt and the flower is offered to the god (3,4). The plant *Butea frondosa* has been indicated in the Indian system of medicine as a plant augmenting memory and as a rejuvenator (5). It was reported that the seed is purgative, anthelmintic, vermifuge, and rubefacient that is

Table 2: The effect of administration of *Butea Frondosa* leaves extracts or estradiol on uterine weight and opening of vagina.

Plasma parameters	Group (Mean ± SE)					
	Control	Estradiol	Non-polar extract	Chloroform extract	MeOH extract	50% MeOH extract
Uterine weight (mg) (Mean±SE)	21.3 ± 0.882	36.7* ± 0.882	30.2* ± 0.703	23.0 ± 0.730	30.7* ± 0.715	21.7* ± 0.759
% Change		72	42	8	44	2
Degree of opening vagina	0/6	5/6	4/6	0/6	4/6	0/6

Values significantly differ from control: *: $p < 0.001$.

Table 3: Nutritional parameters of different experimental groups of the estrogenic test.

Plasma parameters	Group (Mean ± SE)					
	Control	Estradiol	Non-polar extract	Chloroform extract	MeOH extract	50% MeOH extract
Initial body weight (g)	43.3±0.882	42.7± 1.145	43.2±0.872	42.2±1.249	42.7±0.919	43.0±0.730
Final body weight (g)	64.7±1.174	63.7±1.406	64.8±1.352	63.8±1.492	63.0±1.1±25	65.2±1.137
Body weight gain (g)	21.3±0.882	21.0±0.966	21.7±0.558	21.7±0.558	20.3±0.494	21.8±0.601
Total food intake (g)	80.7±2.512	84.7±2.027	87.3 ²³⁸ / ₉₃ 1.686	86.8±2.119	82.2±1.077	86.7±1.725
Food efficiency ratio	0.264±0.004	0.247±0.006	0.264 ²³⁸ / ₉₃ 0.013	0.249**±0.004	0.248**±0.004	0.252*±0.002

Values significantly differ from control: *: $p < 0.05$, **: $p < 0.025$.

taken for destroying all sorts of intestinal worms. It is used in digestive disorders, flatulence, piles, haematemesis, as haemostatic and for increasing micturition (diuretic) and menstrual flux (emmenagogue). The leaf is febrifuge and aphrodisiac. Pasted leaf is taken in indigestion, diarrhea, colic pain, menorrhagia and is applied in glandular inflammation. The flowers and the buds are astringent and diuretic (6). Previous reports noticed that *Butea frondosa* contains flavonoids (7,8), alkaloids (9,10), sterols and triterpenes (11,12).

The aim of the present work is to study the estrogen like activity of the non-polar and polar successive extracts of *Butea frondosa* leaves as well as the phytochemical constituents of the bioactive fractions.

MATERIALS AND METHODS

Materials

Plant materials: The leaves of *Butea frondosa* Roxb. Family Leguminosae (Fabaceae) were collected from the Zoo, Giza, Egypt. Samples were authenticated by Dr. Tereez Labib Consultant of Plant Taxonomy at the ministry of Agriculture, Giza, Egypt. A voucher specimen from each plant was deposited in the National Research Center Herbarium. The collected leaves of the plants under investigation were separately air dried, reduced to No. 36 powdered and kept in tightly closed container.

Animals: Twenty-four female immature white albino rats with average body weight of 44 ± 0.5 g were obtained from the animal house of National Research Centre, Cairo, Egypt. The animals were kept individually in stainless steel cages at room temperature. Water and food were given ad-libitum.

Diets: A balanced diet composed of 10% protein, 10% corn oil, 23.5% sucrose, 47% maize starch, 5% fiber, 3.5% salt mixture (13), and 1% vitamin mixture (14) was prepared for feeding the rats all over the experimental period.

Methods

General experimental procedures: All NMR spectra were run on a Bruker AMX-500, Varian Inova-500. The chemical shifts were reported in δ values (ppm) with TMS as internal standard. ¹H- and ¹³C-NMR spectra were recorded in DMSO. UV spectra were recorded on UV-visible spectrophotometer: labomed, Inc. UVD-3500. Thin layer chromatography was carried out using polyamide 6 S (Riedel-de Haën) for cc, microcrystalline cellulose (E. Merck) for cc and Sephadex LH-20 (Pharmacia, Uppsala, Sweden), paper chromatography was carried out on sheets of whatmann filter paper No 1 for PC.

Preparation of the successive extracts with selective organic solvents: The air-dried powdered of *Butea frondosa* leaves (500 g) were extracted successively in a continuous extraction apparatus (Soxhlet) until exhaustion with the following organic solvents in succession: petroleum ether (40-60°C),

diethyl ether, chloroform, methanol and 50% aqueous methanol. In each case, the solvent was stripped off under reduced pressure and dried to constant weight in vacuum desiccators over anhydrous calcium chloride. The extracts of both petroleum ether and ether were mixed to be the non-polar fraction.

Studying the estrogen like activity of the extracts: The estrogen like activity was determined according to Ali *et al.* (15). Rats were divided into six groups (6 rats/group). Four groups were given daily oral dose from methanol, 50% aqueous methanol, chloroform and non-polar extracts of *Butea frondosa* leaves (500mg/kg rat body weight for 10 days). Rats of the fifth group were daily subcutaneously injected with estradiol benzoate (2mg/kg rat body weight) for 10 days and served as reference group. Rats of the sixth group were run as control where no medication or extract was given. During the experimental period rats were fed on balanced diet. All rats were examined daily for opening of vagina as one parameter of estrogenic activity. Body weight and food intake were recorded once weekly. At the end of the experiment (10 days); total food intake, body weight gain and food efficiency ratio were calculated. Blood samples were withdrawn on heparin from eye vein orbital after an overnight fast. Blood samples were centrifuged for 10 min at 3500 rpm for separation of plasma for determination of 17- β estradiol (16) using ELISA technique (as second parameter of estrogenic activity) and glucose (17), cholesterol (18), total protein (19), urea (20), creatinine (21) and activity of alanine transaminase (ALT) (22) and aspartate transaminase (AST) (22). The uteri of the rats were excised and weighed. Uteri were dried to a constant weight in an oven at 100°C and their dry weights were determined (as third parameter of estrogenic activity). The results obtained were expressed as the Mean \pm SE and the significance of the results was analyzed statistically adopting Student's t-test.

Fractionation of the non-polar extract: The non-polar extract of *Butea frondosa* leaves was subjected to saponification (23) and both the unsaponifiable matter fraction (USM) and fatty acids fraction (as methyl esters) were analyzed using GLC (HP 6890-series GC system).

The unsaponifiable fraction was analyzed by GLC adopting the following conditions: Column: 10% OV-101 packed column; Stationary phase: Chromosorb W-HP; Detector temperature: 290°C; Injector temperature, 28°C; Carrier gas N₂; flow-rate 30 ml/min; air flow-rate: 300ml/min; H₂ Flow-rate 30ml/min; Detector FID; Chart speed: 0.5 cm/min; Oven program: Initial temperature, 70°C; Final temperature, 270°C; programmed

4°C/min. For 35min at 270°C, total time, 85 min. Identification of hydrocarbons and sterols contents of the unsaponifiable matter was carried out by comparison of their retention times with co-injected authentic reference compounds. Quantification was based on peak area integration.

Analysis by GLC of the methyl ester was carried out according to the following conditions: Stationary phase: 10% diethylene glycosuccinate (DEGS) packed column; oven temperature, 170°C; detector temperature, 300°C; injector temperature, 250°C; Carrier gas, N₂; flow-rate, 30ml/min; air flow-rate, 350ml/min; H₂ flow-rate, 350ml/min; detector, FID; Chart speed, 2cm/min. Identification of the fatty acid methyl ester was carried out by direct comparison of retention times of each of the separated compounds with authentic samples of the fatty acid methyl esters analyzed under the same conditions. Quantification was based on peak area integration.

Extraction and isolation of the flavonoidal content: Twenty grams of the dry bioactive successive methanolic extract of the leaves of *Butea frondosa* were applied on 350g of polyamide glass column (100 x 5 cm) using water as eluent then decreasing the polarity by adding methanol gradually to the water till 100% methanol, where no color was obtained at the last fractions. 150 ml fractions were collected and evaporated under pressure at a temperature not exceeding 40°C. All fractions were screened by paper chromatography (Whatman No. 1) using acetic acid: water (15:85) (v/v) and n-butanol: acetic acid:water (4: 1: 5) (v/v/v) as solvent systems, and the chromatograms were examined under UV light before and after exposure to ammonia vapour and spraying with AlCl₃ solution. Then similar fractions (21-30) afford F1 (3.2g) eluted with 20% methanol, fractions (55-60) afford F2 (0.2g) eluted with 70% methanol and fractions (75-90) afford F3 (2g) eluted with 90% methanol. The first fraction, contains two major spots, was chromatographed on 30g of micro crystalline cellulose column (2.5 x 50 cm) and eluted with water, 25ml fractions were collected, evaporated under reduced pressure at a temperature not exceeding 40°C and screened by paper chromatography (Whatmann No. 1) using the previously mentioned solvent systems, and the chromatograms were examined under UV light before and after exposure to ammonia vapors and spraying with AlCl₃ solution. Similar fractions were collected together to yield compounds ViceninII (12mg) and Vitexin (7mg). The second fraction, contains a single major spot, was chromatographed on 10g micro crystalline cellulose and screened as mentioned before to yield compound *Chrysoeriol 7-O- β -D-4C₁-glucuronic*

Table 4: UV spectra data (λ_{max} nm) of the flavonoids isolated from *Butea frondosa* leaves.

Compound number	Band	MeOH	NaOMe	Shift	Na acetate		Na acetate/H ₃ BO ₃		AlCl ₃		AlCl ₃ /HCl	
		Wave length	Wave length		Wave length	Shift	Wave length	Shift	Wave length	Shift	Wave length	Shift
B1	I	331	400	+69	394	+7	334	-	387	+56	383	+52
									352,305(sh)		352,305(sh)	
B2	II	273	283		280		274	-	280(sh), 256		278(sh), 256	
	I	335	399	+64	385	+9	334	-	386	+51	384	+49
B3	II	302 (sh)	335		305		327 (sh)	-	350, 306		343, 303	
	I	273	283		282		275	-	280		280	
B4	I	344.5	403	+58.5	406	-	344	-	387	+42.5	387	+42.5
		296 (sh)	296						359.5, 296		353.5, 296	
B5	II	269	260		259		269	-	274(sh), 256.5		274(sh), 256.5	
	I	336.5	394	+58.5	389		338	-	356	+19.5	356	+19.5
B6	II	271	277		275	+4	272	-	278(sh), 256		278(sh), 256	
	I	348	402	+54	385		372.5	+24.5	418.5	+70.5	385.5	+37.5
B7	II	268, 255	268		272	+17	261.5		302		356.5, 298	
									273		276, 262.5	

acid (35mg). Finally the third fraction contain two major spots was chromatographed on 30g micro crystalline cellulose column (2.5 x 50 cm) and eluted with 50% methanol in water, fractions were collected and screened as with F1 and F2 to yield compounds 6,8 *di-C-rhamnosyl apigenin* (6mg) and luteolin (55mg). All compounds were finally purified by passing over Sephadex LH20 in methanol. The isolated compounds were identified by UV spectroscopy, ¹H NMR, ¹³C NMR and co-chromatography with authentic compounds.

RESULTS

The results represented in Table (1) showed that both non-polar and methanolic extracts of *Butea frondosa* leaves produced significant increase in plasma 17 β -estradiol level ($p < 0.001$) which was 33% and 42% respectively. Both extracts didn't produce any significant change in the other biochemical parameters except a slight increase in plasma AST level produced by the non-polar extract. Although the group of rats that was injected with estradiol showed higher significant level of 17 β -

estradiol, but at the same time it showed an increase in plasma urea, creatinine and AST reflecting that it may have side effect on liver and kidney functions. Regarding the effect of the treatment of immature female rats with different *Butea frondosa* leaves extracts or estradiol benzoate on uterine weight and opening of vagina, the results tabulated in Table (2) revealed that oral administration of non-polar or methanol extract showed a significant increase in uterine weight ($p < 0.001$) and in the rate of opening of vaginal orifice while subcutaneous injection with estradiol benzoate produced higher increase in both parameters. From Table (3) it is clear that all the nutritional parameters except the food efficiency ratio showed non-significant changes. Food efficiency ratio decreased significantly in groups treated with estradiol benzoate, methanol, 50% methanol and chloroform successive extracts of *Butea frondosa* leaves.

The above mentioned results showed that both the non-polar and the methanolic extracts possess significant estrogen-like activity without a significant change in

the plasma biochemical parameters.

The phytochemical investigation of the biologically active non-polar extract of *Butea frondosa* leaves by the aid of GLC analysis revealed that the unsaponifiable matter contains hydrocarbons (eicosane 22.5%), triterpenes (β -amyryn 20.5%) and sterols (campesterol 3.2%, β -sitosterol 2.4%), while the fatty acids fraction contains lauric (4.8%), myristic (3.3%), palmitic (24.9%), linoleic (36.8%) and linolenic (5.1%) acids. Linoleic acid was the major fatty acids.

The phytochemical investigation of the biologically active methanolic extract revealed that it is rich in flavonoids. Chromatographic separation and purification of the flavonoidal content using different chromatographic tools and identification of the purified compounds using spectroscopic techniques resulted in the isolation of five pure compounds (vicenin II (B1), vitexin (B2), chrysoeriol 7-O- β -D-glucuronic acid (B3), 6,8-di-C-rhamnosyl apigenin (B4), and luteolin (B5)) (Table 4), which are different from those previously isolated from the flowers and the stem bark of this plant.

DISCUSSION

Hormones such as estrogen and progesterone play an important role in human growth, and are responsible for regulating the complex cellular events associated with differentiation, function and growth of female reproductive tissues. Women in the menopause suffer from bone density reduction, cardiovascular disease, sweating and anxiety because of a lack of estrogens (24). Exogenous estrogens or hormone replacement therapy (HRT) was introduced to improve the menopausal symptoms, which quickly took effect but caused undesirable side effects such as irregular bleeding and increased risk of breast cancer. Efforts are on worldwide to discover an alternative HRT with minimal risks. It was found that natural compounds from certain plants called phytoestrogens could be used for management of menopausal symp-

toms and have a few side effects (25). Phytoestrogens appear to have both estrogenic and anti-estrogenic effects (26,27). Therefore, these have been considered as a part of selective estrogen receptor modulators (SERMs) and studied as an alternative for hormone replacement therapy (28).

The estrogen-like activity of the non-polar extract of *Butea frondosa* leaves can be attributed to the presence of β -sitosterol which has been previously reported to possess weak estrogen-like activity (29) and linoleic acid which has the ability to bind to estrogen receptors and induce certain estrogen inducible genes (30).

In this study the estrogen-like activity of the successive methanolic extract of *Butea frondosa* leaves may be due to presence of flavonoids. Flavonoids, which are structurally similar to estrogen, are able to bind to the estrogen receptor and possess estrogenic or anti-estrogenic activities (31-33). Flavonoids such as genistein have been shown to inhibit the 17 β -hydroxysteroid dehydrogenase enzyme which is responsible for the conversion of estrone to estradiol (34). Some flavonoids can activate the estrogen receptors which control sexual differentiation and regulate skeletal health in women (35). In the present study, the estrogen-like activity of the successive methanolic extract can be attributed to the presence of luteolin, a flavones which is previously reported to possess estrogenic activity (35-37). This estrogenic activity was explained by the ability of luteolin to bind to and activate the nuclear estrogen receptors.

It was reported previously that alcoholic extract of *Butea frondosa* flowers possess anti-estrogenic activity in immature mice (38,39), while Bhargava (40) reported that the seed extract of *Butea frondosa* exhibited an estrogenic activity in ovariectomized rats.

In conclusion; both non-polar and methanolic extracts of *Butea frondosa* leaves showed estrogen-like activity with different degrees which may be attributed to the presence of sterols and flavonoids.

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