CHAPTER 7

7. Isolation & Characterization of Bio-active Markers from the Selected Plants

Isolation of bio-active markers from the extracts of the active compositions was carried out using various techniques such as column chromatography, preparative Thin layer chromatography (prep-TLC) and semi-preparative-HPLC. Desired bio-active markers to be isolated depended on their stability in *in vitro* simulated gastric fluid (details discussed in chapter 8). The bio-active markers were identified using HPLC (R_t and λ_{max}) and characterized using NMR spectroscopy, mass spectromtery or by comparison with the laboratory standard.

7.1 *Berberis aristata* DC

B. aristata (also known as Indian barberry) belonging to the family Berberidaceae, is a spinous shrub found in Northern Himalayan region (**Figure 42**). It grows at the height of 2000-3000m especially in Kumaon and Chamba region of Himachal Pradesh. It is also found in Nilgiris hills in South India[1].

Taxonomical classification
Kingdom: Plantae
Division: Magnoliophyta
Class: Magnoliopsida
Order: Ranunculales
Family: Berberidaceae
Genus: *Berberis*
Species: *aristata* DC

Figure 42: Taxonomical classification of *B. aristata*

Extract of *B. aristata* is used for cholagogue, antidiarrheal, stomachic, laxative, diaphoretic, antipyretic and antiseptic properties. It is used externally in conditions such as ophthalmia, conjunctivitis, ulcers, sores and swollen gums. It is also used in liver complaints, diarrhoea, dysentery, cholera, gastric disorders, enlargement of spleen and for regulating metabolism. Its root bark has shown anti-inflammatory, hypoglycemic, hypotensive, antiamoebic, anticoagulant, and antibacterial activities [2]. In *Ayurvedic* literature roots, stem and fruits of *B. aristata* are reported to have anti-obesity potential [3]

The roots of *B. aristata* contain isoquinoline alkaloids such as, berbamine (24), berberine (25), oxycanthine (26), epiberberine (27), palmatine (28), karachine (29), taximaline (30), oxyberberine (31), pakistanine (32) as its main constituents (**Figure 43**) [1]. The major alkaloids found in *B. aristata* are berberine followed by palmatine [4].

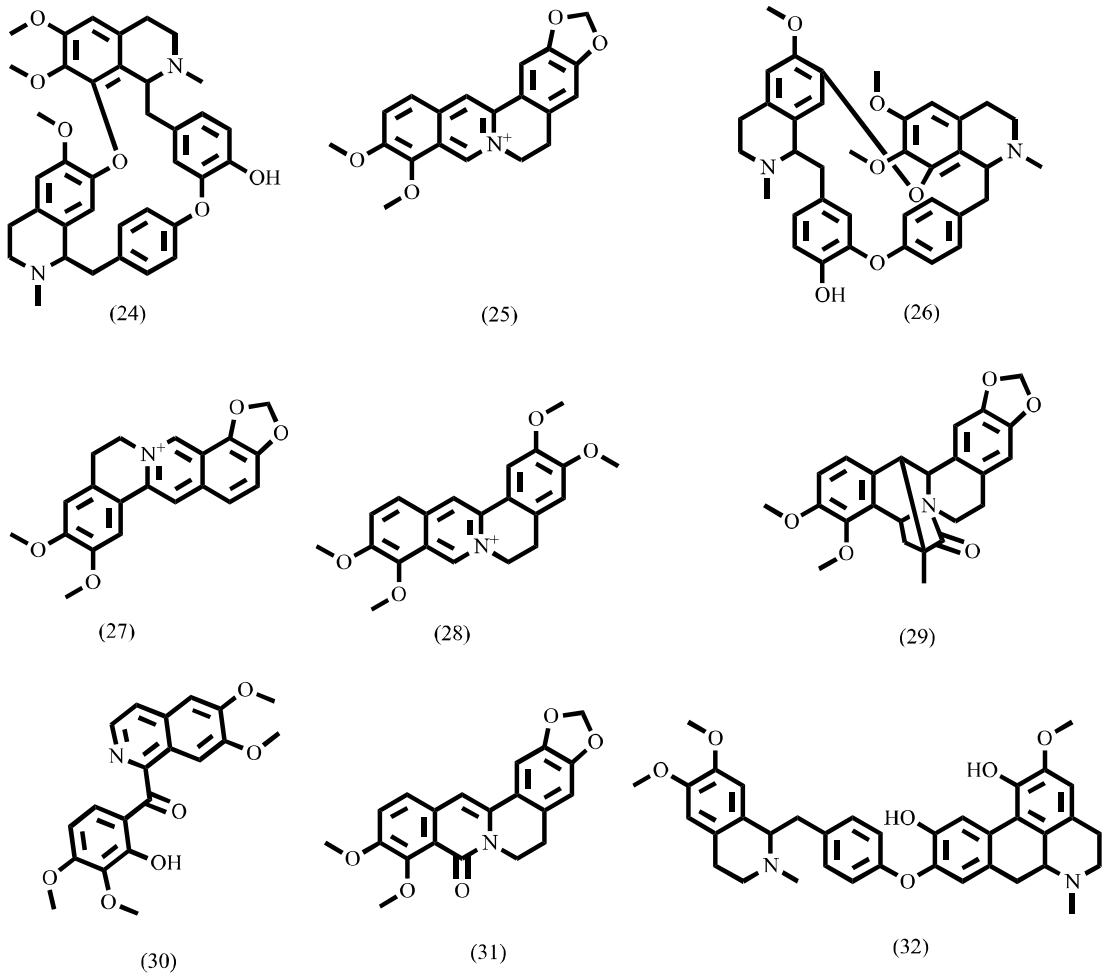


Figure 43: Chemical constituents (24-32) present in roots of *B. aristata*

7.2 *Gymnema sylvestre* (Retz.) R.Br.

G. sylvestre (Retz.) R.Br. is a medicinal herb belonging to the Asclepiadaceae family. It is native to India and Southern China **(Figure 44)**. It is a potent antidiabetic plant and used in *Ayurvedic* system of medicine. It is also used in the treatment of asthma, eye complaints, inflammation, family

planning and snakebite. In addition, it possesses antimicrobial, antihypercholesterolemic and hepatoprotective activities[5].

Taxonomical classification
Kingdom: Plantae
Division: Tracheophyta
Class: Magnoliopsida
Order: Gentianales
Family: Apocynaceae
Genus: *Gymnema*
Species: *sylvestre* R. Br

Figure 44: Taxonomical classification of *G. sylvestre* (Courtesy: https://www.pioneerherbal.com)

The leaf of this plant is used medicinally. The key constituents of *Gymnema* include saponins called gymnemic acids. Gymnemic acids are glycosides of triterpene that suppress sweetness in humans. Hence, this plant is mostly used for diabetes mellitus as it reduces the intake of carbohydrate by blocking the sensation of taste buds [6,7]. Currently, gymnemic acids are being sold in the form of *Gymnema* tea, for curbing obesity. In Japan, there are teas being made from *G. sylvestre* leaves and are being promoted for controlling obesity and diabetes [5].

Several gymnemic acid homologues with different acyl groups have been purified from the leaves of *G. sylvestre* and their structures have been determined. There are various triterpenes glycosides that exists along with a 35 amino acid peptide named gurmarin [8]. The gymnemic acids comprise of several members designated as gymnemic acids I**–**IV (33), gymnemosides A**–**F (34-39), and gymnemasaponins such as gymnemagenin (40) and gymnestrogenin (41) **(Figure 45)**[9].Gymnemic acid contains the acyl group that is responsible for this anti-sweet activity, which was found to be depleted on deletion of this acyl group [10]**.**

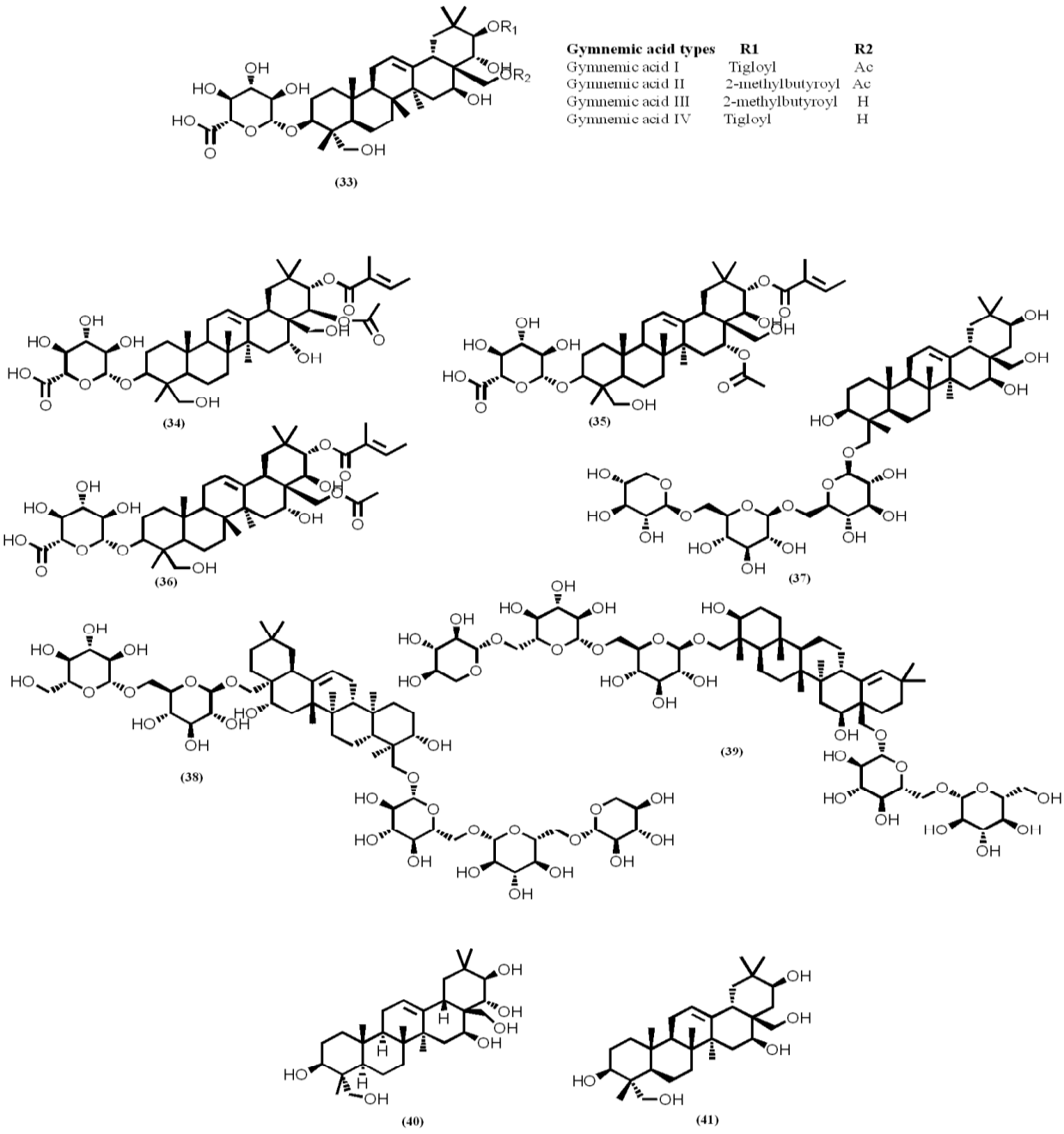


Figure 45: Chemical constituents (33-41) present in leaves of *G. sylvestre*

Gymnemic acids are not available commercially. Therefore, direct quantitative estimation of gymnemic acids is difficult as they easily hydrolyze to their respective gymnemasaponins **(Figure 46)**[11].

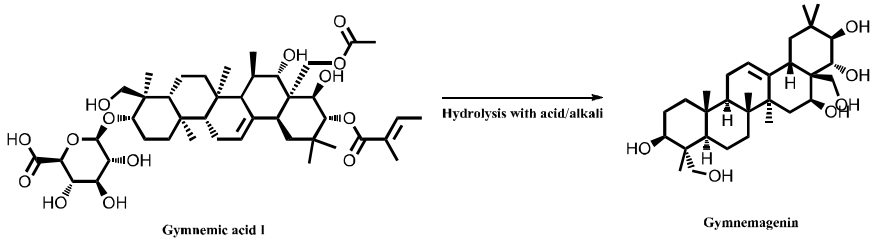
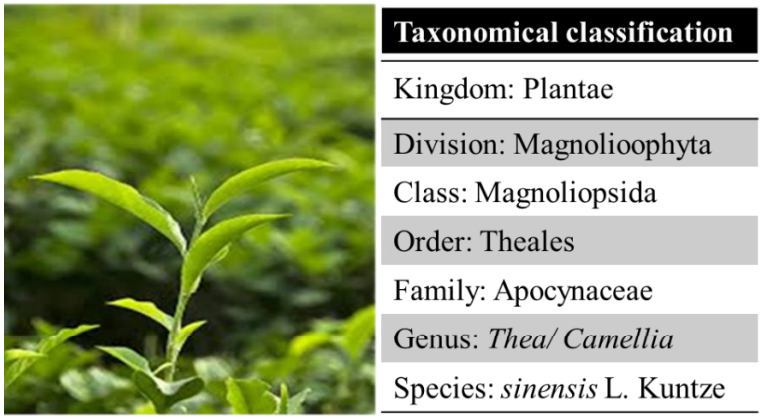


Figure 46: Hydrolysable product (gymnemagenin) from Gymnemic acid I

7.3 *Thea sinensis* (L.) O. Kuntze

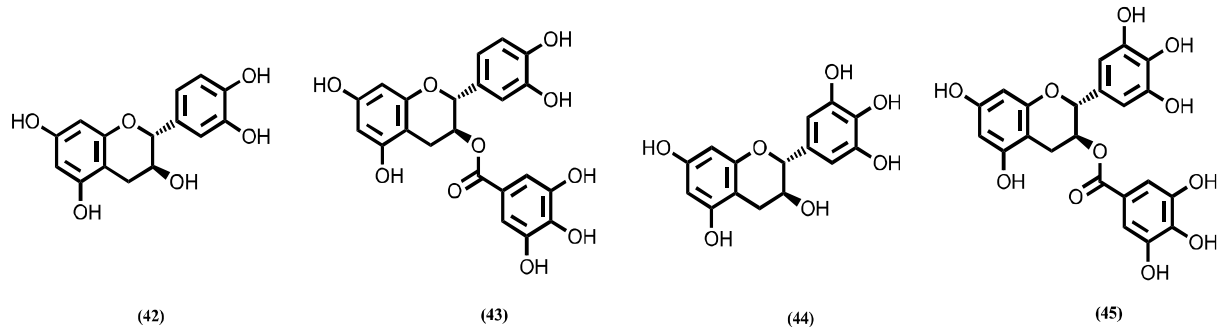
T. sinensis or *C. sinensis* (green tea) belonging to the family Theaceae has been cultivated in China for more than 2700 years (**Figure 47**). In India, the first Chinese tea was cultivated in the 19th century in the region of Darjeeling. It is a shrub that grows to a maximum height of 3 to 4 meters [12]. It is especially suitable for medium climatic zones and can even tolerates frost. Green tea contains caffeine and antioxidant polyphenols. It is useful in a wide variety of conditions, including cancer prevention, cardiovascular disorders and AIDS [13].



Taxonomical classification
Kingdom: Plantae
Division: Magnolioophyta
Class: Magnoliopsida
Order: Theales
Family: Apocynaceae
Genus: *Thea/ Camellia*
Species: *sinensis* L. Kuntze

Figure 47: Taxonomical classification of *T. sinensis* (Courtesy: https://www.sciencelearn.org.)

Polyphenols present in tea includes epicatechin; EC (42), epicatechin gallate; ECG (43), epigallocatechin; EGC (44) and epigallocatechin gallate; EGCG (45) (**Figure 48**) [14].



(42) (43) (44) (45)

Figure 48: Catechins (42-45) present in leaves of *T. sinensis*

Long-term use of tea catechins has been found to be beneficial for the suppression of HFD-induced obesity by modulating lipid and glucose metabolism [15-17].

7.4 *Aegle marmelos* L.

A. marmelos (commonly known as bael) belonging to the family Rutaceae is a species native to the Indian subcontinent and Southeast Asia (**Figure 49**). It is present in India, Sri Lanka, Nepal, Thailand, and Malayasia [18]. The leaves, bark, roots, fruits, and seeds are used in traditional medicine to treat various illnesses. It possesses antidiarrheal, antimicrobial, antiviral, radioprotective, anticancer, antipyretic, ulcer healing, antigenotoxic, diuretic, antifertility and anti-inflammatory properties [19].

Taxonomical classification
Kingdom: Plantae
Division: Tracheophyta
Class: Magnoliopsida
Order: Sapindales
Family: Rutaceae
Genus: *Aegle*
Species: *marmelos* (L.) Correa

Figure 49: Taxonomical classification of *A. marmelos* (Courtesy: https://www.piqsels.com)

Unripe fruits of *A. marmelos* possess anti-obesity properties. They are rich in coumarin and furanocoumarin derivatives namely umbelliferone (46), psoralen (47), xanthotoxol (48), marmesin (49) alloimperatorin (50) imperatorin (51) marmin (52). Alkaloids such as aegeline (53) and skiamminine (54) **(Figure 50)** are also amongst the other chemical constituents present in the fruits [20].

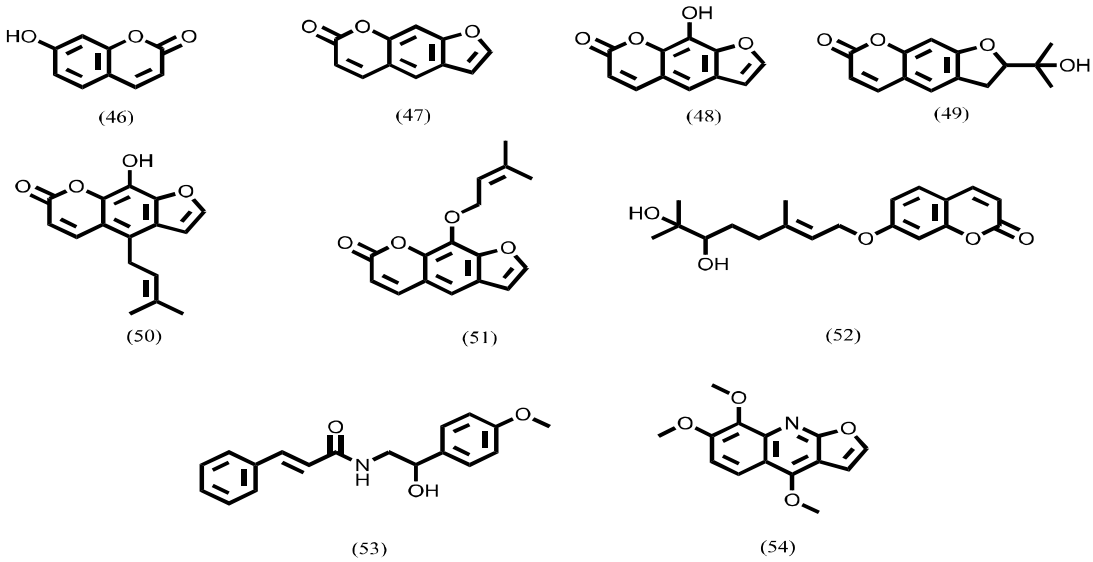


Figure 50: Chemical constituents (46-54) of *A. marmelos*

7.5 *Piper longum* L.

P. longum (also called as Indian long pepper or *pipli*), is a flowering vine in the family Piperaceae. It is usually dried and used as a spice and for seasoning **(Figure 51)**. Long pepper has a taste like that of *Piper nigrum* (black pepper) because of the presence of the phytochemical piperine.

Taxonomical classification
Kingdom: Plantae
Division: Tracheophyta
Class: Magnoliopsida
Order: Piperales
Family: Piperaceae
Genus: *Piper*
Species: *longum* L.

Figure 51: Taxonomical classification of *P. longum* (Courtesy: https://www.123rf.com)

It grows in evergreen forests of India and is cultivated in Assam, Tamil Nadu, and Andhra Pradesh. Fruits of *P. longum* are useful for anti-cancer, hepatoprotective, anti-obesity, antioxidant, antimicrobial, antidepressant, analgesic and cardioprotective properties [21]. Fruits are rich source of isobutyl amide alkaloids namely, Piperine (55), Piperlongumine (56), Pellitorine (57), Retrofractamide A (58), Retrofractamide B (59), Retrofractamide C (60), Guineesine (61) and

Piperlonguminine(62) **(Figure 52)**[22]. The content of piperine in the fruits has been found to be 4-5% [23].

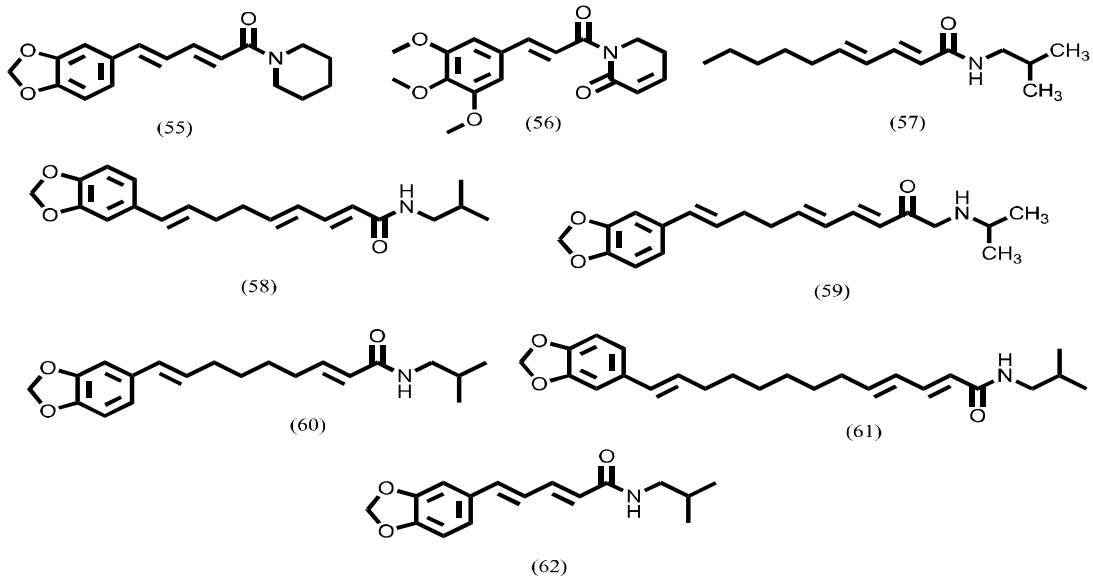


Figure 52: Chemical constituents (55-62) of *P. longum*

7.6. Isolation of berberine and palmatine from *B. aristata*

Berberine and palmatine were isolated from the alkaloid rich fraction (ARF) of BARM-SO extract using the preparative TLC technique. The crude extract (7.5 g) was dissolved in water and further acidified to pH 4 using 5% HCl, to convert the alkaloids into their respective salt derivatives. In a separating funnel, this aqueous phase was extracted with chloroform (3 × 200 ml). The residual aqueous phase was further basified to pH 9 using 37% ammonia solution. The basified aqueous phase was then extracted using chloroform (3 × 200 ml) in a separatory funnel. The alkaloid rich chloroform phase was collected and evaporated to dryness in a rotavapor (Heidolph, Germany) to obtain ARF (0.81). Using the preparative TLC technique, separation of berberine and palmatine from BARM-ARF was performed. The solvent system used in the separation was *n*-butanol: acetic acid: water (14:3:4, % *v/v/v*) [4,24, 25]. The bands separated were marked, and the silica of the respective bands were scraped and collected. The silica scrapped was dissolved in minimum volume of methanol and centrifuged at 10000 rpm. The supernatant was collected and dried. Yellow powder of berberine and palmatine were obtained **(Figure 53)**. The isolated bio-active markers were characterized using NMR spectroscopy and Mass spectrometry **(Annexure I)**.

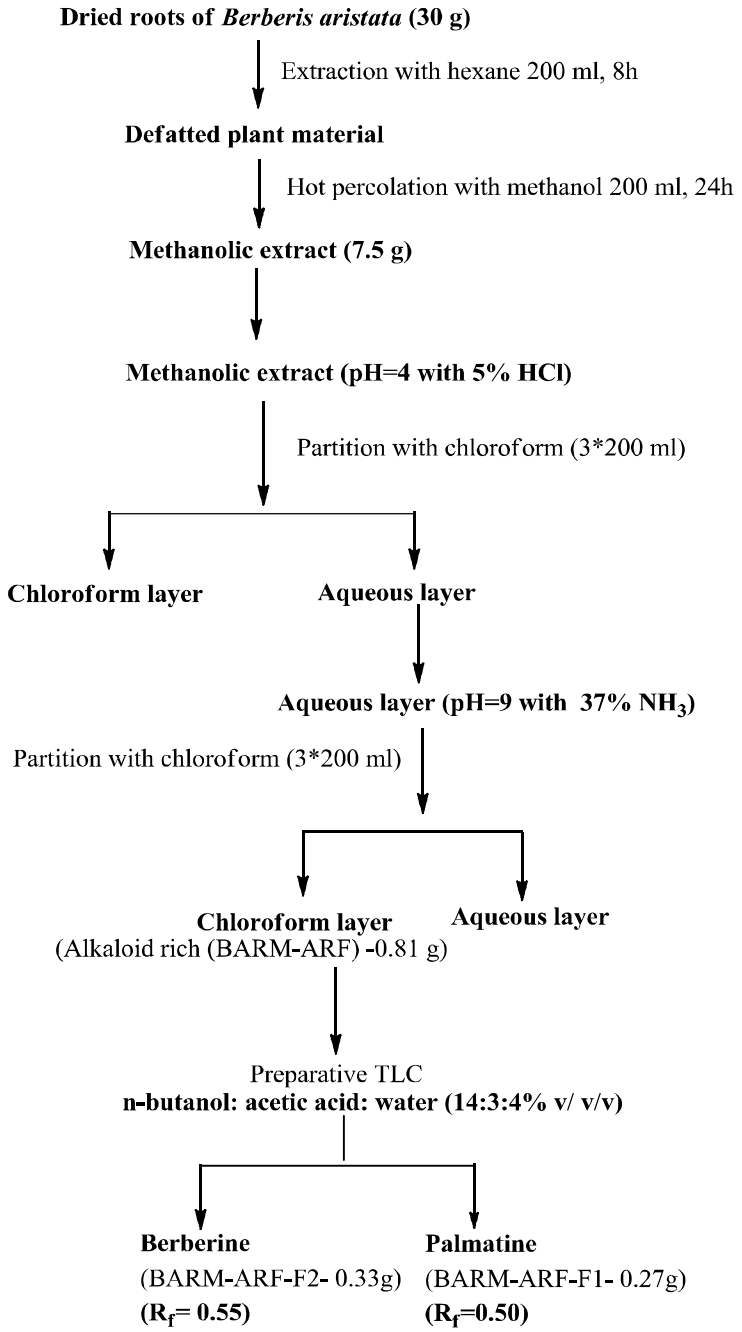


Figure 53: Isolation of berberine (25) and palmatine (28) from roots of *B. aristata*

7.7 Isolation of ECG and EGCG from *T. sinensis*

TSM-SO (20 g) was fractionated using previously reported method to obtain caffeine-rich and polyphenol-rich fractions (TSM-PRF). The TSM-PRF (11 g) was further purified using column chromatography as reported earlier (**Figure 54**) [26]. Fractions 5-7 was further subjected to semi-prep HPLC [Column: C_{18} (250 × 21.2 mm; 10 µm)] – Luna® (Phenomenex); Column Temp: 30 °C; Flow rate:9.0 ml/min; Mobile phase: Methanol and 0.1% formic acid buffer, 25:75 % *v/v*) to obtain ECG and EGCG. The isolated bio-active markers were characterized using NMR spectroscopy and Mass spectrometry **(Annexure I)**.

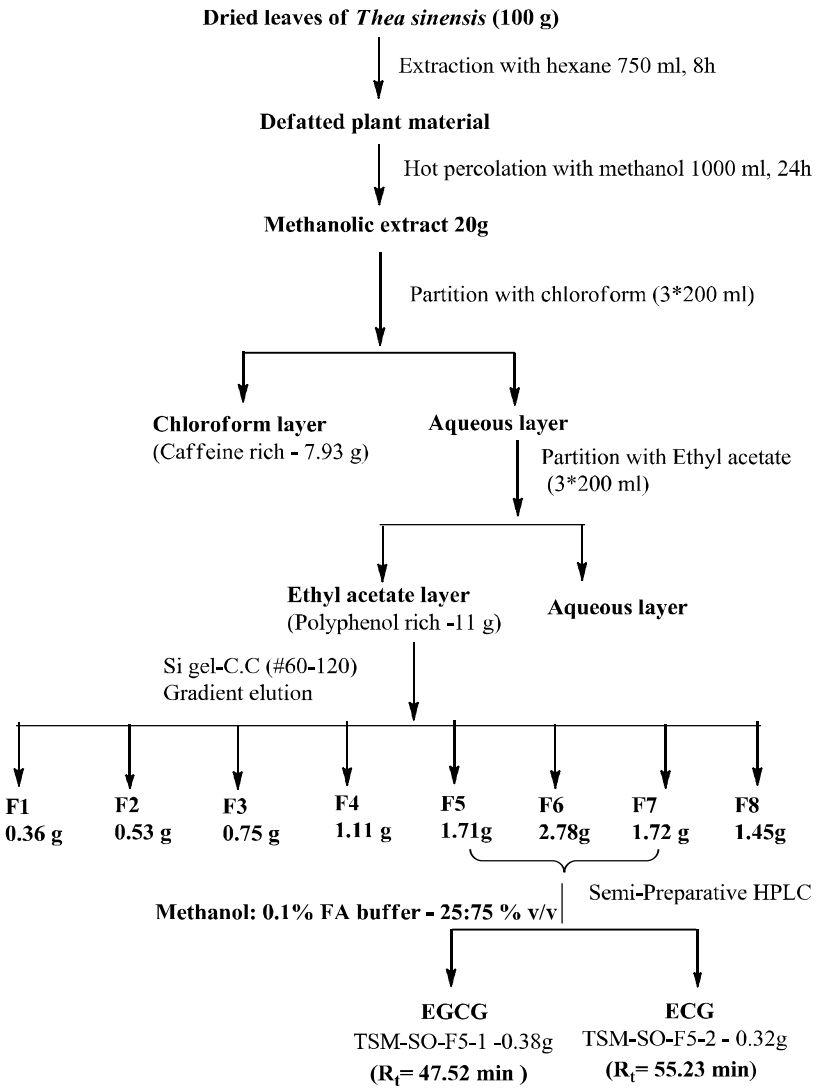


Figure 54: Isolation of ECG (43) and EGCG (45) from leaves of *T. sinensis*

7.8 Isolation of Alloimperatorin from *A. marmelos*

Column chromatography of soluble portion of methanol extract (110 g) was performed using Silica gel (60-200 #) as stationary phase. The mobile phase used was 100% hexane, followed by 25% increment of DCM to 100% DCM. Further, 25% increment of ethyl acetate along with DCM to 100% ethyl acetate, followed by 10 % increment of methanol along with ethyl acetate. The fraction 11 was subjected to semi-prep HPLC { (Column: C-18 (250 × 21.2 mm; 10 µm) – Luna®; Column Temp: 30 °C; Flow rate: 9.0 ml/min; Gradient run using mobile phase consisiting of various ratios of methanol: water such as 50:50 *v/v* (0-15.61 min), 60:40 *v/v* (15.61-31.21 min), 70:30 *v/v* (31.21-46.82 min), 80:20 *v/v* (46.82- 62.42 min), 90:10 *v/v* (62.42-78.03 min) and 50:50 *v/v* (78.03-93.63 min)} for further separation of alloimperatorin (**Figure 55**). The isolated bio-active marker was characterized using NMR spectroscopy and Mass spectrometry (**Annexure I**).

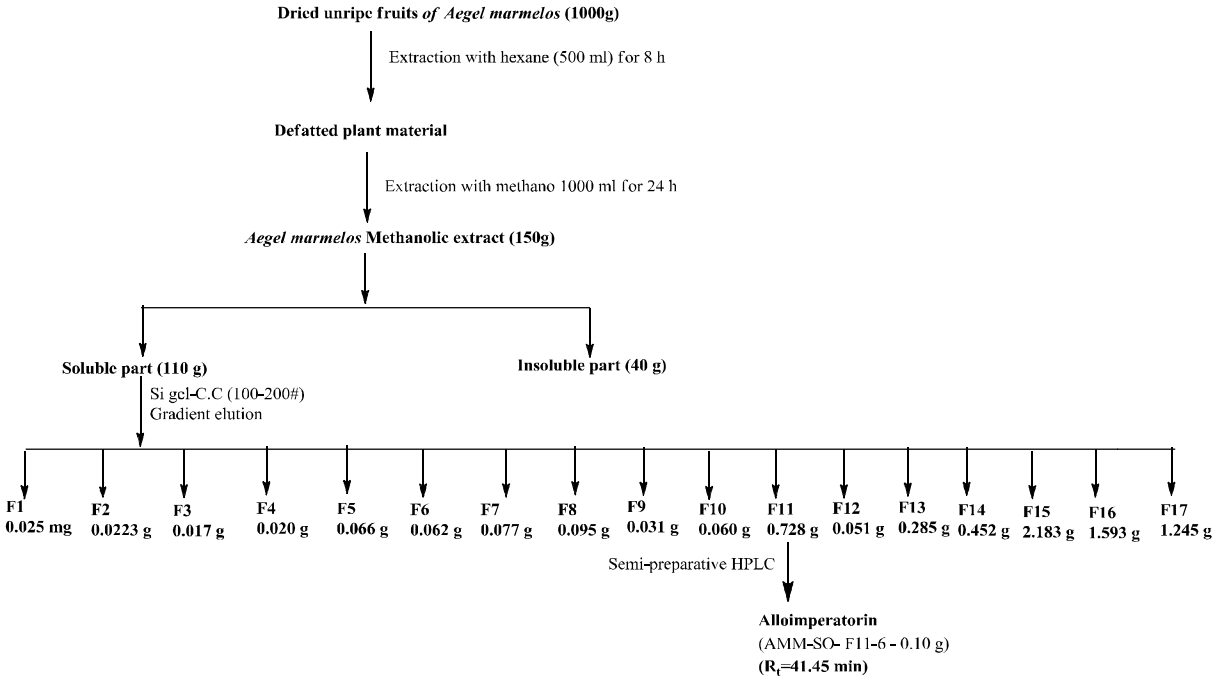


Figure 55: Isolation of alloimperatorin (50) from *A. marmelos*

7.9 Isolation of piperine and pellitorine from *P. longum*

Column chromatography of methanol extract (100 g) was performed using silica gel (200-400 #) as stationary phase. The mobile phase used was 100% hexane, followed by 5% increment of DCM to 100% DCM. Further, 5% increment of ethyl acetate along with DCM to 100% ethyl acetate, followed by 2 % increment of methanol along with ethyl acetate. Fractions 6-17 yielded yellow crystals of piperine. Fraction 10 was subjected to semi prep-HPLC (Column: C_{18} (250 × 21.2 mm;

10 μm) – Luna® (Phenomenex); Column Temp: 30°C; Flow rate: 9.0 ml/min; Mobile phase: ACN and water, 50:50% *v/v*) to obtain pellitorine (**Figure 56**). The piperine was characterized using NMR spectroscopy and Mass spectrometry (**Annexure I**), while pellitorine was identified with R_f in comparsion with the laboratory standard and Mass spectrometry [HRMS: *m/z* 224.2009; M+H].

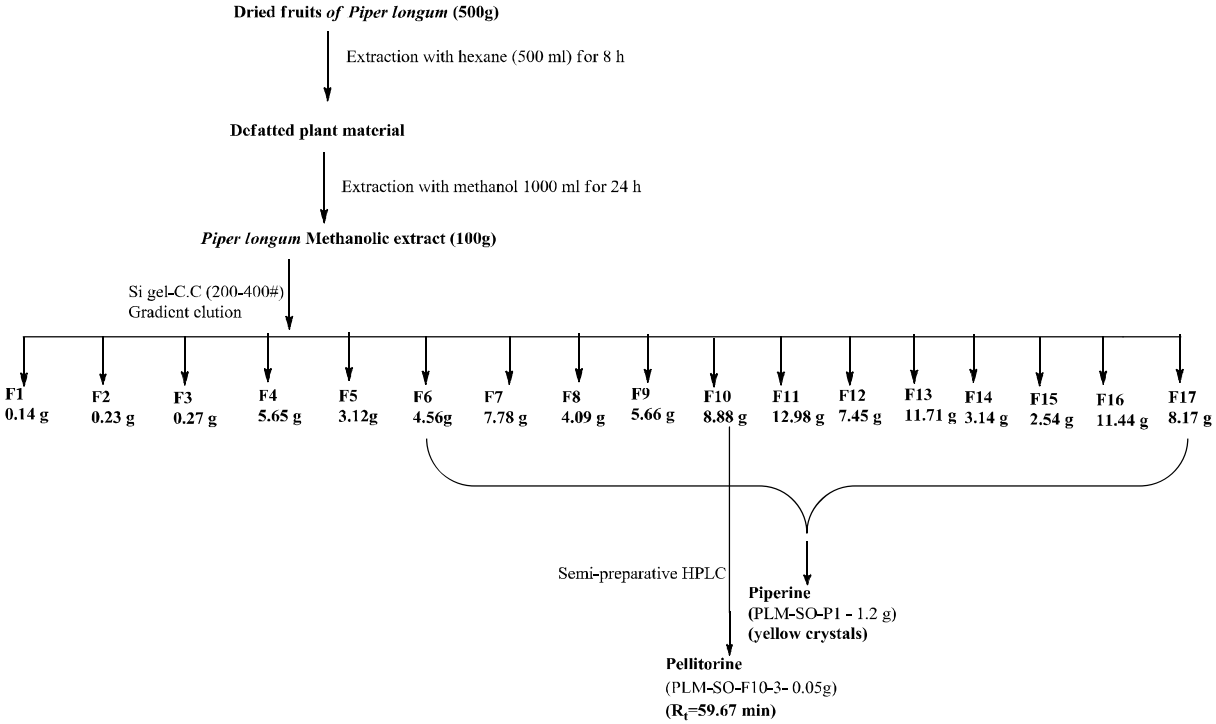


Figure 56: Isolation of piperine (55) and pellitorine (57) from fruits of *P. longum*

In conclusion, berberine, palmatine, ECG, EGCG, alloimperatorin, piperine and pellitorine were successfully isolated from their respective plants, while gymnemagenin was procured for performing further studies mentioned in Chapters 8 and 9.

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