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Fate of Embelin in Pippalyadi Yoga, an Ayurvedic oral contraceptive: Structure of Embelin-borax complex and evaluation of anti-fertility activity

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In the present communication, is reported the existence of embelin **1** as embelin-borax complex **3** in a stoichiometry of 1:2 in an Ayurvedic formulation Pippalyadi Yoga. The structure of the complex is established on the basis of spectral data and it is concluded that both the phenolic oxygens as well as carbonyl groups of embelin are involved in complex formation. Embelin-borax complex is stable under basic conditions; however, it is sensitive to acidic conditions and breaks up to yield embelin and borax on treatment with mild acids. Anti-fertility activity of formulation and individual constituents was evaluated at cellular and hormonal level in Sprague Dawley female albino rats. Pippalyadi Yoga showed better activity than any of the individual constituents in the formulation including the complex.

Keywords: Pippalyadi Yoga, Embelin, piperine, borax, *Embelia ribes, Piper longum*

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Ayurveda, 'the science of life' is traditional system of medicine in India that dates back to ancient times. Most of the formulations used in Ayurveda are compound preparations consisting of more than one ingredient and the useful effects of such formulations are usually supposed to be through synergism. Pippalyadi Yoga is an Ayurvedic formulation used in India since ancient times as an oral contraceptive. It consists of a mixture of equal amounts (1:1:1 w/w) of Vidanga fruits (*Embelia ribes* Burm.f.), Pippali fruits (*Piper longum* Linn.) and Tankana (Borax, $Na_2B_4O_7.10\ H_2O$). All the three ingredients are made into fine powder and mixed together to make tablets as well as capsules.

The individual constituents of the formulation are also reported to possess anti-fertility effects. The studies conducted on anti-fertility effects of *E. ribes* on experimental animals revealed that 50% ethanolic and benzene extracts of *E. ribes* exhibited dose dependent oestrogenic activity. Both the extracts caused a significant increase in the oestrone induced uterine weight of immature rats[1]. Kholkute *et al*. found that administration of powdered berries mixed with diet to female rats prolonged the diestrus phase of estrus cycle and inhibited fertility in 62% animals. Petroleum ether extract and methanol extract prevented pregnancy in 75% animals[2]. In another study it was found that administration of powdered berries of *E. ribes* for three months adversely affected the quantity and quality of semen in male bonnet monkeys (*Macaca radiata*)[3]. It has also been shown that embelin **1** (**Figure 1**), its major constituent, possesses anti-implantation activity when administered postcoitally from day 1 to day 5 of pregnancy at 60 and 120 mg/kg daily dose levels[4] whereas Kholkute *et al.* and others reported that embelin failed to show any postcoital anti-fertility activity[2, 5].

The second ingredient *Piper longum* is used in a large number of Ayurvedic formulations. It is a constituent of well known Ayurvedic formulation Trikatu (*Piper nigrum, Piper longum* and *Zingiber officinalis* in equal proportions) and it is suggested that the use of acrids in Ayurveda is due to their bioavailability enhancing properties. The major constituent of *Piper longum* is piperine **2** (**Figure 1**), and it has been shown to possess among others, CNS stimulant activity[6], antifungal activity[7], anti-pyretic and anti-inflammatory activity against carageenan induced oedema in rats[8].

During the studies on the standardization of Pippalyadi Yoga, it was surprising to see that only

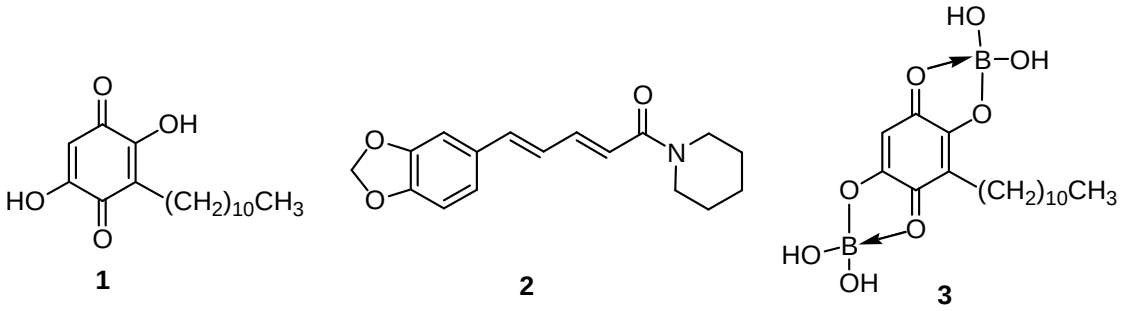

Figure 1 — Chemical constituents of Pippalyadi Yoga formulation

traces of embelin could be extracted from the powdered formulation. *E. ribes* is reported to contain about 2-3% of embelin, so according to formula of Pippalyadi Yoga where all the three ingredients are used in equal amounts, the content of embelin in formulation should be close to 1%. However, the amount of embelin in formulation was negligible (<0.1%) as determined by HPLC studies. In this paper, is reported that embelin forms a complex with borax and exists in the formulation as an embelin-borax complex (**Figure 1**) that could not be extracted with chloroform, the solvent used to extract embelin from *E. ribes*. The complex and the formulation along with its individual constituents were evaluated for anti-fertility activity in Sprague Dawley (S.D.) female albino rats and the results are presented in this paper.

Results and Discussion

Structure of embelin-borax complex: Analysis of the formulation by HPLC revealed that the content of embelin in the formulation did not correspond to the theoretically calculated amount. Different studies have been carried in order to find out the fate of embelin in the formulation. Based on literature reports and structural features of embelin it was thought that embelin has a tendency to form complexes with transition metals and a number of embelin-metal complexes have been reported in the literature[9]. Similar to transition metals, boron has a vacant orbital and can act as lone pair acceptor. So the possibility of embelin interacting with borax was explored.

In order to confirm this assumption, the complex of embelin with borax was synthesized employing method used for synthesizing embelin-metal complexes[9]. The structure of the complex was established based on spectroscopic, elemental analysis and thermal studies. Embelin shows OH stretching frequency at 3310 cm^{-1}. The disappearance of this band in IR spectrum of complex indicates that both phenolic hydroxyl groups participate in complex formation. A broad band of medium intensity observed at 3401 cm^{-1} in the IR spectrum of complex could be ascribed to B-OH group. The carbonyl stretching frequency observed at 1615 cm^{-1} in embelin is shifted to 1525 cm^{-1} in complex suggesting that the carbonyl group also participates in complex formation. Similar shifts in carbonyl stretching frequency have been reported earlier for metal complexes of embelin[9,10]. These data suggest that embelin may be acting as a bidentate ligand coordinating through its carbonyl groups. The mass spectrum of complex showed a prominent peak at m/z 365 indicating loss of one OH group from the molecular ion. The ^{1}H and ^{13}C NMR data also suggest same structural features for the complex. A three proton triplet at δ 0.89 indicated that the undecyl chain is intact, a broad 16 proton signal at δ 1.27 and another two-proton triplet at δ 2.83 accounted for all the protons of the undecyl chain. The aromatic proton which appears at δ 6.00 in embelin could not be observed in the complex. It has been reported earlier that protons in similar chemical environment in cysteinyl-flavan-3-ol conjugates and phloroglucinol derivatives get exchanged with deuterium and are not observed in ^{1}H NMR[11]. The ^{13}C NMR spectrum showed two downfield signals at δ 179.0 and 182.3 that may be ascribed to carbonyl groups. In the ^{11}B NMR spectrum, a broad peak was observed at δ 3.6 typical for $B(OR)_3L$ where L in this case indicates a dative bond formed by carbonyl group. Results obtained from Differential Scanning Calorimetry (DSC) showed five endothermic peaks at 87°C, 198°C, 210°C, 227°C and 413°C. These peaks may be resulting from loss of water, cleavage of dative bond, melting point peak and decomposition. All these data point to a structure for the complex as shown for **3** (**Figure 1**). The complex borate structures such as borax are known to break up when dissolved in water[12]. So it was obvious that embelin-borax interaction could be in the form of embelin attached to

boric acid moieties through phenolic oxygens and the carbonyl groups. In order to confirm this, a complex of embelin with boric acid was synthesized and found to be identical with embelin-borax complex *viz.* melting point, IR, and mass spectra. The stoichiometry of the complex was determined by actual recovery of unreacted material as well as continuous variation method (**Figure 2**) and was found to be 1:2 (embelin:borax)[13]. Investigation for *p*H stability (**Figure 3**) suggested that the embelin-borax complex is stable under alkaline conditions and gets cleaved under acidic environment while under basic conditions, the complex was found to be stable.

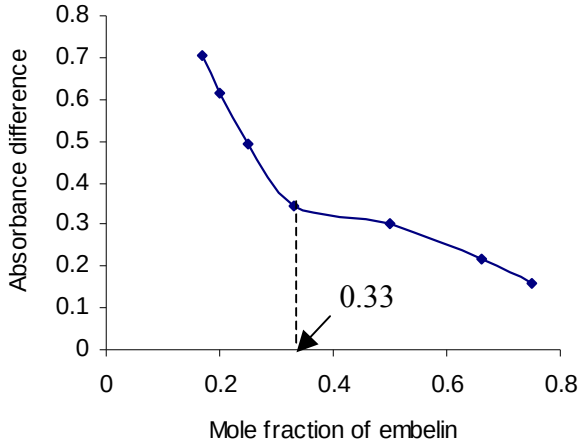

Figure 2 — Plot of mole fraction of embelin versus absorbance (measured at 285 nm) difference

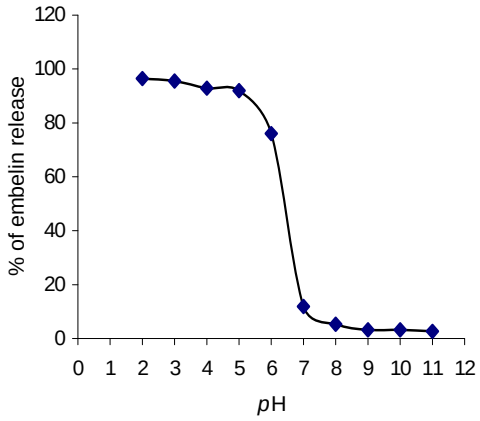

Figure 3 — *p*H versus percentage of embelin release

After attempting isolation on several stationary phases, embelin-borax complex was finally isolated from formulation by gel permeation (Sephadex LH20) chromatography. Isolated complex was found to be completely identical to the synthesized complex *viz.* melting point, UV-Vis, IR, ^{1}H and ^{13}C NMR, ^{11}B NMR, and mass spectral data.

Biological evaluation: The embelin-borax complex, Pippalyadi Yoga, borax, *P. longum*, *E. ribes*, piperine and embelin were subjected to biological evaluation. These studies indicated the irregular estrous cycles in female rats which represented the anti-fertility effect[6]. The length of various stages of estrous cycle was statistically ($P<0.05$) different between the four stages of various groups except in *Piper longum* and *Embelia ribes*. The shortest diestrus cycle and minimum production of progesterone was observed in the animals treated with Pippalyadi Yoga (**Table I**). These data suggest that drug interferes with corpus luteum function in female rats and that the drug would be effective as an interceptive agent. The corpus luteum is an ovarian tissue that produces progesterone necessary for successful pregnancy. In rodents, semi-circadian surges of prolactin secretion are induced by cervical stimuli, which are believed to be responsible for the conversion of corpus luteum of the cycle to the corpus luteum of pregnancy. The corpus luteum of pregnancy secretes a sufficient progesterone amount to maintain

Table I — Different stages of estrus cycle (days) and progesterone production level (ng/mL) in SD female albino rats treated with test drugs for 21 days

Group	Test drug	Proestrus (M ± SEM)	Oestrus (M ± SEM)	Metestrus (M ± SEM)	Diestrus (M ± SEM)	Progesterone (M ± SEM)
Group I	Control	1.85 ± 0.72	1.28 ± 0.60	1.35 ± 0.46	1.57 ± 0.65	26.64 ± 2.56
Group II	Pippalyadi Yoga	3.04* ± 0.72	0.66 ± 0.45	0.85 ± 0.46	1.47 ± 0.52	20.25* ± 2.30
Group III	Borax	0.90* ± 0.40	0.80 ± 0.38	1.40 ± 0.40	2.71*± 0.53	27.67 ± 3.41
Group IV	*Piper longum*	1.38 ± 0.55	0.71 ± 0.53	1.50 ± 0.69	2.47 ± 0.89	24.75 ± 0.73
Group V	*Embelia ribes*	1.28 ± 0.58	1.47 ± 0.55	1.40 ± 0.50	1.85 ± 0.71	23.71 ± 2.03
Group VI	Piperine	0.66* ± 0.29	0.66 ± 0.26	2.55* ± 0.47	2.14 ± 0.55	25.96 ± 2.68
Group VII	Embelin	0.80* ± 0.58	0.61 ± 0.51	0.70 ± 0.52	3.95* ± 0.98	47.92* ± 3.50
Group VIII	Embelin-borax complex	0.90* ± 0.41	0.95 ± 0.51	1.20 ± 0.46	2.95* ± 0.46	54.75* ± 4.19

* $P< 0.05$

pregnancy. It is possible that significantly decreased level of progesterone in the animals treated with Pippalyadi Yoga might be mediated *via* decrease in LH receptor expression that is necessary for maintaining adequate levels of progesterone. The animals of other groups treated with *P. longum, E. ribes* and piperine although showed reduced progesterone level but it was statistically insignificant. The prolonged diestrus and higher production level of progesterone were noted in the animals treated with embelin and embelin-borax complex. This observation suggests that embelin was not effective as anti-fertility agent; same result was also revealed by the Kholkute group[2]. It has also been observed that the body weight reduced significantly in animals treated with compounds like piperine, embelin and complex compared with control animals. This may be related to the reproductive toxicity of piperine which is reported to interfere with several crucial reproductive events in a mammalian model[14].

These studies indicate that Pippalyadi Yoga shows anti-fertility effects by altering the progesterone levels. The embelin-borax complex, the individual ingredients, or the marker compounds as such failed to show any activity. This suggests a synergistic effect of the components. The results also indicate that the complex does not show anti-fertility effect in the said assays, however, it may have a role in stabilizing the formulation with respect to embelin or related constituents. Further acid-labile nature of complex shows that it may be releasing embelin in stomach where acidic conditions prevail.

Experimental Section

Melting points were recorded on capillary melting point apparatus and are uncorrected. NMR spectra are recorded on 300 MHz Bruker FT-NMR (Avance DPX300) spectrometer using tetramethylsilane as internal standard and the chemical shifts are reported in δ units. Mass spectra were recorded on LCMS (ESI). Absorption spectroscopic analysis was done on UV-Vis spectrophotometer (Model: Beckman DU-7000). IR was done using Nicolet spectrometer and thermal studies using Mettler Toledo Differential Scanning Calorimeter. Elemental analyses were recorded on Elementar Vario EL spectrometer.

Fruits of *Embelia ribes* were purchased from local market and were authenticated by the botanist of Department of Natural Products. Borax used for studies was also purchased from local market and was dehydrated before use. All the solvents purchased from commercial sources were of analytical grade, and were used without further purification unless otherwise stated.

Isolation of embelin from fruits of *Embelia ribes*: The crushed fruits of *Embelia ribes* (2 kg) were extracted with chloroform (5 L) using hot soxhlet extraction. The extract was concentrated to 500 mL and kept in freezer at 4°C for 10-12 hr. Precipitate was filtered and the yellow solid was recrystallised from ethanol to yield embelin as orange crystals (50 g, 2.5%). It was characterized by comparison of melting point, NMR, MS and IR spectra with literature values[15].

Preparation of embelin-borax complex. To a solution of embelin (1.0 g, 3.4 mmole) in ethanol (25 mL) was added dehydrated borax (1.37 g, 6.8 mmole) and reaction mixture was refluxed for 1 hr. Reaction mixture was cooled, filtered and filtrate concentrated to yield brownish violet solid. It was washed with ethyl acetate and solid residue was dried and recrystallised from ethanol to yield brownish violet colored crystalline solid (1.27 g, 98%). m.p. 250°C (decomposed); UV λ_{max} (EtOH) (ε): 303 nm (40000); IR (KBr): 3401, 1603 (weak), 1525, 1475, 1387, 1244 cm^{-1}; ^1H NMR (CD_3OD): δ 2.29 (2H, *t*, *J* = 6.4 Hz), 1.27 (20H, *brs*), 0.89 (3H, *t*, *J* = 6.3 Hz); ^{13}C NMR (D_2O): δ 182.3, 179.0, 115.4, 100.1, 31.2, 28.9, 28.7, 28.5, 22.7, 22.0, 13.4; ^{11}B NMR (D_2O): δ 3.6 (brs); ESIMS: m/z 365 [M-OH]. Anal. $C_{17}H_{28}B_2O_8$ (382.20). Calcd C, 53.45; H, 7.39. Found. C, 54.42; H, 8.06%.

Preparation of embelin-boric acid complex. To an ethanolic solution of embelin (**1**, 1.0 g, 3.4 mmole) was added aqueous solution of boric acid (0.422 g, 6.8 mmole, *p*H was adjusted to 9.0 with 1N KOH). Reaction mixture was refluxed for 1 hr. Reaction mixture was then cooled, filtered and the filtrate concentrated. The solid obtained was washed with ethyl acetate and recrystallised from ethanol to yield brownish violet solid (1.2 g, 93%).

Isolation of embelin-borax complex from formulation. The Pippalyadi Yoga powder (20 g) was shaken at RT for 5-10 min with methanol and filtered. Filtrate was concentrated to get crude extract (3.5 g). Methanolic extract was washed several times with ethyl acetate in order to remove piperine and other non-polar components. The remaining extract (3.3 g) was then dissolved in 5 mL of methanol and charged on Sephadex LH20 column, packed in methanol and eluted with methanol. Fractions of 10 mL were collected. Brownish violet colored fractions

were pooled and concentrated to obtain brownish violet solid (0.177 g, 0.88%; theoretical 0.198 g).

Stoichiometry of complex. Recovery method - Mixtures of embelin and borax in different molar ratios were refluxed in ethanol for 1 hr. Stoichiometry was determined from the amounts of unreacted borax and embelin.

Continuous variation method. Eight reaction mixtures containing embelin-borax in different ratios were refluxed for 1hr and then allowed to cool. Absorbance was measured at 285 nm after proper dilutions. Absorbance difference was calculated from the difference in the absorbance before and after complexation. A graph was plotted between mole fraction of embelin versus difference as shown in **Figure 2**.

***p*H stability determinations**. A solution of 5 mg of complex in phosphate buffers of different *p*H was sonicated for 5 min. The solutions were extracted with 1 mL of ethyl acetate. Aliquot (50 µL) of ethyl acetate layer was diluted to 5 mL with ethyl acetate and absorbance was measured at 285 nm. The percentage of embelin released was calculated from the below mentioned formula. Graph was plotted between the *p*H versus percentage embelin release as shown in **Figure 3**.

$$\% \text{ Embelin release} = 100 - [(A-B) \times 100/A]$$

where, A = Absorbance of the pure embelin; B = Absorbance of the embelin released from complex

Biological evaluation. Female SD albino rats weighing 120-180 g were used for anti-fertility experiments. The rats were procured from Central Animal Facility (CAF) of the institute and housed in a 12 hr day/night cycle at 22°C and 50% relative humidity with food and water *ad libitum*. Experimental designs and procedures were approved by Institutional Animal Ethics Committee (IAEC) of NIPER. Dose administered to animals was derived from clinical dose and converted for animals according to the standard protocol.

The animals were divided into eight groups of six female rats each. Group-I was control administered with vehicle (normal saline). Group-II was treated with Pippalyadi Yoga (600 mg/kg; p.o.). Animals of group-III were treated with borax. Groups IV and V were treated with fine powder of *P. longum* and *E. ribes*, respectively at dose of 200 mg/kg p.o. Groups VI-VIII received piperine, embelin and complex at a dose of 100 mg/kg p.o. Vaginal smears were collected every day for 21 days. Smears were streaked on slides and estrous cycle stages were determined under microscope[16]. On day 22, the blood was collected for serum from tail vein of animals under mild anesthesia. Progesterone was measured by ELISA kit (Tanya Biotech, Mohali, India). The absorbance measured at 450 nm was proportional to the concentration of progesterone present in the samples. A standard curve was obtained by plotting known concentration of progesterone versus absorbance and the concentration in experimental samples was determined from standard curve. The results are reported as mean ± S.E.M. Statistical analysis was carried out by using ANOVA and Dunnett's multiple comparison tests. The results are shown in **Table I**.

Conclusions

Synthesis of embelin-borax complex and its isolation from Pippalyadi Yoga formulation have been reported for the first time. The structure of complex has been determined on basis of spectral data and the stoichiometry of the complex is determined to be 1:2. Thus, the existence of embelin in complexed form in Pippalyadi Yoga formulation has been found to be the reason for drastic decline in the content of embelin in HPLC analysis of formulation. The complex is stable in alkaline conditions, however it is acid labile. This suggests that at acidic *p*H in stomach, the complex may release embelin. Formulation as such showed anti-fertility activity by altering the progesterone level and the estrous cycle, whereas the complex and individual constituents failed to show changes at the hormonal and cellular level.

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