# SUMMARY

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## 6.1 INTRODUCTION

The very first step in process of obtaining secondary metabolites from biogenic material is to release them from the matrix by means of extraction. The choice of extraction method is of great importance because of the very complex composition of the material and the minute amount of some of the constituents present. This work is an attempt to develop a modern technique with significant advantages over conventional method(s) for the extraction and analysis of the active constituents.

### 6.1.1 EXTRACTION METHODOLOGIES

The extraction methods can be conveniently divided into older methods, conventional/traditional methods and non conventional methods. The older methods include Mechanical pressing, Hydro distillation and Enfleurage. The conventional/traditional methods include Infusion, Decoction, Digestion, steam Distillation, Maceration and Percolation. Each method has its advantages and dis-advantages. In order to overcome the shortcomings of older as well as conventional methods, the improved/non conventional methods are used for the extraction.

#### 6.1.1.1 NON CONVENTIONAL/IMPROVED METHODS OF EXTRACTION

The conventional extraction processes are time consuming, *e.g.*, maceration done for 2-7 days; involve bulk amount of solvents and ultimately there might be thermal decomposition of the target molecule

like in the case of Soxhlet extraction. The demand for new extraction techniques has encouraged the development of alternative extraction techniques such as ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), supercritical fluid extraction (SCF) and accelerated solvent extraction (ASE), solid phase extraction (SPE) method, solid phase micro extraction (SPME) Method, circulatory extraction, rotary film evaporator, spouted bed extraction, bio-chelation, phytonic extraction, forced flow solid-liquid extraction, electric discharge extraction technique. These techniques have enabled automation, shortened extraction time and reduced organic solvent consumption. Thus all efforts in developing improved extraction methods need to be focused in order to increase the extraction efficiency, which leads to increased yield and/or shorter extraction time.

### 6.1.2 DIABETES MELLITUS

Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia, glycosuria, hyperlipemia, negative nitrogen balance and sometimes ketonemia.

Two major types of Diabetes mellitus are:

Type I: It is also known as Insulin dependent diabetes mellitus (IDMM) and juvenile onset diabetes mellitus. It is probably an autoimmune disorder as the antibodies that destroy β cells of islets of Langerhans in pancreas are often detectable in blood.

Type II: It is also known as Non insulin dependent diabetes mellitus and maturity onset diabetes mellitus. There is no loss of β cells and the insulin in circulation is normal and even high.

There are more than 125 millions persons with diabetes in the world today, and by the end of 2010 this number is expected to approach 220 millions. Type 1 and 2 are both increasing in frequency. The reason for the increase of Type 1 diabetes mellitus is not known. Diabetes mellitus is a chronic disease which is difficult to cure. Management concentrates on keeping blood sugar levels as close to normal as possible without presenting undue patient danger. This can usually be done with close dietary management, exercise, and use of appropriate medications. Herbal remedies for effective treatment of diabetes have attracted the attention of physicians and medicinal chemist because of holistic treatment of mind and body. Herbs are non-toxic and found to be useful for effective rehabilitation and rejuvenation therapies. Herbal drugs differ in many ways from conventional drugs. The insulin and oral hypoglycaemic agents suffer from many side effects and reactions, and in order to have a safe and effective treatment the herbal treatment is gaining attention. There are large numbers of plants and plant extracts which possess marked hypoglycaemics. There have been many comprehensive reviews focusing on plant hypoglycaemic actions.

### 6.1.3 *PTEROCARPUS MARSUPIUM*

*Pterocarpus marsupium* Roxb is grown in deciduous and evergreen forests of central, western and southern regions of India. It is found mostly in the states of Gujarat, Madhya Pradesh, Bihar and Orissa. The *Pterocarpus marsupium* heartwood has been used since ages to treat diabetes. It is golden yellowish- brown in colour with darker streaks. It is very hard and brittle. In water it gives yellow coloured solution with blue fluorescence. The beakers made from heartwood are filled with water and allowed to stand overnight to give 'Beeja Wood Water.'

## 6.2 LITERATURE REVIEW

### 6.2.1 *PTEROCARPUS MARSUPIUM*

The genus is widely distributed on the Earth and the astringent drug from this genus is known as 'kino'. The phloem of stem contains red astringent fluid present in secretory cell which exudes after given incision. Kino is odourless but has astringent taste and sticks in the teeth, colouring the saliva red in colour. As astringent it is used in diarrhoea, dysentery, *etc.* Bruised leaves are applied on skin diseases, sores and boils. Wood is useful in treating diabetes. The *Pterocarpus marsupium* heartwood has been extracted by using the conventional methods. The Infusion method has been used since ages, as the beakers made up of heartwood are filled with water and allowed to stand overnight. The Ayurvedic Pharmacopoeia (1990) recommends 50-100 grams of the drug for decoction. The wood has been extracted with water as well as alcohol using maceration. The central heartwood was dried and crushed into coarse powder. The extract

was prepared with the addition of 95 % alcohol in a percolator in the strength of 1:6. Some scientists have even prepared extract using Hot water extraction. The extracts of *Pterocarpus marsupium* have been used and various fractions were prepared. These fractions have revealed the presence of various constituents like, pterostilbene, epicatechin, liquiritigenin, isoliquiritigenin, marsupsin, pterosupin, lupeol, sitosterol, stigmasterol and naringenin. The antidiabetic action of *Pterocarpus marsupium* has been well substantiated. Besides this, it also possesses antihypertriglyceridaemic, cardio tonic, anti-cataract, COX-2 inhibition, hepatoprotective activities. *Pterocarpus marsupium* is one of the drugs mentioned in the Ayurvedic formulary and is extensively being used as Antidiabetic drug in herbal formulations. Although preliminary work has been done, but keeping in view the importance of *Pterocarpus marsupium*, efforts were made to increase the yield of active constituents as well as efficacy of drugs by using the two non conventional methods.

### 6.2.2 MICROWAVE-ASSISTED EXTRACTION METHOD

Microwave-assisted extraction (MAE) is a relatively new extraction technique that utilizes microwave energy to heat the solvent and the sample to increase the mass transfer rate of the solutes from the sample matrix into the solvent. MAE has been used as an alternative in the extraction of organic compounds from plant materials and foods. It is based upon the selective and rapid localized heating of moisture in the

sample by microwaves. Due to the localized heating, pressure builds up within the cells of the sample, leading to a fast transfer of the compounds from the cells into the extracting solvent. In the case of extraction; due to dipole rotation there is disruption of the weak hydrogen bonds in the solvent. Furthermore, the migration of dissolved ions increases solvent penetration into the matrix and thus facilitates the solvation of analytes. There are two types of commercially available instruments, which use different approaches,

- Closed extraction vessel, under controlled pressure and temperature.
- Open extraction vessel, under atmospheric pressure.

There are various factors which affect the MAE, these are, Solvent, Extraction Time, Power, Temperature and Matrix. The affects of these factors have been comprehensively reviewed. The various Optimization strategies like orthogonal array design have also been reviewed. The applications of the MAE have also been reviewed. There are many studies in which the MAE has been compared with the conventional technique. The MAE has been considered as a potential alternative to traditional methods of extraction. Some of its potential advantages are highlighted below:

- Decreased extraction time.
- Decreased solvent consumption.
- Better reproducibility.
- Increased extraction yield.

### 6.2.3 ULTRASOUND-ASSISTED EXTRACTION METHOD

Ultrasound refers to mechanical vibrations which are essentially the same as sound waves but of a higher frequency. Such waves are beyond the range of human hearing & can therefore also be called ultrasonic. Ultrasound, *i.e.*, frequencies above 20,000 Hz, may be produced with:

- Magnetostrictive ultrasonic transmitters.
- Piezoelectric ultrasonic transmitters.

Ultrasound causes rapid extraction due to:

- increase in the permeability of the cell wall,
- spontaneous formation of bubbles in the liquid below its boiling point, *i.e.*, cavitation effect, due to dynamic stressing and
- increase in the mechanical stressing, *i.e.*, internal friction of the cells.

There are mainly four types of commercially available apparatus for analytical chemist:

(a) Whistle reactors (b) Ultrasonic cleaning bath (c) Probes (d) Cup-horn devices. Out of these ultrasonic bath and probes are most commonly used. The various factors like, Extraction Time, Solvent, Temperature, Pulsation, Intensity and Matrix characteristic have been reviewed. UAE is influenced by many factors as described above and there exists interaction among these factors, thus attention has to be focused on optimization of procedures of sample preparation, extraction conditions, and optimization of analysis itself. UAE has been considered as a potential alternative to

traditional methods of extraction. Some of its potential advantages are highlighted below:

- Increased extraction yield.
- Volatile compounds can be efficiently extracted at lower temperature.
- Significant reduction in the extraction time.
- A relatively fast method, a significant reduction in the extraction time is seen.
- The amount of solvent used is drastically reduced.
- This method is suitable for thermo labile constituents.

## 6.3 CONVENTIONAL EXTRACTION METHODS

The powdered heartwood of *Pterocarpus marsupium,* was extracted using aqueous and ethanol solvents by the four conventional extraction methods, *i.e.,* infusion, decoction, maceration and percolation. The maximum yield was obtained using aqueous solvent and percolation as the extraction method.

## 6.4 NON CONVENTIONAL EXTRACTION METHODS

### 6.4.1 MICROWAVE-ASSISTED EXTRACTION (MAE) METHOD

- The powdered heartwood of *Pterocarpus marsupium,* was extracted using aqueous and ethanol solvents using microwave oven. The maximum yield was obtained with the use of aqueous solvent.
- The two important factors, power of the microwave and the irradiation time were studied using the factorial design of experiments. The experiments proved that there was a negligible interaction between the two

factors. And out of the two, microwave irradiation time was more significant.

- The MAE was optimized by obtaining the yield at different time interval. It was found that the maximum extraction yield was obtained using microwave at 100 % power for 30 min.
- The temperature of the microwave cavity increased with the increase in irradiation time.
- The extraction yield of *Pterocarpus marsupium* using MAE method was significantly higher as compared to that obtained using percolation.

### 6.4.2 ULTRASOUND-ASSISTED EXTRACTION (UAE) METHOD

- The powdered heartwood of *Pterocarpus marsupium,* was extracted using aqueous and ethanol solvents using ultrasonic waves. The maximum yield was obtained with the use of aqueous solvent.
- The two important factors, temperature and time were studied using the factorial design of experiments. The experiments proved that there was a negligible interaction between the two factors.
- The Simplex Optimization method was used to optimize the extraction yield using UAE. This technique led to an optimized condition of temperature of 47 °C and time period of 26 min.

## 6.5 COMPARATIVE EVALUATION USING THE EXTRACTION YIELD (%)

The powdered heartwood of *Pterocarpus marsupium* was extracted using different extraction methods, the extraction yields obtained using aqueous

percolation, and optimized MAE and optimized UAE were compared. The results clearly show that the microwave-assisted extraction produced the best extraction yield as compared to that obtained by using ultrasound-assisted extraction method and other conventional methods.

## 6.6 QUALITATIVE CHEMICAL EXAMINATION

The three extracts prepared by using three different extraction methods, *i.e.,* percolation, optimized MAE, optimized UAE showed the presence of carbohydrates, phenolic compounds and tannins, saponins, flavonoids, acidic compounds and proteins and free amino acids. All the three aqueous extracts showed the absence of alkaloids, glycosides, sterols and oils and fats.

## 6.7 COMPARATIVE EVALUATION BY USING THIN LAYER CHROMATOGRAPHY

The ethyl acetate soluble fraction of the three extracts prepared by using three different extraction methods, *i.e.,* percolation, optimized MAE, and optimized UAE contained the reference compound pterostilbene. The chemical composition of the above fraction was similar.

## 6.8 COMPARATIVE EVALUATION BY USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The pterostilbene was used as the reference compound. The high performance liquid chromatography analytical method was developed and

validated. The method was validated using ICH guidelines on the various validation characteristics. The experiment clearly demonstrated the **specificity** of the analytical method. The **Linearity** of the method was determined. A linear relationship was observed, and correlation coefficient calculated to be 0.996. The system suitability tests were conducted. The **calibration range** falls between 25 mcg-220 mcg. The number of **theoretical plates** comes out to be fairly high, 1, 41,877 ensuring that the column was reasonably efficient. The **tailing factor** of 0.4 % indicates that the peak was sufficiently asymmetric. The **relative standard deviation** of the retention time and peak area was 0.022 % and 0.306 % respectively. This ensures the repeatability of the method. The **limit of detection** of the peak area and concentration was 6.39 and 0.758 ppm respectively. This implies that the minimum concentration of the pterostilbene which can be detected was 0.758 ppm. The **limit of quantification** of the peak area and concentration is 21.3 and 2.526 ppm respectively. This implies that the minimum concentration of the pterostilbene which could be quantified was 2.526 ppm. The extract obtained using optimized MAE method contained the maximum percentage of pterostilbene (0.667 %). The amount of pterostilbene obtained by using percolation and optimized UAE method was nearly same.

## 6.9 COMPARATIVE EVALUATION OF THE ANTIDIABETIC ACTIVITY OF THE EXTRACTS OBTAINED FROM CONVENTIONAL AND NON-CONVENTIONAL METHODS

### 6.9.1 NORMAL FASTED RATS (SPRAGUE-DAWLEY)

The hypoglycaemic effect of the three extracts of the plant, using gliclazide as standard drug on the normal fasted rats, was demonstrated. At the end of 3rd hour of treatment, the standard Gliclazide, *Pterocarpus marsupium* UAE extract (aqueous) and percolation extract (aqueous) showed statistically significant hypoglycaemic effect. The UAE extract and gliclazide have highly effective hypoglycaemic action when compared with the other plant extracts

### 6.9.2 HYPERGLYCAEMIC RATS (SPRAGUE-DAWLEY)

The induction of diabetes was good in all alloxan treated rats.

#### 6.9.2.1 Acute treatment

The oral administration of gliclazide, UAE aqueous extract and percolation aqueous extract led to statistically significant blood glucose lowering effect in alloxan-induced hyperglycaemic rats. The gliclazide and UAE aqueous extract have highly effective antihyperglycaemic action when compared with other extracts.

#### 6.9.2.2 Sub acute treatment

In sub-acute treatment the administration of extract/standard/vehicle was continued for 10 days, once daily. The oral administration of gliclazide

and UAE aqueous extract led to consistent and statistically significant ($p<0.001$) reduction in the blood glucose level in alloxan-induced hyperglycaemic rats. The percolation aqueous extract also showed significant reduction in the blood glucose level but the response was inconsistent.

## 6.10 ESTIMATION OF BODY WEIGHT CHANGES

There was no abnormal change in weight of the hyperglycaemic animals after 10 days of administration of plant extracts and gliclazide, when compared to the change in weight of the normal animals.

## 6.11 SUMMARY

The *Pterocarpus marsupium* was extracted using conventional and non conventional methods (UAE and MAE). Both the non conventional methods were optimized by $2^2$ factorial method (MAE) and simplex method (UAE). The results showed that the extraction efficiency using MAE was much higher, than that for percolation or UAE (although there was considerable reduction in time of extraction -- < 30 mins for UAE, as compared to percolation -- > 12 hours). All the three extracts showed similar phytochemical constituents (such as carbohydrates, phenolic compounds and tannins, saponins, flavonoids, acidic compounds and proteins and free amino acids.) and TLC indicated the presence of pterostilbene (reported to be one of the marker compounds) in all the three extracts. The proportion of pterostilbene was much higher in MAE, as compared to lesser proportion of pterostilbene in UAE or percolation (no

significant difference between the proportion of pterostilbene in UAE or percolation).

Antidiabetic activity of UAE extract was found to be statistically significant and consistent, comparable to that of the gliclazide. However, the antidiabetic activity of MAE was found to be non-significant, as compared to UAE or gliclazide.

The results indicate that, both UAE and MAE are better methods of extraction, so far as the time of extraction, and efficiency of extraction are concerned, as compared to the conventional methods.

UAE results in an extract which has significant antihyperglycaemic activity, comparable to that with gliclazide. But MAE extract did not result in such antihyperglycaemic activity. This difference can be attributed to unique mechanism of UAE, which results in increased cell membrane permeability, as compared to MAE, wherein temperature has a predominant effect. As a result, it can be postulated that the UAE results in a better (or rather complete extract), as compared to MAE or percolation.

Hence, UAE proved to be a successful and complete non conventional method of extraction, yielding a product which

1) Could be produced in a much lesser time,
2) Could be produced using a small volume of the solvent,
3) Could exhibit significant pharmacological activity.

In comparison, MAE resulted in a product, which

1) Could be produced in a much lesser time,

2) Could be produced using a small volume of the solvent,

3) Did not result in significant pharmacological activity.

Thus, there exists further scope of exploring such non conventional methods of extraction, as compared to the conventional methods.