TCABSE-J Research Article
ISSN: 2583- 2557, *Volume 1, Issue 6, pp4-8. 2023.*

TCABSE-J
Interdisciplinary Sciences Journal

Proton NMR-based comparative analysis of aqueous vs. organic extractions from *Ficus religiosa* leaf with potential applications in the treatment of polycystic ovarian syndrome

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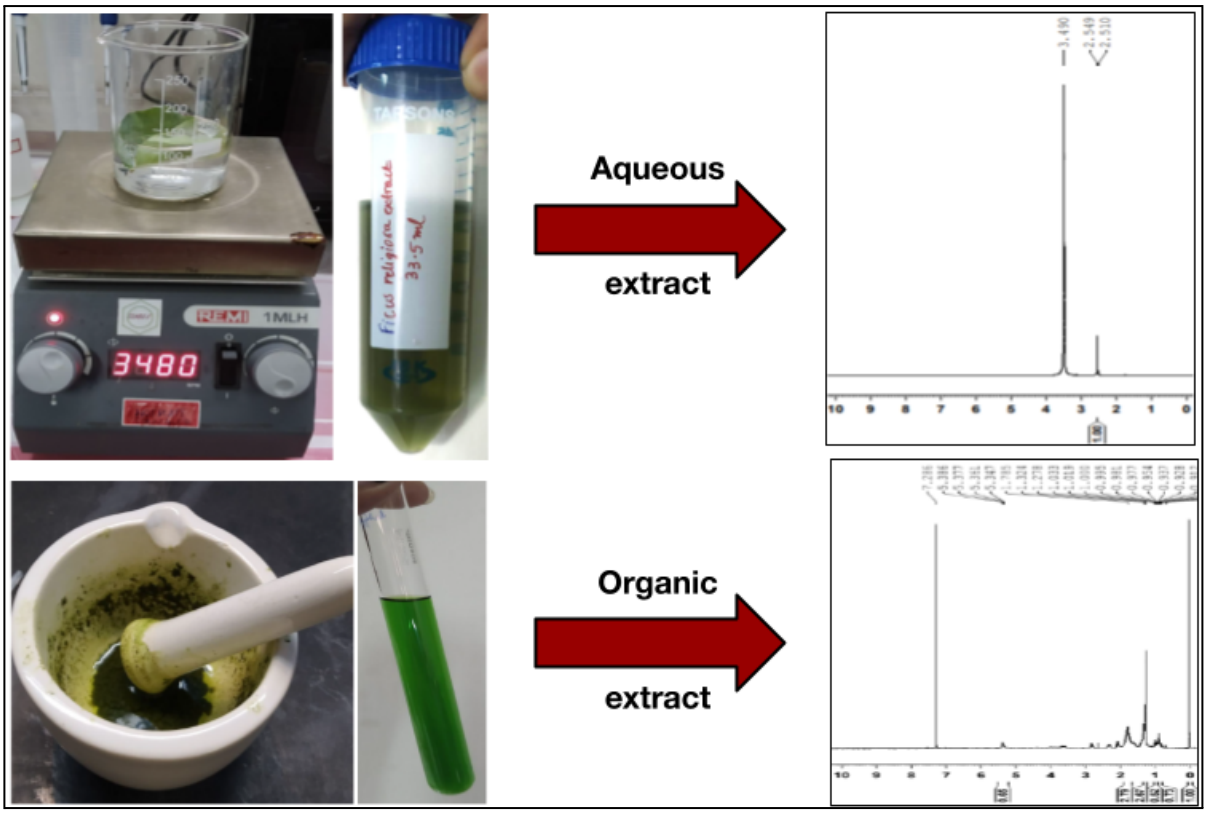
Keywords: PCOS, *F. religiosa*, proton NMR, solvent extraction, aqueous extracts, goat liver extracts, cytochrome P450.



Graphical abstract: Overview of the current study is outlined in this graphical abstract.

Citation: Karri, L., Sodasani, M., Addala, S., Grandhi, A.V.K.S., Chintalapati, J. and Yedidi, R.S. (2023). Proton-NMR-based comparative analysis of aqueous vs. organic extractions from Ficus religiosa with potential applications in the treatment of polycystic ovarian syndrome. *TCABSE-J*, Vol. 1, Issue 6:4-8. Epub: Aug 15th, 2023.

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Polycystic ovarian syndrome (PCOS) prevalence has tremendously increased in recent years in India. Many women in their early teens are facing PCOS issues primarily due to the stress that they go through. Diabetes, obesity, etc. are a few co-morbidities that are commonly observed in PCOS women along with the upregulated secondary sexual characteristics such as facial hair, etc. Recently it has been proposed that the boiled leaf aqueous extracts of *Ficus religiosa* exhibit therapeutic effects on PCOS. In order to understand the importance of the aqueous extracts, we obtained boiled *F. religiosa* leaf aqueous extract and analyzed it using proton-NMR spectrum. In parallel, a methanolic extract of the same was also prepared. The proton NMR spectra of both aqueous and organic extracts were compared. Significant difference was seen in both spectra, as expected, indicating that the extraction method influences the final NMR profile of the sample suggesting that the active ingredients are to be naturally extracted by boiling rather than using the harsh organic solvents. Further phytochemical characterization is currently ongoing to delineate the possible anti-PCOS therapeutic effects of *F. religiosa.*

***F**icus religiosa* is a native fig and considered as sacred plant in India. Under the order Utricales, it belongs to Moraceae, also known as the Mulberry family consisting of diverse trees, shrubs, vines, epiphytes, hemiphytes and rarely herbs [1]. *F. religiosa* is commonly called as peepal tree, bodhi tree in English, Ashvattha tree in India/Nepal or Asathu in Sinhala. This tree is considered as a sacred tree by Hindus, Jains and Buddists [2]. This species is named so because of its religious importance and this is considered as the tree under which Gautam Bhudda was enlightened [2]. Generally *F. religiosa* starts its life cycle as an epiphyte and can live up to 3000 years [1] and has aerial roots which later become vestigial after it starts growing on land. According to the Global Compendium of Weeds this species is listed as "Environmental weed/naturalized weed". This can grow in various climatic conditions and can survive better in tropical rainforests. The only wasp species that pollinate *F. religiosa* is *Blastophaga quadriceps* [1]. This tree is not only treated as sacred due to religious beliefs in India but also due to several medicinal values such as antioxidants in the leaves of peepal trees [3]. Thus, all of its parts viz. leaves, bark, fruits, roots etc. have a lot of importance in the Indian Ayurvedic medicine that has been followed for ages. It is used to treat several disorders related to the central nervous system, endocrine system, gastrointestinal problems, reproductive disorders and some other infectious diseases [4]. It is also believed to have antibacterial, anthelmintic, antioxidant, immunomodulatory, anticonvulsant, hypolipidemic, hypoglycemic and wound healing activities [5-10].

In particular, *F. religiosa* is used in treating reproductive disorders including polycystic ovarian syndrome (PCOS), menorrhagia, infertility in females etc. in the Indian Ayurvedic medicine [11]. Freshly boiled leaf extracts of *F. religiosa* have been used in Ayurveda for PCOS treatment. It has been previously reported that the 3-acetoxy-3-hydroxy propanoic acid (AHPA) in the fresh leaf extracts of *F. religiosa* may have therapeutic effects on PCOS in rats [11]. However, it is not clearly known whether the AHPA has a direct effect on controlling PCOS or its metabolites produced by various CYPs present in the liver during AHPA metabolism that may possess the therapeutic effects.

In this study we performed a comparative analysis of aqueous and organic extract from *F. religiosa* leaf using proton NMR spectra. The leaf was boiled in water and the water was taken as aqueous extract. In order to obtain the organic extract, the lead was homogenized in a mortar and pestle in the presence of methanol.

Materials & Methods:

Collection of F. religiosa leaves: By studying the morphological characteristics of *F. religiosa*, fresh young leaves are collected from the garden of the Botany department of Andhra University.

Preparation of Boiled leaf extract of F. religiosa leaves: This was performed systematically as described below: Day 1: Collected *F. religiosa* leaf is sterilized properly using deionized (di)-water. This leaf is then soaked overnight in a beaker with 100 ml di-water. Day 2: The overnight soaked leaf is then boiled on a hot plate till the water reduces to half. The extract obtained is about 33.5 ml which is left to dry on a plate placed on a hot plate at lowest temperatures till the water has evaporated. We use lower temperatures for the water to evaporate because higher temperatures may affect the constituents present in the extract. The plate with residue of the extract is carefully covered and then placed aside to cool and then this is used for NMR spectroscopy.

Preparation of organic extract of F. religiosa: A fresh young leaf of *F. religiosa* is collected and that is surface sterilized using di-water. This leaf is crushed using mortar and pestle by adding methanol drop by drop till the leaf is completely crushed. Now this extract is filtered using whatmann's filter paper and the filtrate is collected in the test tube. This extract is now left for air drying in the boiling water.

Solubility test of the leaf extracts residue: The dried residue after evaporating the di-water is tested for solubility before NMR spectroscopy. Small amount of the residue is scraped with spatula and a small portion of it is dissolved in dimethyl sulphoxide (DMSO) and another small portion is dissolved in chloroform ($CHCl_3$).

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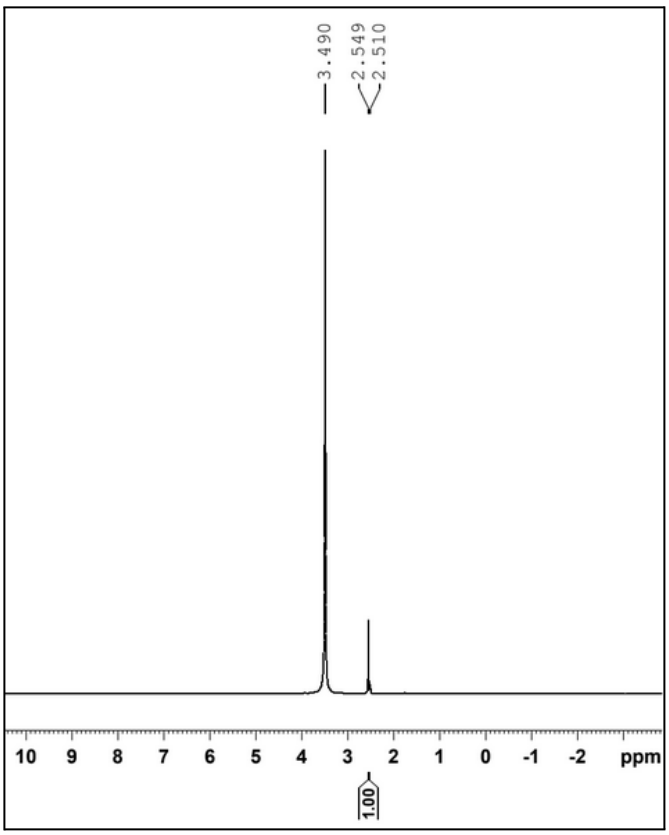

Figure 1. [1]H-NMR spectrum of aqueous extract.

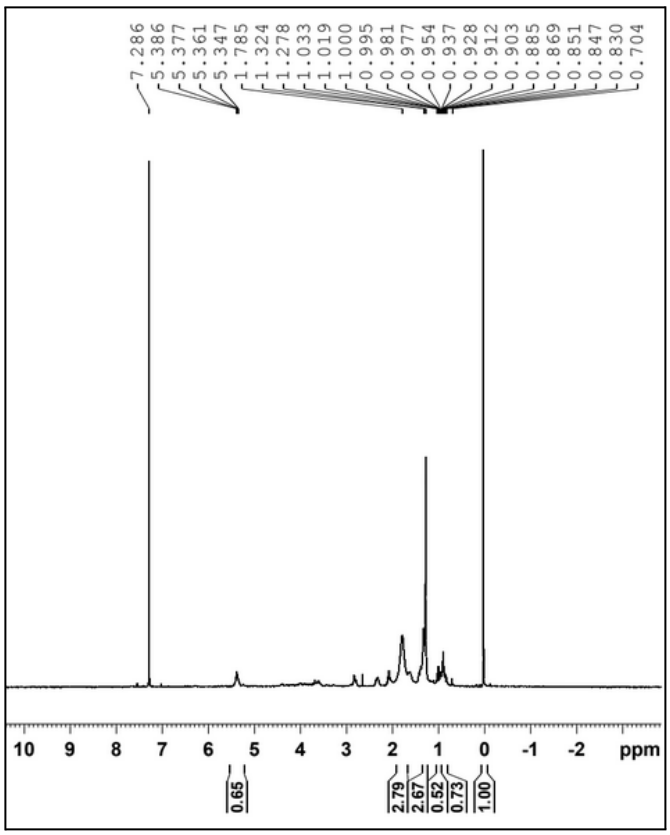

Figure 2. [1]H-NMR spectrum of organic extract.

The residue is completely soluble in DMSO but not in $CHCl_3$. We tested for solubility to decide the heavy solvent used for NMR spectroscopy. Similarly, the solubility of the dried residue from organic solvent was also tested for solubility with DMSO and $CHCl_3$ and found to be soluble in $CHCl_3$.

NMR Spectroscopy: NMR spectroscopy was performed as described previously [12]. Briefly, BRUKER Ascend 400 MHz magnet was used for the acquisition of [1]H-NMR spectra for both samples. The FIDs were deconvoluted and fourier transformed into the individual spectra using TopSpin software. The final spectra are shown in Figures 1 and 2.

Results and Discussion:

Proton NMR spectrum of aqueous extract contains only a few peaks: The proton NMR spectrum of aqueous extract, as shown in Figure 1, contains only three peaks at 3.49 ppm, 2.549 ppm and 2.510 ppm. These peaks are all in the aliphatic region suggesting the absence of any aromatic compounds containing benzene rings specifically. Previously AHPA was reported within *F. religiosa* aqueous leaf extracts.

However, the proton NMR spectrum shown in Figure 1 does not perfectly match AHPA predicted spectrum (Figure 3) from the NMRDB [13-15]. It is noteworthy that the aqueous extract was slightly warmed to dry so that it can be dissolved in heavy solvent for NMR analysis and this warming may cause changes in the AHPA, if at all it was present in the aqueous extract.

The proton NMR spectra show different profiles as expected: The two proton NMR spectra shown in Figures 1 and 2 show different profiles in the context of number of peaks and their positions (ppm) as expected. Normal boiling of the leaf in di-water may only selectively extract the ingredients that are hydrophilic and are easy to extract without damaging the leaf. This may include compounds (may or may not be volatile) that are naturally secreted or exuded by the leaf. Although the AHPA is completely water soluble, comparative analysis of Figures 1 and 3 suggest that the active ingredient that is extracted simply by boiling the leaf in di-water is not AHPA. However, the peaks obtained in Figure 1 are in the aliphatic region suggesting that they could be closely related analogs of AHPA. Along these lines, as shown in Figure 2, the multiple peaks obtained in the organic

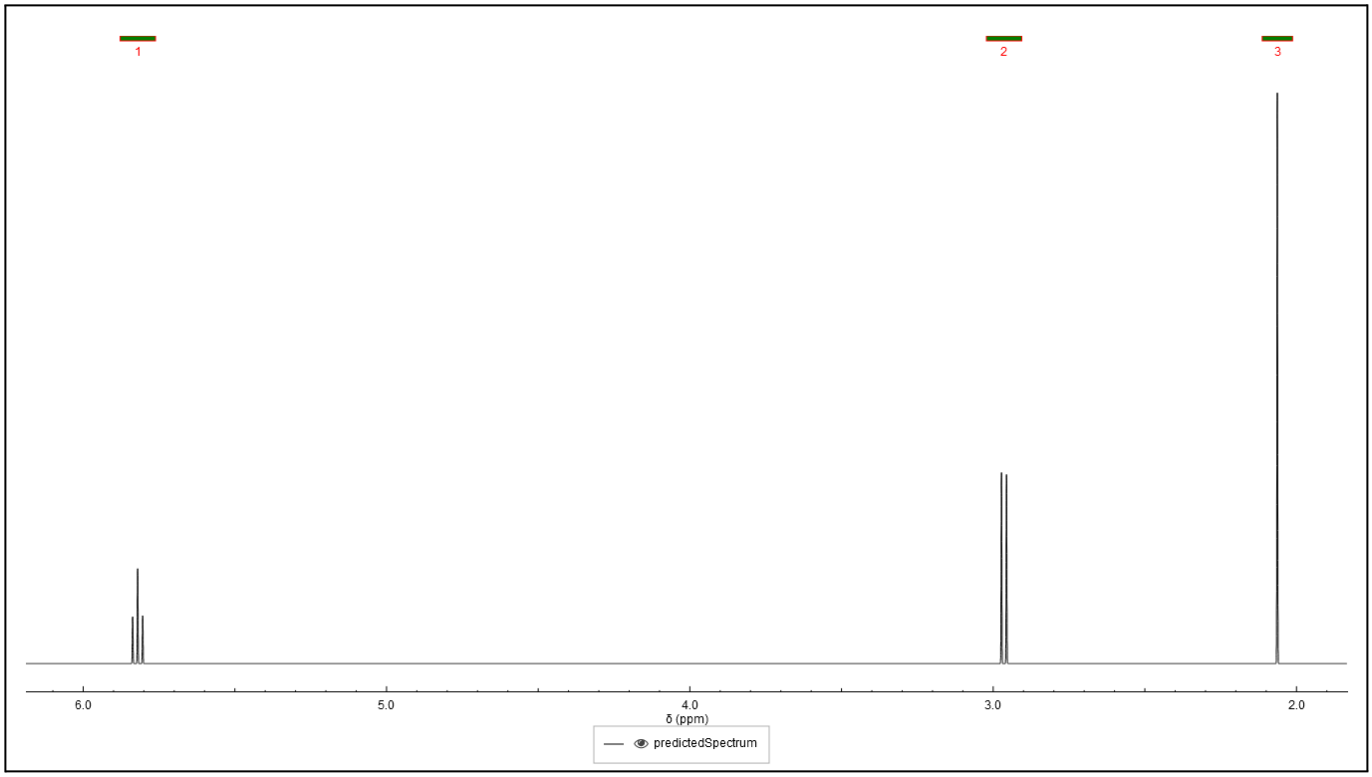

Figure 3. Predicted ^{1}H-NMR spectrum of AHPA.

solvent extract are mostly populated in the aliphatic region suggesting the closely related analogs of AHPA might be present in this extract.

Conclusion and Future directions:

The current study confirms that the AHPA that is believed to be the active ingredient in boiled leaf extract of *F. religiosa* that has anti-PCOS activity is absent based on the proton NMR spectrum. However, this does not conclude the complete absence of AHPA in *F. religiosa*. Maybe the extraction method is not just simply boiling the leaf in water.

Acknowledgements: We thank The Yedidi Institute of Discovery and Education, Toronto for scientific collaborations.

Conflict of interest: The authors declare no conflict of interest in this study. However, this research article is an ongoing project currently at TCABS-E, Visakhapatnam, India.

Author contributions: L.K. collected leaves, prepared aqueous and organic extracts, prepared NMR samples. M.S. and S.A. assisted L.K. in preparation of extracts and solubility tests. A.G. and J.C. assisted L.K. in preparation of NMR samples, data acquisition and analysis. R.S.Y. is the principal investigator who designed the project, trained L.K., M.S., S.A., A.G. and J.C., secured required material for the project, provided the laboratory space and facilities needed, wrote and edited the final version of the manuscript.

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Full figure legends:

Figure 1. ^{1}H-NMR spectrum of aqueous extract. A total of 3 peaks are seen in this spectrum and none of these peaks match the predicted spectrum of AHPA.

Figure 2. ^{1}H-NMR spectrum of organic extract. Multiple peaks are seen in this spectrum with most of them populated in the aliphatic region.

Figure 3. Predicted ^{1}H-NMR spectrum of AHPA. Peaks are seen at three different ppms. One singlet and one doublet are seen in the aliphatic region while one triplet is seen at 5.8 ppm.