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Eichhornia crassipes (Mart.) Solms. - An alternate renewable source for Shikimic acid, a precursor for Tamiflu, a swine flu drug

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Abstract
Shikimic acid is a lead compound in the manufacture of the drug oseltamivir phosphate, commercially known as Tamiflu®, which is an oral antiviral used to treat influenza viruses, as a H5N1(avian influenza) and a H1N1(swine influenza). The major bottleneck in Tamiflu production is the availability of shikimic acid. The only source for the extraction of shikimic acid is fruits of Chinese star anise, *Illicium verum* (Hook. f.). However, it takes almost six years after plantation to get fruits of star anise. Therefore, search of alternative source for shikimic acid is warranted. The water hyacinth, *Eichhornia crassipes* (Mart.) Solms. (Pontederiaceae) is considered as one of the most productive plants on earth, and an aquatic weed, which causes serious environmental problems. In this study, *E. crassipes* is considered as an alternative renewable source of shikimic acid since, n-hexane extract of leaves of *E. crassipes* was found to have more shikimic acid content (3.25%) than that of star anise (1.77%), hence, it may be considered as an alternate renewable source of shikimic acid.

Keywords: Shikimic acid, water hyacinth, *Eichhornia crassipes,* Tamiflu, Chinese star anise, and *Illicium verum*

1. Introduction
Shikimic acid is the key intermediate in the common pathway of aromatic amino acid biosynthesis (the shikimate pathway). The benzene ring, the basic unit of all aromatic compounds, is formed in plants and microorganisms through the shikimate pathway and the intermediate shikimic acid is an extremely essential compound in plants and microbes, is absent in mammals. It is the precursor for the synthesis of the drug oseltamivir (commercially called Tamiflu), an efficient inhibitor of the human influenza virus H1N1 of swine origin, seasonal influenza virus types A and B, and avian influenza virus H5N1 [1]. Currently, Roche produces majority of the world's supply of shikimic acid. Their method of extraction involves isolating the compound from Chinese star anise, *Illicium verum* (Hook.f.). In addition to being an inefficient extraction method, the harvest itself is labour intensive and highly polluting [1]. Furthermore, as demonstrated by the Tamiflu® shortages announced by Roche in 2005, a bad harvest will inevitably lead to mass shortages in drug supply [1]. Currently most of the world's demand for shikimic acid is met only from fruits of Chinese star anise [1]. Since, star anise is the primary source of shikimic acid, it takes almost six years after plantation to bear fruits. Efforts need to be made to explore alternate sources of shikimic acid to meet the demands of the world market. Therefore, we aimed at search of shikimic acid from Invasive Alien Weed in aquatic ecosystem, the water hyacinth, *Eichhornia crassipes* (Mart.) Solms. (Pontederiaceae) as an alternate renewable source of shikimic acid.

2. Materials and methods
2.1 Collection of plant material and extraction
Leaves of *Eichhornia crassipes* (Mart.) solms, (Pontederiaceae), were collected from Kurichi Kulam (Kurichi lake), Coimbatore city, Tamil Nādu, India. It is situated between 10°57'57.6" latitude and 76°57'48.96" longitude. Kurichi Kulam in Coimbatore city is one of the major water bodies enhances the ground water level around this area. It is often polluted due to invasion of alien weed, water hyacinth.

The Coimbatore city municipal corporation made efforts to remove them periodically with considerable budget expenditure. Hence, attempt was made to utilize it potentially an alternate source for shikimic acid.

The whole plant was removed from the lake and brought into the laboratory at Institute of Forest Genetics and Tree Breeding, Coimbatore, Tamilnadu. The root portion was cut off and the aerial parts of the plant were washed thoroughly under running tap water to free from debris. The leaves of the fresh plant material was chopped into small pieces and air dried. The aerial parts of the plant was dried, ground, extracted with methanol and n-hexane in heating mantle using soxhlet apparatus. After completion of extraction, the solvents were removed by distillation in rotary evaporator. The crude extracts were fractioned using methanol and n-Hexane. The extracts were concentrated to dryness at reduced pressure. Fractionates of the extracts were stored in refrigerator for further studies.

2.2. Isolation and identification of shikimic acid

2.2.1 Sample preparation

100mg of residue (plant extract) was mixed with 5ml of 0.04% of orthophosphoric acid followed by sonication. After sonication, the sample was evaporated to dryness at 40 ºc.10 ml of HPLC grade methanol was added to the sample and filtered through 0.45µm filter (c-18 solid phase extraction column), 20 µl were injected directly into HPLC.

2.2.2 HPLC analysis

HPLC analyses were performed on a system with Hitachi L-6200 intelligent pump equipped with a Hitachi L-4000 UV detector. The mobile phase was degassed in an ultrasonic cleaner 250W bath for 60 minutes prior to use.

HPLC analyses of the extracts were performed using 0.04% of orthophosphoric acid in water as solvent A with the pH of 2.8 and methanol as solvent B at a flow rate of 0.5mL/minute. The mode of operation was set to be isocratic. Detection wavelength was achieved at 210 nm. The identification of shikimic acid in the samples was based on comparison of its retention time and UV-spectra with those of the standard compound.

3. Result and discussion

In the present study, methanol and n-hexane extracts of leaves of *E. crassipes* were prepared using Soxhlet extraction method. The extracts were analyzed by HPLC. The identification of the peak corresponding to the compound shikimic acid in the samples was achieved comparing t_R and UV spectra with the standard compound (Fig. 1-4). A semi-quantitative analysis of these extracts data evidenced that the leaves of *E. crassipes* contain higher shikimic acid concentrations (1.45%-3.25%) than the *I verum* (1.77%); and n-hexane is a better solvent for extraction of shikimic acid than methanol (Fig. 5). The fast growth and high biomass production of *E. crassipes* may prove the use of *E. crassipes* more advantageous. It was reported that 0.03-2.7% of shikimic acid content in aerial parts of water hyacinth in Brazil [2]. However, in the present study, it is found that 3.27% of shikimic acid in leaves of water hyacinth. The content of shikimic acid in plant species varies and depends on the synthesis rate of aromatic amino acids [3]. In 2009, prospecting for alternate sources of shikimic acid from plants of the Western Ghats, a mega diversity hotspot in South India, was carried out [4]. Analysis was performed on 210 plant species, out of which a total of 193 angiosperms belonged to 59 families and 17 gymnosperms belonged to five families. The highest level of shikimic acid (5.02%) was found in *Araucaria excels* R.Br. belonging to the family Araucariaceae. The present study evidenced that *E. crassipes* is a promising source of shikimic acid since *E. crassipes* aerial parts yielded 3.25 percent of shikimic acid when compared to that of seasonal *Illicium verum* pods (1.77%). Moreover, the commercial use of this plant could be an alternative for the management of water hyacinth, contributing to solve environmental and economic problems caused by it.

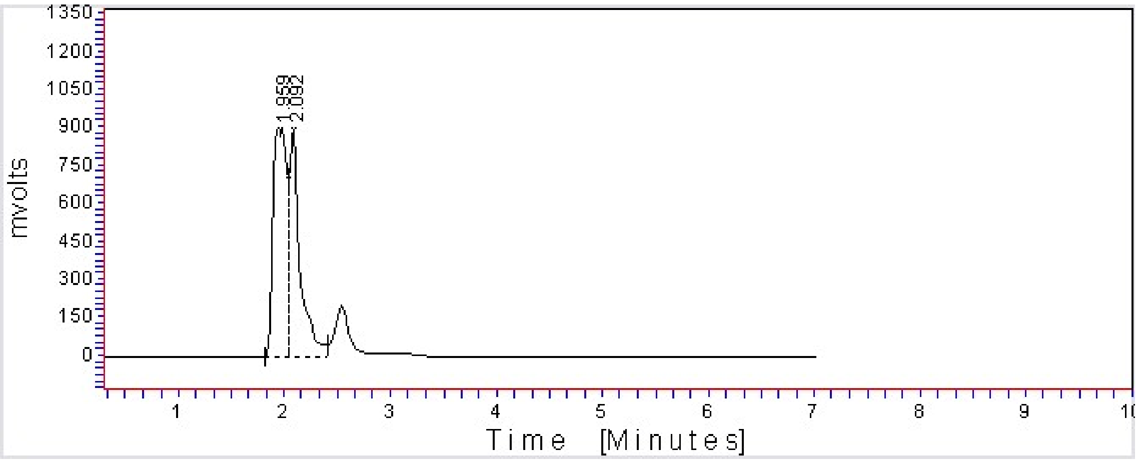

Fig 1: HPLC chromatogram of standard Shikimic acid

Peak No	Retn. Time	Area %	Height %	Name
1	1.958	56.381	50.078	-
2	2.092	43.619	49.922	Shikimic acid standard (10000ppm)
Total		100	100	

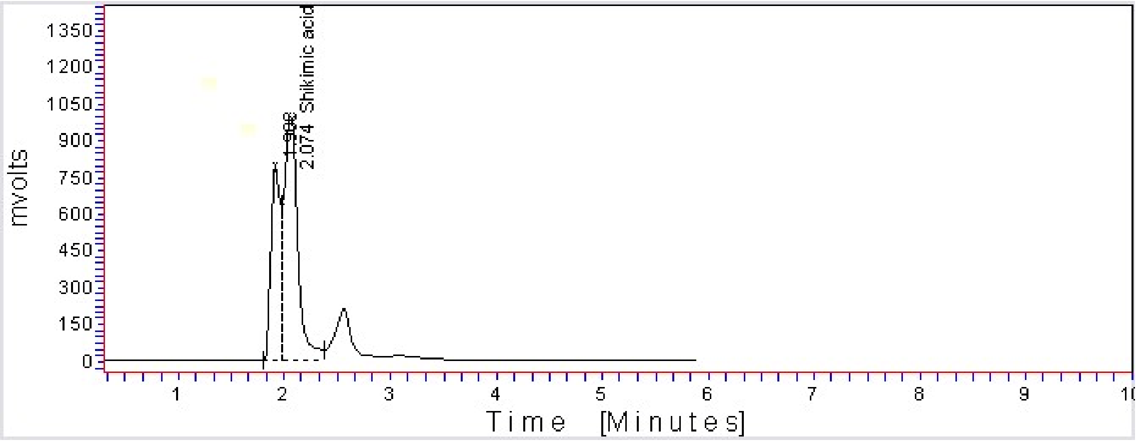

Fig 2: HPLC chromatogram of extract of fruits of *I. verum*

Peak No	Retn. Time	Area %	Height %	Name
1	1.908	30.019	44.733	-
2	2.074	69.981	55.267	Shikimic acid (*I. verum)*
Total		100	100	

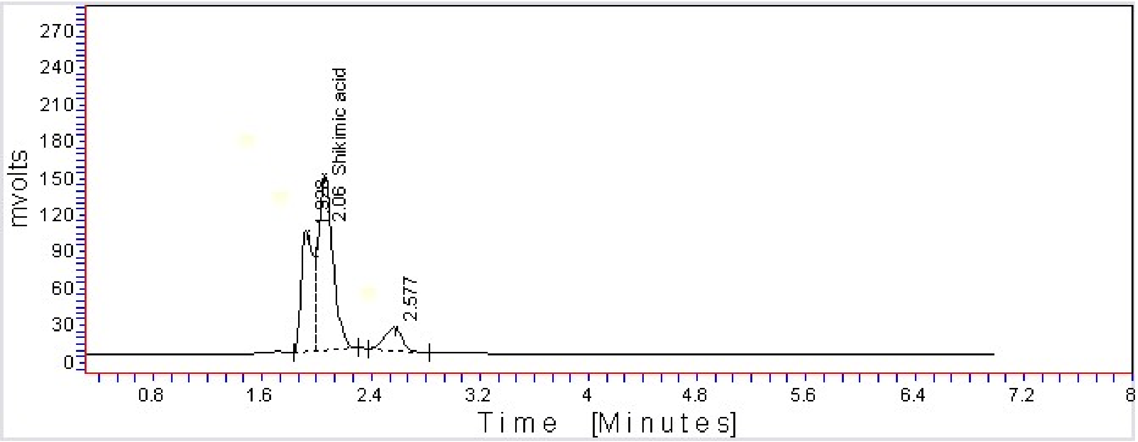

Fig 3: HPLC chromatogram of methanol extract of leaves of *E. crassipes*

Peak No	Retn. Time	Area %	Height %	Name
1	1.928	33.853	37.852	-
2	2.061	57.503	55.065	Shikimic acid (*E. crassipes* –Methanol extract)
3	2.577	8.644	7.083	-
Total		100	100	

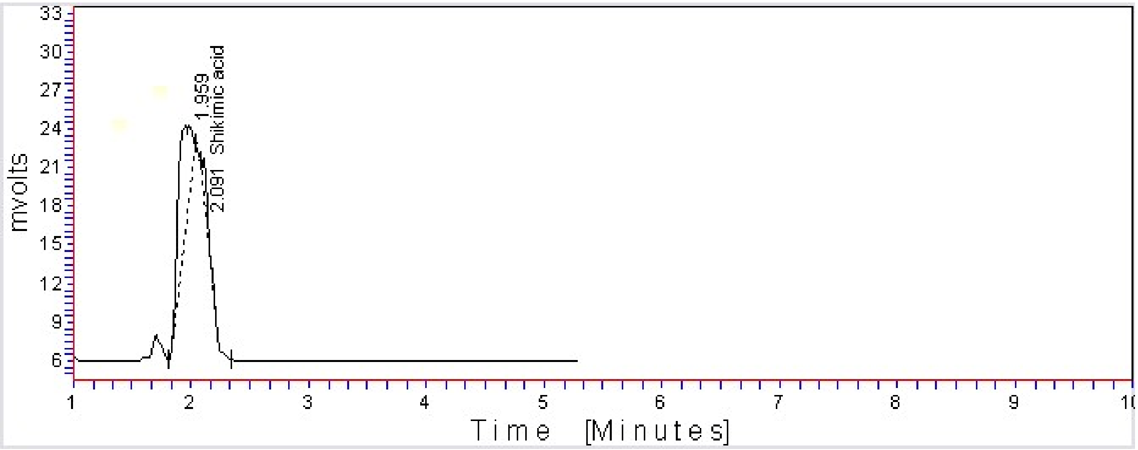

Fig 4: HPLC chromatogram of n hexane extract of leaves of *E. crassipes*

Peak No	Retn. Time	Area %	Height %	Name
1	1.959	14.044	17.644	-
2	2.092	85.956	82.356	Shikimic acid (*E. crassipes* –n hexane extract)
Total		100	100	

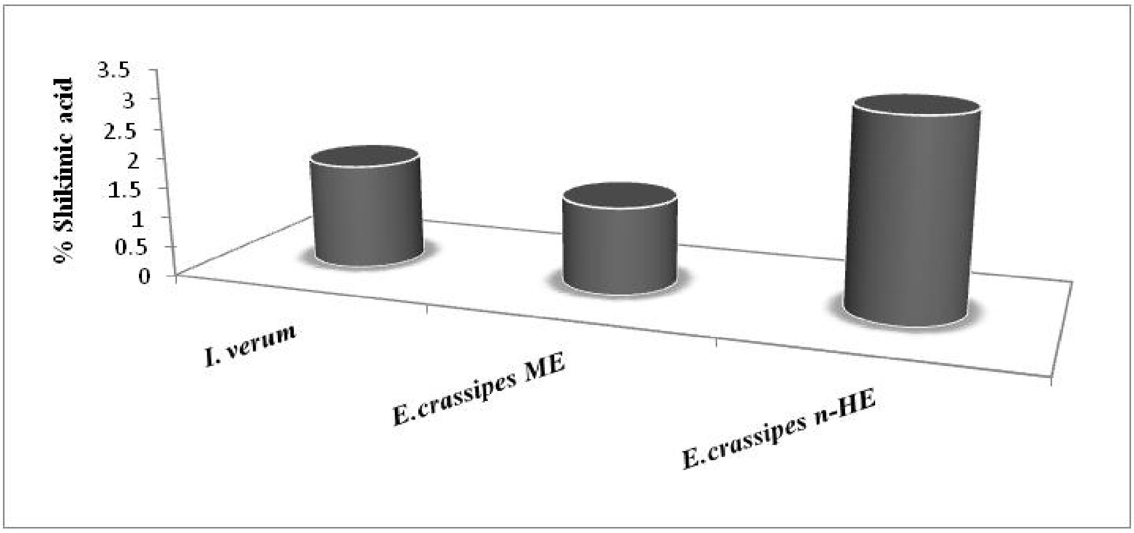

Fig 5: Shikimic acid content in star anise and water hyacinth

4. Conclusion

The shikimic acid is the key intermediate in the common pathway of aromatic amino acid biosynthesis. It is an economically important chiral compound used as a building block for the synthesis of different pharmaceutical products. However, the most important use of (–)-shikimic acid is as substrate for industrial synthesis of Tamiflu. Forecast by WHO pandemic influenza preparedness and response guidance (2014) suggests that threats of influenza pandemics will continue to emerge. Tamiflu is the only orally administered approved drug for treatment of influenza. Unfortunately, currently available Tamiflu is sufficient only for 2% of the world population. Hence, more attention is being paid for the development of efficient technologies for the production of shikimic acid as well as to find alternative sources of shikimic acid. It is evident from the present study that *E. crassipes* is a promising alternate renewable source of shikimic acid since *E. crassipes* aerial parts yielded 3.25 percent of shikimic acid when compared to that of seasonal *Illicium verum* pods (1.77%). Moreover, the commercial use of this plant could be an alternative for the management of water hyacinth, contributing to solve environmental and economic problems caused by it.

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