Determination of Oligomeric Proanthocyanidins (OPCs) from *Tamarindus indica* Seed Coat (of Indian origin) using LC/MS/MS

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1: Introduction

1-1: *Tamarindus indica*

Tamarindus indica is used in Indian traditional medicines as blood and skin cleanser and for treatment of stomach disorders, peptic ulcers, jaundice, yellow fever etc. The seed (shown in Figure 1a) consists of 30% hard brown seed coat (as shown in Figure 1b) and 70% kernel (as shown in Figure 1c). The kernels contain polysaccharides (jellose) having very good sizing property and employed as a sizing agent for jute and textile yarns[(1),(2)].

a. Whole seeds b. Seed coat c. Kernel

Figure 1. Seeds of *Tamarindus indica*

Classification of *Tamarindus indica*

Kingdom	: Plantae
Division	: Magnoliophyta
Class	: Magnoliopsida
Order	: Fabales
Family	: Fabaceae
Subfamily	: Caesalpinioideae
Tribe	: Detarieae
Genus	: *Tamarindus*
Species	: *indica*

Proanthocyanidins refer to a larger class of polyphenols called as flavanols. Oligomeric Proanthocyanidins (OPCs) are the simplest flavanols belonging to this group. More complex polyphenols, having the same polymeric building block, form the group of tannins.

1-2 : Oligomeric proanthocyanidins (OPCs)

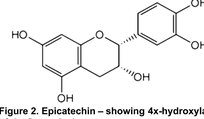


Figure 2. Epicatechin – showing 4x-hydroxylation of the flavan-3-ol core.

Colorless OPCs are a strictly defined group of 3 flavanol naturally occurring as a mix of monomers, dimers, and trimers of the catechin building block, which is a 4x-hydroxylation of the flavan-3-ol core (shown in Figure 2). These procyanidins are formed from the condensation of monomeric units. They differ in their position and configuration of their monomeric linkages, with the dimers B1, B2, B3 and B4 being most

OPCs have been shown to have powerful "free-radical" scavenging activity. They are non toxic, bioavailable and water soluble. Oligomeric Proanthocyanidins (OPCs) have other beneficial effects such as helping reduce inflammation in the tissues and stabilizing the walls of different blood vessels in the body. OPCs also positively affect the proteins found in cartilage, in the tendons and in the blood vessels. OPCs aid muscle growth and maintenance and also keep skin young and elastic. OPCs supplements have also been used in alleviating high blood pressure and decreasing the risk of stroke. OPCs can also be used in treating swollen ankles, cuts and scrapes as well as bruises. They have also been used to treat lymphedema, nosebleed, tendinitis, and varicose veins.

Seed coats of this plant are discarded as waste after removal of the kernels from the seeds. It was, therefore, decided to use this waste product as a raw material for extraction of OPCs. OPCs from the seed coat of *Tamarindus indica* from India were extracted and their presence was confirmed using HPLC and LC/MS/MS. They were tested further for their antimicrobial activity as described ahead.

2: Method of Analysis

2-1: Extraction of OPCs from *Tamarindus indica* seed coat

Tamarindus indica seeds were collected from Virar area in the state of Maharashtra in India. The seed coats were separated from the kernels by roasting the seeds and breaking the seeds before cooling down. The seed coats were then ground finely using a mixer. To 10g of the ground seed coat powder 100mL of distilled water was added (1:10 w/v). This mixture was heated for 1hour to produce an aqueous solution containing all proanthocyanidins. The solution was cooled and filtered using muslin cloth to remove the residual solid material. This filtrate was further subjected to Solid Phase Extraction (SPE) technique to extract OPCs as described in the following flowchart.[3]

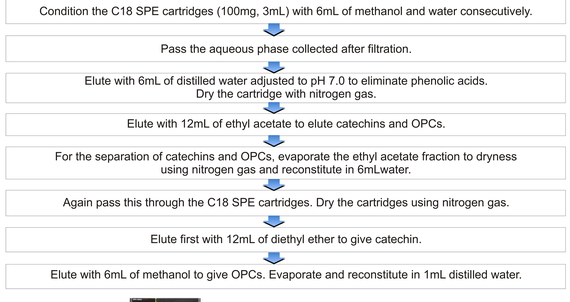


Figure 3. LCMS-8030 triple quadrupole mass spectrometer by Shimadzu

2-2: LC/MS/MS analysis

The extract was analyzed by LC/MS/MS triple quadrupole system (shown in Figure 3) for identification and confirmation of OPCs. The analytical conditions are as follows:

- Column : Shim-pack XR ODS II (100mm L x 3mm I.D. x 2.2 µm)
- Flow rate : 0.4mL/min
- Oven temperature : 40ºC
- Detector : UV detector at 280nm
- Mobile phase : A: Water containing 1% acetonitrile and 0.1% formic acid
  B: Acetonitrile
- Gradient program (%B) : 0 - 5min -> 3 - 9%; 5 - 15min -> 9 - 16%;
  15 - 25min -> 16%; 25 - 26min -> 16 - 3%
- Injection volume : 20µL
- MS interface : Electro spray Ionization (ESI)
- Nitrogen gas flow : Nebulizing gas 3L/min; Drying gas 15L/min
- MS temperature : Desolvation line 250°C; Heat Block 400°C

2-3: Antimicrobial Sensitivity Testing (AST)

The aqueous extract of OPCs obtained from *Tamarindus indica* seedcoat was subjected to AST to qualitatively confirm the antimicrobial activity.

Two methods[4] were adopted for AST which are described below:

a) Antibiotic Sensitivity Test by Agar Ditch Method for insoluble compound

Four bacterial cultures were used for this method namely *Staphylococcus aureus* ATCC 6538 P and *Bacillus subtilis* as representative Gram positive bacteria and *Escherichia coli* and *Salmonella typhi* as representative Gram negative bacteria. All the cultures were prepared in 0.85% sterile saline with optical density (O.D.) of the culture solutions adjusted colorimetrically to 0.1AU at 540nm.

A ditch of the dimensions 6cm x 1cm was made on Muller-Hinton (MH) agar (Himedia). 100mg of the seed coat powder was then mixed with 5mL of sterile molten MH agar butt and poured into the ditch and allowed to set. Each of the above mentioned culture suspension were streaked onto the set agar plate perpendicular to the ditch ensuring that streaking is done on the ditch. The plate was incubated at 37°C for 24 hours .

b) Antibiotic Sensitivity Test by Agar cup Method for soluble compound

Based on the results obtained by the above method, the agar cup method was carried out using *Staphylococcus aureus* ATCC 6538 P culture (O.D. adjusted to 0.1AU at 540nm).

0.625mL of this culture suspension was mixed with 25mL of sterile molten MH agar butt and poured onto petridish to make an agar plate.

Once the agar was cooled and set, the plate was divided into four quadrants and a well was dug in each quadrant using a sterile cork borer. Samples namely blank (distilled water), crude aqueous seed coat extract and aqueous OPCs extract introduced in the wells.

The plates were then refrigerated at 4°C for 20min for prediffusion and then incubated at 37°C for 18 hours.

3: Results

3-1: LC/MS/MS results

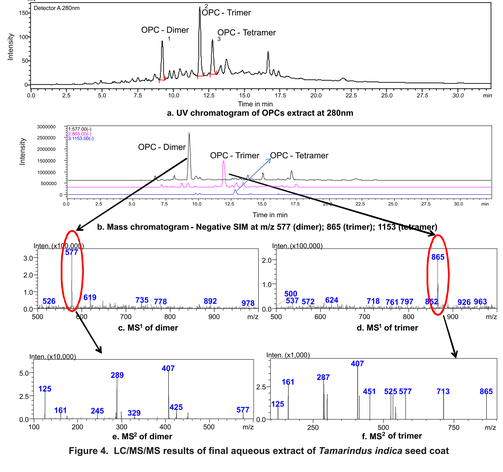


Figure 4. LC/MS/MS results of final aqueous extract of *Tamarindus indica* seed coat

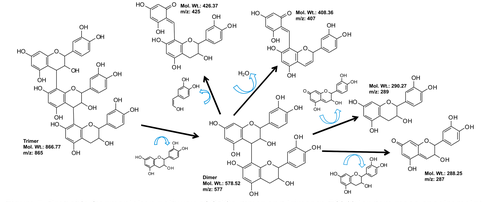
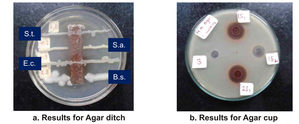


Figure 5. Probable fragmentation pathway of OPCs dimer and trimer in ESI (-) tandem mass spectrometry[5]

The aqueous extracts obtained from *Tamarindus indica* seed coats were subjected to LC/MS/MS for identification. The UV chromatographic profile and mass chromatograms are shown in Figures 4a and 4b respectively. In ESI negative ionization mode, m/z 577 for dimer and 865 for trimer were observed (shown in Figures 4c and 4d respectively). Their MS^2 product ion scan spectra shown in Figures 4e and 4f respectively provided further confirmation. The m/z of 1153 (shown in Figure 4b) indicates the likely presence of tetramer units as well. The probable fragmentation pathway of OPCs dimer and trimer has been elucidated in Figure 5.

3-2: Antimicrobial Sensitivity Testing (AST) results



a. Results for Agar ditch b. Results for Agar cup

Figure 6. Results for AST of *Tamarindus indica* seed coat extracts

S.a. - *Staphylococcus aureus* ATCC 6538 P; S.t. - *Salmonella typhi*
E.c. - *Escherichia coli* ; B.s. – *Bacillus subtilis*
B - Blank
1S, 2S, 1S, - Crude aqueous extract
- OPCs aqueous extract

Absence of growth on the agar ditch (shown in Figure 6a) indicates that the seed coat powder inhibits the growth of *Staphylococcus aureus* 6538 P and *Bacillus subtilis* (representative Gram positive organisms).

In the Agar cup method, zone of inhibition of microbial growth (shown in Figure 6b) is not observed around the blank water (B) but is seen around the crude aqueous extract and around the OPCs aqueous extract. This shows that the extract has antimicrobial activity.

4: Conclusion

- The MS^1 and MS^2 results obtained from LC/MS/MS triple quadrupole system, helped in confirming the presence OPCs in *Tamarindus indica* seed coat even in the absence of standards.
- In antimicrobial sensitivity testing, the aqueous extract showed inhibition to the growth of Gram positive organisms whereas Gram negative organisms were unaffected. This indicates a narrow spectrum antimicrobial activity confined to Gram positive bacteria.
- The crude aqueous extract showed higher zone of inhibition than the final OPCs aqueous extract. Therefore, one may also conclude that in addition to OPCs, there is a synergistic action of other components that aid in antimicrobial activity exhibited by *Tamarindus indica* seed coat.

5: References

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