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# Detection of flavones in plants using a PCR based approach

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**ABSTRACT**

*Therapeutic molecules from natural sources were identified mainly through conventional extraction/analysis procedure. In this study we used a PCR method to predict the presence of an end product in the metabolic pathway of flavone biosynthesis. Specific primers designed for chalcone synthase (CHS) (PCR control), 18S rRNA (template control) and flavone synthase (FNS) (target gene) were used to deduce the presence of flavones in the plants (Asparagus, Tea and different parts of lemon) studied. Results obtained by molecular method were comparable with the biochemical method. We propose that, this technique possesses great potential to be used with much sensitive realtime PCR to quantify minute amounts of secondary metabolites in unexplored plants and to foresee whether it is worthwhile to explore the possibilities of large scale extraction.*

**Key words**: Therapeutic molecule, Polymerase chain reaction, metabolic pathways, flavanoid biosynthesis.

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## INTRODUCTION

Conventional way to identify plant based therapeutic molecule follows, laborious, elaborated and systematic modus operandi. The main drawbacks of these routine methods are; time consuming, less accurate and various biochemical analysis and standards are required to arrive at a conclusion [1]. More over, these conventional biochemical analyses will be confirmative only if the amount of target molecule is detectable by the biochemical test used. The expected negative outcome is that, the raw material which is available in plenty may be missed out as the amount of target molecule is not detectable. At present there are synthetic compound libraries serve as source of active molecules [2]. But, natural products continue to provide greater structural diversity than the molecules generated through combinatorial chemistry and offer major opportunities for finding novel lead structures, active against a wide range of assay targets [3]. As less than 10% of the world's biodiversity has been tested for biological activity, many more useful natural lead compounds are awaiting discovery. The PCR based approach we propose uses the concept that, the transcript levels of crucial enzymes in each biosynthesis pathway could be used as an indicator for the presence/amount of the end product, ie; secondary metabolite.

Metabolic pathways are highly conserved among different groups of plants and the enzymes catalyzing different steps in different pathways are highly specific. All flavonoids derive their 15-carbon skeletons from two basic metabolites, malonyl-CoA and *p*-coumaroyl- CoA. Basically, flavonoids are derivatives of 1,3-diphenylpropan- 1-one (C6–C3–C6). The crucial biosynthetic reaction is the condensation of three molecules malonyl- CoA with one molecule *p*-coumaroyl-CoA to form a chalcone intermediate. Chalcones and dihydrochalcones are classes of flavonoids that consist of two phenolic groups which are connected by an open three carbon bridge [4]. Chalcone synthase (CHS) is the key enzyme and entry point into flavonoid biosynthesis [5]. The metabolic pathway after the

enzymatic reaction of chalcone synthase continues through a series of enzymatic modifications to yield various flavanoids (Figure-1). Flavone synthase (FNS) facilitate the control of a biosynthetic step at an important junction of flavonoid biosynthesis pathway leading to various flavonoids classes, such as flavones, isoflavones, flavonols, flvanols, and anthocyanins [4]. FNS converts flavanones to flavones by introducing a double bond between second and third carbon atom [6]

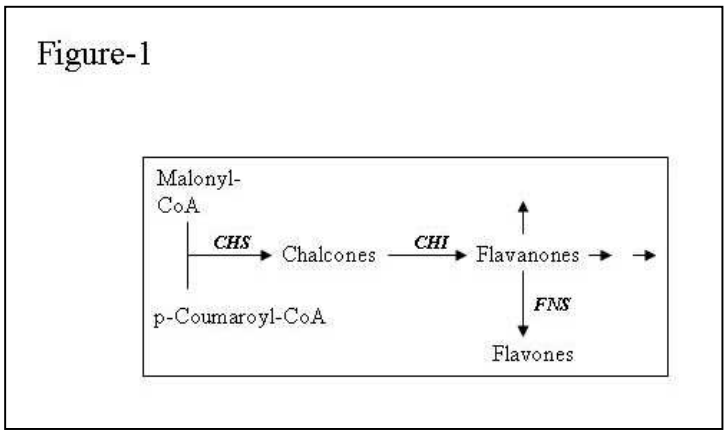


**Figure-1 Biosynthesis pathway of flavones**
*CHS-Chalcone synthase, CHI-Chalcone isomerase, FNS-Flavone synthase*

In this study, we have analyzed the possibility to predict whether the end product of a secondary metabolite biosynthesis pathway (flavons) is present or not by analyzing the expression profile of the enzyme concerned in the biosynthesis (FNS) using a PCR based approach. This method is found to be discriminating well between different plants/tissues in terms of the presence of flavone by analyzing the expression of its biosynthetic enzyme- FNS.

## MATERIALS AND METHODS

*Plant materials:*
The plants/plant parts of Asparagus, Lemon and Tea were collected from the area in and around Vellore, Tamilnadu, India. The plant parts were washed thoroughly in tap water followed by sterile distilled water. A part of the material was used for RNA extraction/cDNA synthesis and the other part was used for biochemical assays.

*RNA preparation and cDNA synthesis:*
100mg of tissue was ground to powder using liquid nitrogen in a motor. The ground material was transferred into a fresh, autoclaved eppendorf tube and the RNA was extracted using plant RNA isolation kit (Himedia Laboratories, India). cDNA was synthesized using cDNA synthesis kit (Promega Corporation, USA).

*Designing of primers:*
Primers used in the study were designed from the conserved region of chalcone synthase and flavone synthase cDNAs from 6 eukaryotic plants. The cDNA sequences each for flavone synthase and chalcone synthase (Flavone synthase (*Petroselinum crispum* Acc. No. AY230247, *Cuminum cyminum* Acc. No. DQ683349, *Apium graveolens* Acc. No. AY817676, *Aethusa cynapium* Acc. No. DQ683350, *Daucus carota* Acc. No. AY817675 and *Angelica archangelica* Acc. No. DQ683352), Chalcone synthase (*Arabidopsis thaliana* Acc. No. AY044331, *Senna tora* Acc. No. EU430077, *Malus x domestica* Acc. No. EU872158, *Rudbeckia hirta* Acc. No. EF070339, *Bromheadia finlaysoniana* Acc. No. AF007098 and *Vitis rotundifolia*, Acc. No. FJ644942) were selected and multiple sequence alignment was performed using *clustalW* at ebi.ac.uk. The primers (FNS-F 5'- GGGGCATTTTTCAGGT -3', FNS-R 5'- GCACGGTAATCCAGGTT- 3', CHS-F 5'- G GTGGTGGAAGTGCCGA -3', CHS-R 5'- GCAGCAGATGAAAGGTC- 3', 18S-F 5'- GTGACGGAGAATTAGGGTTCG-3' and 18S-R 5'- ATCGCCGGCAGAAGGGACG A GA- 3') designed from the most conserved sequence of cDNAs were used in the study.

*Densitometry analysis:*
The intensity of the PCR amplicons was analyzed using the programme, Quantity 1 (Biorad) and used to compare the extent of expression of target genes from various sources.

Estimation of total flavonoids: 1g each of lemon leaf, peal, bark and root, tea leaf and asparagus leaf were extracted with 5ml of 80% ethanol, centrifuged at 7000 rpm for 40 minutes and collected the supernatant. The total flavonoid content present in each extract was determined by aluminium chloride colorimetric assay [7]. Quercetin was used as the standard for calibration curve. Total flavonoid content of the extract was expressed as mg of quercetin equivalent in per gram of sample.

## RESULTS AND DISCUSSION

The conventional method to screen for active biomolecues from a plant source follows the pathway of extraction, purification and biochemical analysis [3]. The main drawbacks of these routine methods are; time consuming, less accurate and various biochemical analysis and standards are required to arrive at a conclusion about its biochemical and functional properties [1]. More over, the conventional biochemical analysis will be confirmative only if the amount of the target molecule is detectable by the biochemical test used.

We propose that, if the transcript level of an enzyme, which catalyzes the biosynthesis pathway of a therapeutic molecule is more, there is more possibility that the target molecule concentration also will be high in that plant. The flavone biosynthesis pathway selected to prove this hypothesis was analyzed for the presence of flavone synthase (target gene) transcript level. The constitutively expressing enzyme, chalcone synthase was used as the control. Expression of chalcone synthase and flavone synthase in various parts of lemon like leaf, peel, bark and root, asparagus leaf, and tea leaves was analysed with specific primers for chalcone synthase and flavone synthase. Amplification of 700bp band indicates the presence of expression of chalcone synthase and 500bp indicates the presence of expression of flavone synthse in the samples.

As the first step, cDNA samples were normalized for concentration with 18S rRNA specific primers and the experimental PCR was performed with the normalized cDNA samples in an Eppendorf thermal cycler (Eppendorf, USA) with an annealing temperature of 47ºC for chalcone synthase primers (Figure 2A) and 51ºC for flavone synthase primers (Figure 2B). The PCR amplicons (1kb corresponds to CHS and 0.7kb corresponds to FNS) were resolved on a 1.5% of agarose gel. The normalized cDNA amplified more or less same intensity amplicons when used with CHS primers, but the intensity of the PCR amplifications varied considerably with the FNS primers. The difference was distinct for FNS in the lemon samples used.

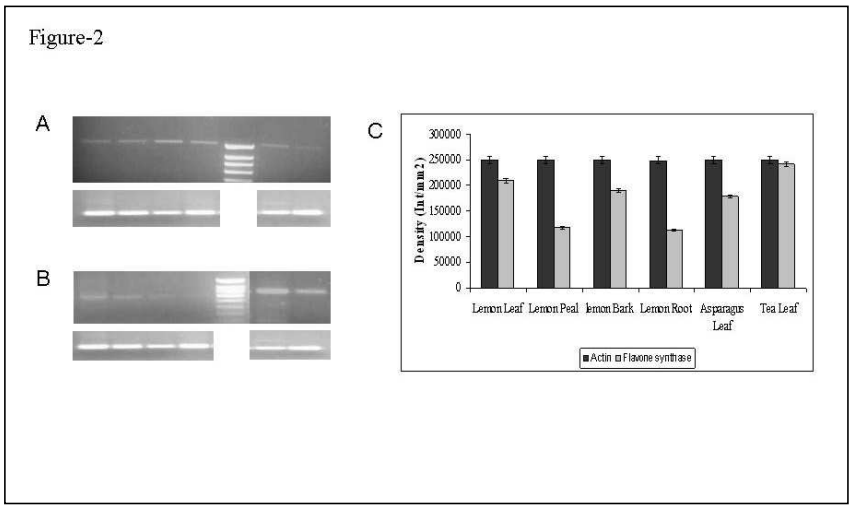


**Figure-2 Detection of Flavones in different plants/parts by molecular methods**

*(A) PCR amplification of CHS and (B) PCR amplification of FNS in different experimental materials used (Lane 1-lemon leaf, lane 2-lemon bark, lane 3- lemon peal, lane 4 lemon root, Lane 5-100bp marker, lane 6 Asparagus leaf, lane-7 Tea leaf). (C) Densitogram of PCR amplifications.*

The intensity of PCR amplicons of FNS were in an ascending order of root, peel, bark and leaf. Two other samples, tea and Asparagus used in the study showed almost similar intensity for the PCR Amplicon. We have taken it as an indication of the expression level of the enzymes concerned and propose that, this may be used to predict whether flavons are synthesized/present in the plants/parts studied.

In order to prove the hypothesis, we have analyzed the flavanoid content by biochemical methods given in the methods. The biochemical estimations showed that, the total flavonoid content of tea leaf was highest followed by lemon leaf, bark and asparagus leaf (Figure-3). There were very little amount of flavonoids estimated in the biochemical methods in root and peel of lemon. The minor mismatches present between the experiments could be because of the fact that, the biochemical estimation targeted the whole range of flavanoids but the PCR was specific to flavones. This makes it more significant as the protocol could be defined according to the end product.

Figure- 3

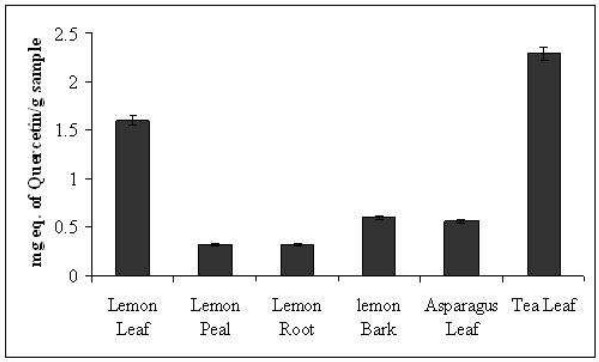


**Figure-3 Estimation of total flavanoids using the biochemical methods**
*Values are the average of three independent experiments with SD.*

In most of the cases, the transcript levels indirectly predict the level of proteins in a living system. In order to find an application for this phenomenon, a PCR based analysis has been developed, which can predict the occurrence of the final products in a metabolic pathway. Compared to the conventional methods, this approach is fast, time saving and cheap. The commercial exploitation of this method will yield defined set of primers to screen for specific end products in plants.

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