DEVELOPMENT OF AN ELECTROCHEMICAL SENSOR BASED ON MOLECULARLY IMPRINTED GO/ZnO/PPy COMPOSITES ON PENCIL GRAPHITE ELECTRODE FOR DETERMINATION OF ANDROGRAPHOLIDE

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by

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LIST OF ABBREVIATIONS

A. paniculata	*Andrographis paniculata*
CV	Cyclic voltammetry
DPV	Differential pulse voltammetry
EIS	Electrochemical impedance spectroscopy
EDX	Electron dispersive X-ray
FT-IR	Fourier Transform Infrared Spectroscopy
GO	Graphene oxide
GQDs	Graphene quantum dots
GO/PPy/PGE	Graphene oxide, polypyyrole coated pencil graphite electrode
GO/ZnO/PPy/PGE	Graphene oxide, zinc oxide polypyyrole coated pencil graphite electrode
GQDs/PPy/PGE	Graphene quantum dots polypyyrole coated pencil graphite electrode
K_3 [Fe $(CN)_6$]	Potassium ferricyanide
KCl	Potassium chloride
LOD	Limit of detection
MIP	Molecularly imprinted polymers
NIP	Non imprinted polymers
PGE	Pencil graphite electrode
PPy	Polypyyrole
PL	Photoluminescence
R.S. D	Relative standard deviation
SEM	Scanning electron microscope
S. D	Standard deviation
XRD	X-ray diffraction
ZnO	Zinc oxide

LIST OF SYMBOLS

cm^{-1}	per centimeter
μM	Micro molar
μA	Micro ampere
mM	millimolar
R_p	Polarization resistance
R_u	electrolyte resistance
R_2	correlation coefficient
V/s	Volt per second
W_d	Warburg element
Y_0	pseudo-capacitance
%	Percentage
°	Degree

PEMBANGUNAN PENDERIA ELEKTROKIMIA BERASASKAN KOMPOSIT MOLEKUL TERCETAK GO/ ZnO/ PPY PADA ELEKTROD PENSIL GRAFIT BAGI PENENTUAN ANDOGRAFOLID

ABSTRAK

Kajian ini menyorot pembangunan penderia elektrokimia berasaskan terbitan grafen dengan lapisan molekul tercetak polipirol (PPy) pada elektrod pensil grafit (PGE) bagi penentuan andrografolid, komponen bioaktif tumbuhan purba yang dikenali sebagai *Andrographis paniculata*. Secara konvensional, pengesanan andrographolide berdasarkan instrumentasi yang membosankan dan berat. Menggunakan kaedah elektroanalitik dengan prinsip elektropolimerisasi untuk melaksanakan kaedah mudah untuk mengesan andrographolide. Beberapa terbitan grafen telah dienap pada PGEs. Terbitan grafen ini dicirikan dengan teknik spektroskopi Raman, spektroskopi pembelauan sinar-X, spektroskopi inframerah transformasi Fourier, spektroskopi ultrajingga-nampak dan spektroskopi pendarcahaya. Kemudian, elektrod terubah suai ini diolah dengan PPy dengan menggunakan teknik molekul tercetak. Elektrod terubah suai seterusnya dicirikan dengan teknik voltammetri berkitar (CV), spektroskopi elektrokimia impedans dan voltammetri denyut pembezaan (DPV). Didapati PGE grafen oksida/ zink oksida/ polipirol (GO/ ZnO/ PPy) menghasilkan prestasi analisis terbaik berbanding elektrod terubah suai lain. Tambahan, elektrod terubah suai GO/ ZnO/ PPy menghasilkan puncak redoks tertinggi dalam analisis CV. Pada parameter optimum, rangsangan linear yang baik telah diperoleh bagi penentuan andrografolid dengan DPV, iaitu julat 50 to 145 μM dan had pengesan 42.6 μM. Sisihan piawai relatif bagi tiga pengukuran ialah 1.47%, yang mana ia mengesahkan kebolehulangan penderia elektrokimia ini.

Aplikasi penderia elektrokimia ini ditunjukkan dengan menentukan andrografolid dalam sampel air sebenar dan keputusan yang memuaskan diperoleh. Penderia elektrokimia GO/ZnO/PPy ini turut menunjukkan kesensitifan, kestabilan dan kebolehulangan yang cemerlang, membuatkan ia berpotensi diguna untuk penentuan lakton dan herba diterpena.

DEVELOPMENT OF AN ELECTROCHEMICAL SENSOR BASED ON MOLECULARLY IMPRINTED GO/ZnO/PPy COMPOSITES ON PENCIL GRAPHITE ELECTRODE FOR DETERMINATION OF ANDROGRAPHOLIDE

ABSTRACT

This research highlights the development of an electrochemical sensor based on graphene derivatives with a molecularly imprinted polypyyrole (PPy) layer on a pencil graphite electrode (PGE) to detect andrographolide, which is a bioactive component of an ancient plant known as *Andrographis paniculata*. Conventionally, andrographolide detection based on tedious and heavy instrumentation. Utilizing the electroanalytical method with electropolymerization principles to implemented the easy method to detect andrographolide. Different graphene derivatives such as graphene oxide (GO) and graphene quantum dots (GQDs) have been deposited on PGEs. The graphene derivatives were characterized by Raman spectroscopy, X-ray diffraction spectroscopy, Fourier-transform infrared spectroscopy, ultraviolet-visible spectroscopy and luminescence spectroscopy techniques. After deposition of GO and GQDs, the modified electrodes were altered with PPy by using a molecularly imprinted technique. The modified electrodes were characterized by cyclic voltammetry (CV), electrochemical impedance spectroscopy and differential pulse voltammetry (DPV) techniques. It was found that graphene oxide/ zinc oxide/ polypyyrole (GO/ ZnO/ PPy) PGE produced the best analytical performances compared to other fabricated electrodes. Furthermore, the GO/ ZnO/ PPy modified electrode produced the highest redox peaks in CV analysis. Under optimized parameters, a good linear response was obtained for andrographolide detection by

DPV, in the range of 50 to 145 μM and a detection limit of 42.6 μM. The relative standard deviation of the three measurements was 1.47%, which confirmed the excellent repeatability of the electrochemical sensor. The usefulness of the electrochemical sensor was demonstrated by determining andrographolide in real water samples, and the produced results were satisfactory. The GO/ZnO/PPy based electrochemical sensor exhibited excellent sensitivity, stability and reproducibility, making it a potential tool for instant lactones and diterpenes herbs detection.

CHAPTER 1

INTRODUCTION

1.1 Overview

Sensors are modules used in every field to determine every parameter and change in its environment to provide more feasible services to humanity. One of the sensors, electrochemical sensors, provides precise information about analytes, concentration determination even in a minimal amount. Electrochemical modifications disclosed a method to fabricate more bio-compatible and high-resolution electrodes (Bollella et al., 2017; Lahcen et al., 2020). Electrochemical sensors provide many advantages over conventional sensors. Initially, two-electrode electrochemical sensors (working electrode and either reference or counter electrode) was used. It is preferable to use a three-electrode system over two-electrode electrochemical sensors to reduce and compensate the potential changes caused by large currents passing through the working and counter electrodes for better control and measurement of the current and potential going through the cell during an electricity-driven chemical reaction. Now, three-electrode electrochemical sensors consisting of working, reference and counter electrodes are widely used. It is highly appreciable to modify the working electrode area surface (Maduraiveeran & Jin, 2017). Research on the fabrication of electrochemical sensors with different modifications has increased the diversity of detection techniques in various fields such as biochemistry for biomolecular interactions, nano-technology and clinical analysis (Faridbod et al., 2011).

Natural herbs and their artificial modified derivatives are spreading approximately 70-80% bioactive constituents for beneficial purposes for humanity throughout many years (Tilburt & Kaptchuk, 2008). *Andrographis Paniculata* (*A.*

paniculata), a versatile natural herb, has various bioactive constituents (Figure 1.1). Among them, andrographolide ($C_{20}H_{30}O_5$) is one of the most important bioactive compounds from a herbal plant known as *A. paniculata*, belonging to the Acanthaceae family (Khan et al., 2018). *A. paniculata* is used for many centuries in China to cure diseases like gastric disorders, colds, influenza and infectious diseases and is also known as the "King of bitters" (Zhang et al., 2020).

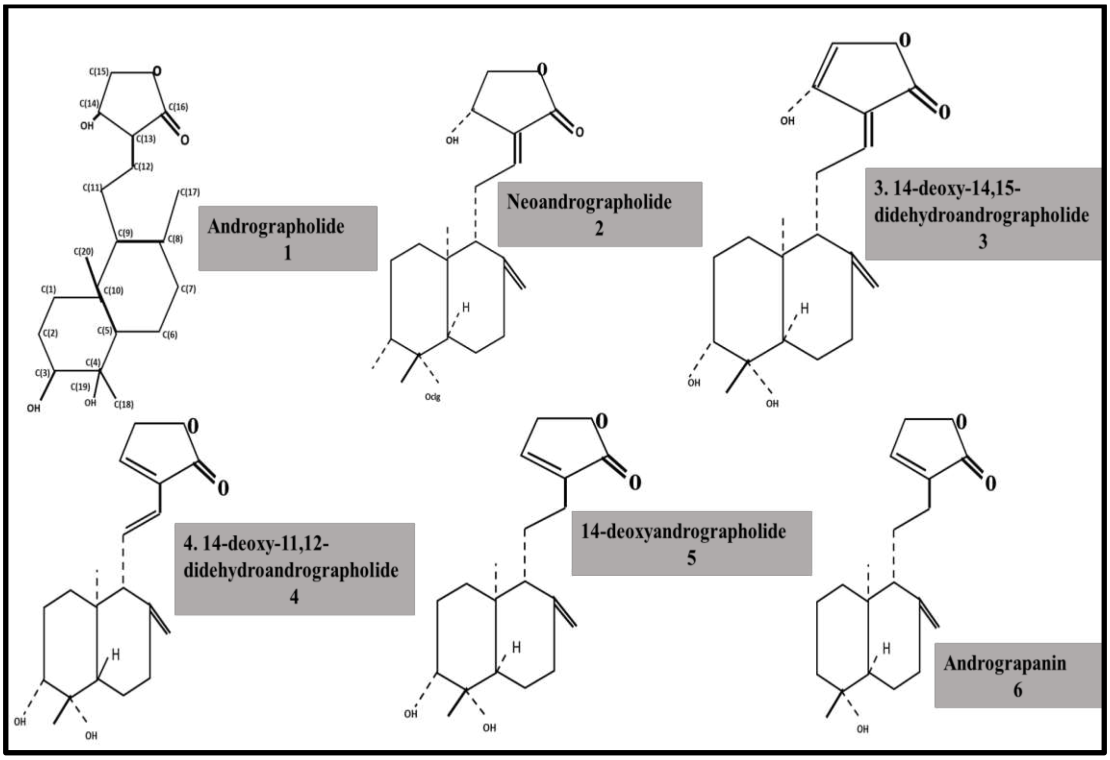

Figure 1.1 Extracts of *A. paniculata* (Kumar et al., 2020).

Molecularly imprinted polymer (MIP) is an emerging technology and have gained much interest in recent decades. It is the affixation of artificial binding sites with a facsimile shape and chemical-physical characteristics of template molecule (Whitcombe et al., 1995). In recent years, MIP technology is widely spread in electrochemical sensors to detect the target molecules with high selectivity, excellent robustness and adequate chemical stability. It is easy to prepare and also can be applied as a biological recognizing element. This technique becomes preferable due to its unique features, such as the convenient adherence of polymeric films to conducting electrode surfaces and the ability to control the thickness of films under various deposition parameters (Lingxin Chen et al., 2016; Martín-Esteban, 2013)

Graphene oxide (GO) is the most useful graphene derivative in electrochemical sensors incorporating different metallic nanoparticles. Moreover, the graphene derivatives with different nanoparticles such as Ni, Au, Pt, Cu, ZnO, have been used to increase the electroconductivity properties of the working electrode, helps in the detection of different analytes (Karimi-Maleh et al., 2018; Rao et al., 2018; Shetti et al., 2019; Wang et al., 2014). It is also observed that the interaction of graphene derivatives composites with metal nanoparticles improves the performance of the electrochemical sensor (Jothi Ramalingam et al., 2021).

PGE is considered a promising material due to its ease of manipulation, and it is inexpensive. The electrode modifications are an essential technique to enhance electrode working by creating a suitable template for immobilization with biomolecules and lowering the charge transfer resistance at surfaces (Dehnavi & Soleymanpour, 2020). In addition, PPy and many metal oxides are easily incorporated at the surface of PGE (Akbari Hasanjani & Zarei, 2021).

1.2 Problem statement

Researchers have reported many analysis reports of bioactive material known as andrographolide (Ajaya Kumar et al., 2004; Zhang et al., 2020). Moreover, many studies prove it is a beneficial drug against many diseases like influenza, hepatoma cancer cells, human immunodeficiency virus (HIV) and hepatitis C (Khan et al., 2018). However, despite the beneficial properties of andrographolide, the detection of andrographolide has still seldom reports through electrochemical techniques. Therefore, there is a need for a simple, sensitive and cheap method to detect andrographolide derived from A. paniculata to utilize it to serve humanity.

Many analytical methods are introduced in early work, such as high-performance liquid chromatography, high-performance thin-layer chromatography, column preparative chromatography, and solid-phase extraction to detect andrographolide. However, these techniques seemed to have many shortcomings, such as tedious methods for sample preparation, time-consuming and low reproducibility. Moreover, the upgraded electrochemical techniques were easier than conventional methods. Therefore, there is a need for promising construction of portable and low-cost electrochemical sensor with good selectivity and sensitivity for detection of andrographolide derived from *A. paniculata*.

1.3 Hypothesis

Pencil Graphite Electrode (PGE) material has a larger active electrode surface area and is valuable for detecting a low analyte concentration. PGE is used to check the improvement in the sensitivity and selectivity of molecular recognition sites on the electrode to detect andrographolide. In addition, to check the molecular recognition site can play a vital role in response specifically and quantitatively to the target molecule.

The GO/ZnO composites coated on PGE to check the enhancement in the electrochemical response in detecting andrographolide. The proposed sensor will be fabricated via electro polymerization of pyrrole in the presence of andrographolide template molecules onto the surface of a PGE modified with GO/ZnO to check the influence of imprinting technology with nanocomposites in detection of bioactive herbs. The sensitivity, selectivity, good reproducibility, stability and detection limit of the sensor will be studied. Furthermore, the fabricated electrochemical sensor will be applied to detect andrographolide in real samples to check the feasibility of sensor.

1.4 Research objectives

The current work introduces an easy, less expensive and fast method of determining diterpenes like andrographolide. The electrochemical analysis is one of the better options compared to complicated and expensive methods for this purpose. In this study, an electrochemically modified electrode with graphene derivatives and polymer (polypyyrole) was fabricated. In the first section, synthetic materials were deposited onto the working electrode (PGE) to increase its surface area and conductivity. In the next section, the modified PGE were characterized, and its electrochemical behavior was studied using CV and DPV techniques. Finally, the last section of this study consists of GO/ZnO/PPy/PGE modified electrode performance in the presence of interferences and detection of andrographolide in water samples.

Objectives of this study are:

i. To prepare molecularly imprinted GO/ZnO/PPy by using electro polymerization technique.
ii. To evaluate the performance of the developed GO/ZnO/PPy-PGE towards andrographolide.
iii. To evaluate applicability of the developed sensor for the detection of andrographolide in real sample.

CHAPTER 2

LITERATURE REVIEW

2.1 Medicinal plants

Regardless of incredible innovations in the recent field of science and technology, man cannot provide quality healthcare to all. Therefore, traditional medicinal herbs are considered a significant part of our lives for quality healthcare. Many of us rely on these medicinal herbs for our healthcare (Sen & Chakraborty, 2017). Medicinal plants have been used to treat most of our illnesses with minimal or no industrial processes. As reported in literature (Banning, 2005), in the general population, daily consumption of certain herbs has also been shown to have curing effects over different illnesses.

Although medicinal plants played a significant role in global health and have shown a favorable outcome on injuries or any illness, most of these are unverified in terms of use and purity. Therefore, the World Health Organization (WHO) has investigated in researching these medicinal plants worldwide to provide quality-based medicinal herbs (Tilburt & Kaptchuk, 2008).

2.2 *Andrographis paniculata*

Andrographis paniculata (A. paniculata), known as "hempedu bumi" in Malaysia," chuanxinlian" in China, and "maha tikta" in India. Geographically, it was originated from India, China, and other Southeast Asian countries. Moreover, it is primarily grown in wastelands and grasslands of tropical areas and adjusts itself in various soil conditions (Chen et al., 2020). In Ayurveda, *A. paniculata* is quite possibly the most utilized plant in the definition of polyherbal blends. Around 26 formulations were known that has *A. paniculata* as an essential ingredient (Abd Aziz et al., 2021).

Andrographolide, dehydroandrographolide, and neoandrographolide are the different contents of A. paniculata (Xu et al., 2015). Among them, andrographolide is the most found content of the plant (Abd Aziz et al., 2021). The content of andrographolide in *A. paniculata* is more than other components of *A. paniculata* (Chen et al., 2020).

Many studies on animals prove that the extract of *A. paniculata* is biologically actives (Li et al., 2020). These biologically active components are diterpenes lactones, collectively known as andrographolide, with other components such as flavonoids and polyphenols. The andrographolide extract from *A. paniculata* has the main constituents of this traditional medicinal plant. Several bio-activities of andrographolide have been reported, such as anti-inflammatory effect (Kumar et al., 2020), antimicrobial activity (Khamphaya et al., 2016), anticancer effect (Kumar et al., 2020) and stimulation of immune system effect (Ajaya Kumar et al., 2004).

2.3 Andrographolide

Andrographolide seems to be a versatile bioactive tool that has various pharmacological properties such as antiviral (Iwu et al., 2020), anti-inflammatory (Tran et al., 2020), anticancer (Khan et al., 2018), immunomodulatory (Ajaya Kumar et al., 2004), antibacterial (Li et al., 2018), hepatoprotective (Khamphaya et al., 2016) and hypoglycemic (Zhang et al., 2020). Andrographolide structure provided the chance for modifications and made its application wider. Literature shows that modifications at 3, 14, 19 positions as shown in figure 2.1 of andrographolide make it more effective in sense of biological activity to improve physiochemical and pharmaceuticals properties of andrographolide (Kumar et al., 2020). By modern means such as 3D computer modelling, more avenues will be discovered for future aspects. Due to its medical importance in the sense of curing of different illnesses with its unique bioactivity on humans increases the demand of andrographolide. That's why, the researchers work on different techniques isolation of andrographolide. Liquid chromatography (LC-MS/MS), high performance thin layer chromatography (HPTLC), column chromatography and solid phase extraction (SPE) were reported for the analysis of andrographolide (Kumar et al., 2014; Xu et al., 2015; Yin et al., 2011). These methods seem to be expensive, time consuming and come with complicated procedures. Therefore, a simple, inexpensive, and easy method of analysis of andrographolide is preferred.

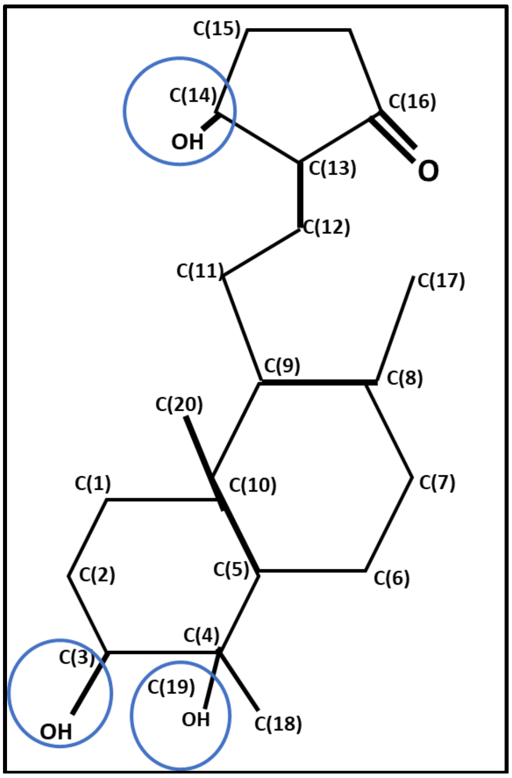

Figure 2.1 Molecular structure of andrographolide with its modified positions.

2.4 Bioactivities of andrographolide

With the advancements in pharmacological research, most of the traditional medicinal uses of *A. paniculata* have been seen beneficial for humanity. Thus, a brief description of the various bioactive properties of *A. paniculata* is provided in this section.

Literature shows that andrographolide has broad antiviral activity against different viruses such as influenza A, chikungunya virus, human immunodeficiency virus (HIV), dengue virus, hepatitis B virus, and hepatitis C virus, herpes simplex virus, and Ebola virus (Iwu et al., 2020). Paemanee et al. (2019) described the proteomics-based approach using andrographolide against the dengue virus. In response, there is an enhancement of phosphorylation of eIF2α (protein), which is evidence of anti-dengue virus activity due to andrographolide. The GRP78 is functioning as a chaperone and critical modulator of unfolded protein response. Dengue virus controls this machinery to replicates its viral production. Andrographolide has a powerful effect on the conciliate GRP78 gene expression (Paemanee et al., 2019). Here, the andrographolide plays an essential role for GRP78 and unfolded protein response by mediating the anti-dengue virus activity.

Many studies have shown that andrographolide takes part in various cellular processes (Iwu et al., 2020). One of them is autophagy that is induced by andrographolide. Dengue virus also induced autophagy that causes virus replication. The cells treated with andrographolide show an increase of LC3 (Microtuble-associated protein light chain 3), a marker of autophagy that increases induction of autophagy which is the cause of anti-dengue virus replication. Besides the dengue and chikungunya virus, andrographolide derivatives with quinoline moieties seem effective for zika virus and various arbovirus types (Li et al., 2020)

Many research reports demonstrated that andrographolide seems to have an anti-inflammatory and anti-oxidative response against lead-induced kidney injury and LPS- induced lung injury (Tran et al., 2020). They illustrated the development of a new anti-inflammatory agent by using natural products known as andrographolide. By now, this approach needs to be exploited to develop covalent inhibitors with natural products for anti-inflammatory action. It seems to be shown that enone 17 with andrographolide converts irreversible inhibitor to reversible inhibitor that has the advantage of more potency and selectivity (Tran et al., 2020). Thus, the therapeutic agent (enone 17) plays a vital role during inflammatory conditions in LPS-induced lung injury models by coupling with andrographolide (Tran et al., 2020).

It is known that TNF-α and IL-1β (proteins) production causes the generation of lead in the kidney. Andrographolide work as a shield to inhibit their production and activate the Nrf2 signaling pathway in common carp (Zhang et al., 2020). It also demonstrated the analysis of glutathione (GSH), superoxide dismutase (SOD), myeloperoxidase (MPO), and malondialdehyde (MDA) by using andrographolide on control and inflammatory kidney cells. Furthermore, a recent report explains the protective role of andrographolide in improving arthritis inflammation induced by complete Freund's adjuvant by inhibiting neutrophils chemotactic factors that are causing rheumatoid arthritis (RA) (Luo et al., 2020)

Recently, Khan et al. (2018) have reported the anticancer nature of andrographolide by studying the intracellular generation of reactive oxygen species, mediation of mitochondrial membrane depolarization, nuclear condensation, an assay of caspase-3 activity, and DNA fragmentation. The reports explained that andrographolide is a cause of cell cycle arrest and induced apoptosis in colon cancer cells. Ajaya Kumar et al. (2004) explained that andrographolide with an anticancer nature could mediate our immune system by inhibiting tumor growth and stopping metastasis. It was explained in paper through results of dichloromethane fraction of andrographolide which works against the spreading of HT-29 (colon cancer cells) and increases the proliferation of human peripheral blood lymphocytes. In a recent report, the andrographolide new derivative known as AGS-30 has been synthesized and seems to possess an anti-angiogenic effect compared to its pure form (Li et al., 2020). Thus, the AGS-30 seems to be an efficient and robust therapeutic agent for cancer.

With the features of anti-inflammatory and antiviral, the andrographolide works as a natural antibiotic in terms of antibacterial activity. Li et al. (2018) discussed the antibacterial activity with the immunosuppressant nature of andrographolide derivatives through structure-activity relationship (SAR) analysis. It restricted inflammatory bowel disease by inhibiting the production of biofilms and blocking the quorum sensing system of *P. aeruginosa* (Ma et al., 2012). Furthermore, it was reported that 14-aryloxy andrographolide (a derivative of andrographolide) works against *E. coli, S. aureus, E. faecalis* as antibacterial (Li et al., 2018).

Khamphaya et al. (2016) evaluated the in vivo hepatoprotective nature of andrographolide and provided evidence through rats examining intrahepatic cholestatic liver disease. The pretreated rats with andrographolide show evidence of avoidance of liver fibrosis and prevention of intrahepatic cholestatic liver disease with the help of analysis of protein expressions in the liver. In a recent report, XYLDF (Xiaoyan Lidan Formula) treatment based on metabolomic study shows an effective result against intrahepatic cholestatic liver disease due to the presence of three constituents known as herba andrographis, herba rabdosia and folium picrasmae (Zhang et al., 2020). It effectively regulates the homeostatic metabolic conditions of cholic acid. This treatment also reversed biomarkers to control levels that perform various essential functions such as excretion, bile acid metabolism, steroids and retinol metabolism.

Verma et al. (2020) has reported that the extract of andrographolide worked as a nano-Phyto vesicles delivery system and enhanced the antihyperglycemic activity in blood plasma. Moreover, Kumar et al. (2020) also proved by experimentation on Wistar rats that andrographolide works as an indoleamine 2,3-dioxygenase (IDO) inhibitor and provides considerable stress changes. It also works as an agent for stabilizing diabetic complications (Zhang et al., 2020).

2.5 Conventional methods to detect andrographolide

The detection and profiling of bioactive compounds is of utmost importance, in view of the various ways of adulteration and blending of drugs. The diterpene lactones of A. paniculata are determined using a variety of analytical methods. For the determination of active constituents in A. paniculata, chromatographic methods such as thin-layer chromatography (TLC) (Jain et al., 2010), gas chromatography (GC) (Kalaiselvan et al., 2012), high-performance liquid chromatography (HPLC) (Ligang Chen, Jin, et al., 2007), micellar electrokinetic chromatography (MEKC) (Safaei & Shishehbore, 2021), and capillary electrophoresis (CE) (Sareer et al., 2014) have been standardized. These methods are currently widely used for detection and measurement of this miracle herb's active ingredients. A brief description of the various methods to determine of andrographolide is provided in the later sections.

2.5.1 Thin layer chromatography

Thin layer chromatography in its improvised form known as high performance thin layer chromatography (HPTLC) has been used for quantifying the active principles of A. paniculata such as 14-deoxy-11, 12-didehydroandrographolide, andrographolide and neoandrographolide. Andrographolide has also been quantified effectively using toluene, ethyl acetate and formic acid in a ratio of 5.0:3.5:1.5 as the mobile phase, and the silica gel 60 F254 pre-coated aluminium plate as the solid phase (Vijaykumar et al., 2007). Another experiment involving the same stationary phase used chloroform: methanol (7:1, v/v) as the mobile phase. Andrographolide displayed an R_f value of 0.41. The limits of detection and quantification were 30 ng and 100 ng, respectively (Mamatha, 2011).

Andrographolide has also been quantified using a mobile phase of toluene: ethyl acetate: formic acid (5:4.5:0.5, v/v/v). The chromatogram on the silica gel 60 F254 pre-coated aluminium plate was developed with anisaldehyde þ sulphuric acid, followed by heating at 1108C for 10 min. Quantification was carried out densitometrically by UV detection at 235 nm. A 99.92% recovery was obtained with this method. A detection range of 40 ng for andrographolide was found with a mobile phase of chloroform: methanol (27:3, v/v) on a silica gel 60 F254 pre-coated aluminium plate (Sareer et al., 2014). In another HPTLC method developed for the estimation of andrographolide, samples were applied on a silica gel 60 F254 plate and a solvent combination of chloroform: methanol was used (A. Kumar & Pareek, 2007).

HPTLC is better for quality control than the conventional methods because of its simplicity, ease of operation, efficacy, less solvent consumption and possibility of analyzing several samples in a short time. But it has seldom reported due to variation of outputs based on differences in the stationary and mobile phases, the solvent-flow rates.

2.5.2 High performance liquid chromatography

This technique of analytical chemistry extensively used for the quantification of individual components of a mixture still remains the most popular because of its simplicity, precision and sensitivity. Several HPLC methods have been used for the purification and quantification of andrographolide. A Supelco Discovery C18 column was used in a reverse HPLC experiment to quantify andrographolide. The mobile phase consisted of a graded concentration of acetonitrile and water. Detection was done with an evaporative light-scattering detector (ELSD) providing a light detection limit of 50 ng for andrographolide. The calibration curves revealed an excellent linear relationship (Hossain et al., 2021).

The HPLC-diode array detector (DAD) was used to compare the conventional and improved fingerprint profiles of andrographolide and dehydroandrographolide. As reported, there were more peaks within the improved fingerprint. The signals were also more powerful than those produced by traditional fingerprints (Sareer et al., 2014). As in literature, it has been observed that these methods have higher consumption of solvents and lower extraction efficiency. Moreover, for this reason, HPLC coupled with microwave-assisted extraction to allow the on-line determination of andrographolide and dehydroandrographolide in *A. paniculata*. It can therefore be considered that the traditional methods with coupling of different approaches make them promising and may be a good alternative.

2.5.3 Gas chromatography

Gas chromatography, one of the famous chromatographic techniques is very useful in the study to identify the components present in the extract of bioactive herbs. Therefore, as we know that andrographolide is one of the active constituents of *A. paniculata.* As reported (Kalaiselvan et al., 2012) successfully isolated the andrographolide from extract of *A.paniculata* by using a Perkin Elmer GC Claurus 500 system and gas chromatograph interfaced to a mass spectrometer (GC–MS) equipped with Elite-1 fused silica capillary column (100% dimethyl polysiloxane). For GC–MS detection, electron-ionization-energy system with ionization energy of 70 eV was used. Helium (99.999%) was used as the carrier gas at a constant flow rate of 1 mL/min, with an injection volume of 2 mL (Split ratio of 10:1). Injector temperature was 250°C and oven temperature was programmed from 110°C. The total GC running time was 36 min.

In another experiment, 13 compounds were isolated by GC–MS from the leaf ethanolic extracts of the plant in which one of the components is andrographolide. A column packed with Elite-1 (100% dimethyl polysiloxane, 30 nm £ 0.25 nm ID £ 1mf df) was used. The carrier gas used was helium. A flow of 1 mL/min was maintained. Detection was done by turbo mass gold mass detector. Oven temperature was 110°C. MS detection was completed in 36 min (Yanfang et al., 2006). This method is somehow troublesome method due to heavy instrumentation and maintenance of temperatures. However, more dedicated research is needed to characterize fully the bioactive compounds of this plant.

2.5.4 Capillary electrophoresis

Capillary electrophoresis is emerging because of its advantages in terms of speed, efficiency, reproducibility and ease. Such innovations include micellar electrokinetic chromatography (MEKC) and capillary gel electrophoresis. It can be used in isoelectric focusing and affinity electrophoresis. A high-performance CE (HPCE) method has been developed to separate the bioactive constituents (andrographolide and dehydroandrographolide) of A. paniculata. The MEKC has also been used for the determination of neoandrographolide, deoxy-andrographolide and andrographolide in *A. paniculata*. For this, the mobile phase was 20mM borate buffer in 20mM SDS (pH 8.3); a temperature of 35°C and a voltage of 25KV were applied. A fused silica capillary tube of 57 cm length was used. UV detection was done at 214 nm (Safaei & Shishehbore, 2021).

In another experiment, the maximum extraction of compounds was achieved by changing the experimental conditions. Molarity and pH of buffer were varied to 15mM SDS in 30mM borate buffer (pH 9.5); the voltage was decreased to 16KV and the temperature to 25°C. This method yielded a good recovery with stable and repeatable separation. Recovery was done in 13 min only (Vijaykumar et al., 2007). An ethyl-acetate-based microemulsion electrokinetic capillary chromatography with short-end injection method has also been developed for the determination of andrographolide and dehydroandrographolide in A. paniculata. Calibration curves revealed an excellent linear relationship of 0.9984 for andrographolide and 0.9993 for dehydroandrographolide (Sareer et al., 2014).

2.6 Molecular imprinting

Molecular imprinting technology offers the creation of cavities that can recognize and attract a template molecule of particular interest in terms of shape, size, and chemical functionality. Moreover, its principle involved a lock and key model consisting of a molecular lock with its molecular key as a template to synthesize MIPs with artificial binding sites harmonizing the template molecules in shape, size, and functional groups. Thus, it is an easy method of molecular recognition. The following steps are as follows: (1) The imprinted molecule is known as template added with functional monomer and a high proportion of cross-linkers, (2) polymerization is done suitable conditions, (3) polymeric chains organized themselves around imprinted molecules through functional groups, and (4) Washing and removal of imprinted molecules to develop highly bonded vacancies with affixed geometrical structure (Martín-Esteban, 2013; Vasapollo et al., 2011). A schematic presentation of a molecular imprinting process is shown in Figure 2.2.

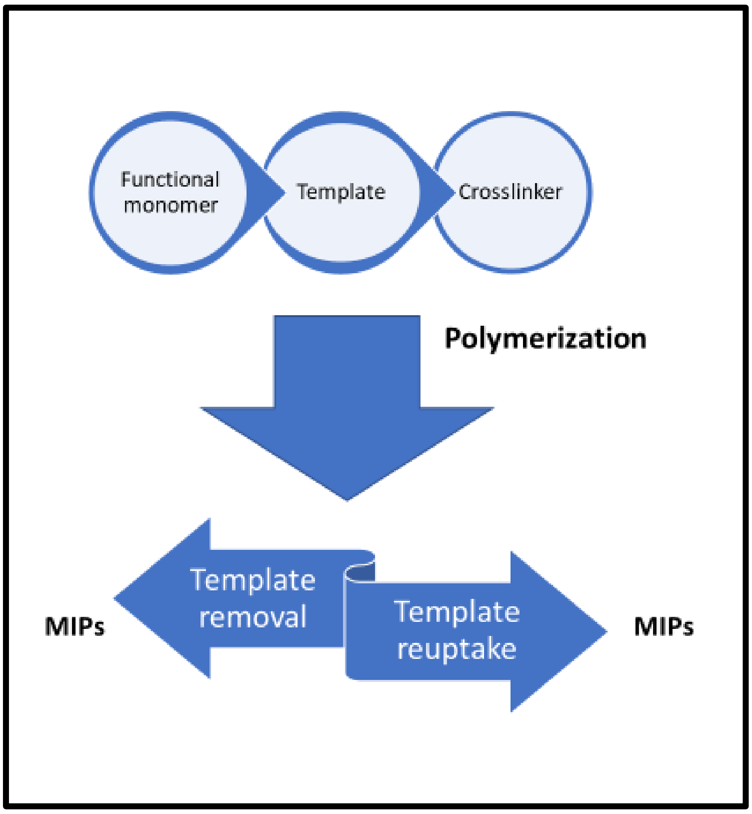

Figure 2.2 A schematic of molecular imprinting process technology.

Many polymers such as polyphenols, poly aminophenyl boronate, polyaniline and polypyyrole have been utilized in various applications. Polymers are considered an innovative class of functional materials (Dong et al., 2021). Among them, polypyyrole (PPy) has proven to be a potential candidate in sensor application for potentiometric, amperometric, conductometric and optical measurement of chemical species (Nezhadali et al., 2018). PPy was first reported in 1916, and it can be prepared through chemical and electrochemical polymerization (Imani et al., 2013). Chemical polymerization is a simple method, but electrochemical polymerization gained more attention due to high-quality films with high conductivities. PPy is a promising polymer used in several applications such as biosensors, batteries, computer displays, supercapacitors, fuel cells, corrosion shielding and antistatic coatings (Zeng et al., 2021). It is the most studied conducting polymer due to its unique features like stability in oxidized form, ease of preparation, high electrical conductivity and good redox properties (Zeng et al., 2021). PPy can be prepared as nanoparticles such as PPy nanorods and PPy nanofibers. Incorporating graphene derivatives for example GQDs and GO with PPy shows enhanced electrochemical performance instead of using them separately in their pure forms (Tan et al., 2016). PPy monomer is shown in Figure 2.3.

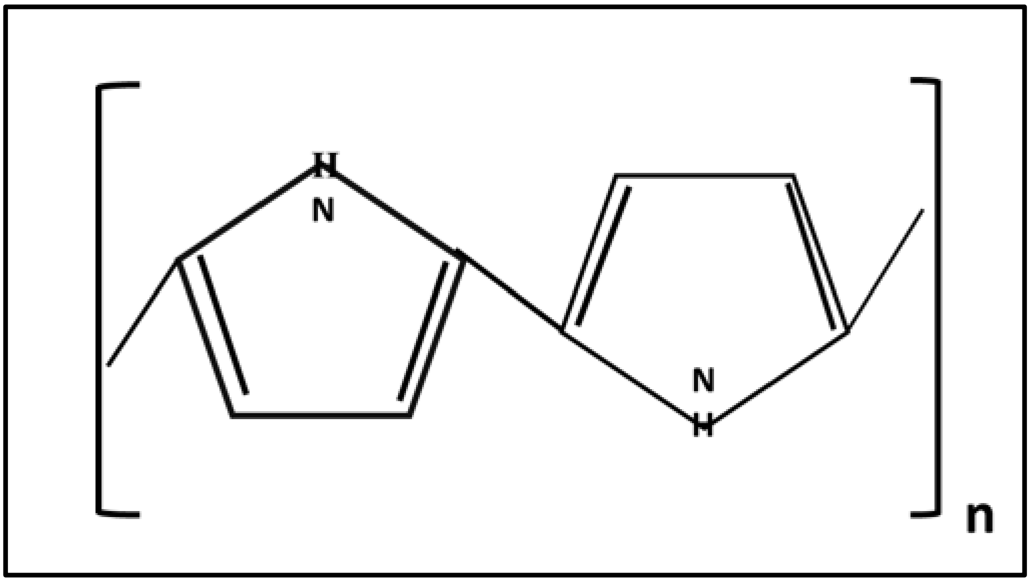

Figure 2.3 A representation of monomer of polypyyrole.

2.6.1 Fundamentals involved in the imprinting process

Fabrication of a MIP is an easy process, but it needs consideration of critical experimental variables. Template, functional monomer, cross-linker, and physical-chemical properties are the experimental variables that must be considered during the fabrication of MIP.

2.6.1(a) Functional monomer

For stable template-monomer complexes, the choice of functional monomer is significant according to its chemical properties in the imprinting process. The stability of the imprinting process depends on the relationship between the functionality of the monomer and the functionality of the template. In addition, it depends on the structure and functional groups of template and monomer. According to the literature, it is stated that templates with acidic groups correlate with monomers of basic groups and vice versa (Martín-Esteban, 2013). Moreover, the poly-monomer imprinted polymer has higher retention and resolution than one monomer due to the enhancement of the attraction property of MIP. The two co-monomers reactivity also ensures that co-polymerization is stable and feasible (Scorrano et al., 2011; H. Yan & Kyung, 2006)

2.6.1(b) Template

A template is considered a target molecule used as an example for fabricating a lock (molecularly imprinted polymer). It works as a key for a specific receptor present in MIP. It marks the physical and chemical analogues on the polymer matrix to detect the same template in different samples. Nowadays, imprints of various templates species have been designed for various ranges of molecular sizes and functional groups. The template choice depends on various factors such as soluble in organic solvents, electrostatic functionalities, and chemically inert under polymerization conditions (Chen et al., 2016).

2.6.1(c) Cross-Linker

The cross-linker in polymerization is used to fix the template-monomer interactions. Therefore, the selectivity of the template is highly dependent on the structure and amount of cross-linker in the fabrication process of MIP. In addition, it provides mechanical stability and rigidity to the polymer matrix and supports the imprinted binding site (Vasapollo et al., 2011).

MIP is also an emerging method in the analytical chemistry field. Now, it has greatly expanded in the materials and application approach also. It has gained attention due to its attractive properties of sorbent with high sensitivity and affinity. In addition, it possesses physical robustness, strength, resistance to high temperature and pressure (Vasapollo et al., 2011). Many researchers reported the analysis of various templates through MIP in chemical, biological, and pharmaceutical fields, leading to advancements in immunoassays, separation, adsorption, catalysis, biosensor, and drug delivery (Çimen et al., 2021; Ma et al., 2021)

2.6.2 Removal of the Template

Removal of templates from the fabricated MIP is done by various methods. Some standard methods are the soxhlet extraction method, washing online, and solid-phase extraction. In addition, various reports with supercritical fluid extraction, microwave-assisted extraction, and ultrasonic-assisted extraction have also reported removing the template. Recently, for environmentally-friendly purposes, pressurized hot water extraction method is also used for template removal (Batlokwa et al., 2011).

2.7 Classes of MIP

The conventional techniques of imprinting are still used but with different modifications. Moreover, there are three types of MIP providing a preferable result of synthesizing of MIPs. Classification with further sub-branches of MIP is shown in Table 2.1.

Table 2.1 Classes of MIP according to different processing (Chen et al., 2016).

Indigneous imprinting technology	Special imprinting technology	Stimuli-responsive imprinting technology
• **Surface imprinting technology**	• Multi template/functional imprinting technology	• Magnetic/thermoresponsive technology
• **Nanoimprinting**	• Dummy segment imprinting technology	• Dual/multi responsive technology
• **Hollow porous polymer synthesis technology.**	• Composite imprinting material strategy	• pH-responsive technology
• **Click chemistry cycloaddition reaction technology**	• Segment imprinting technology	• Photoresponsive technology
• **Living/controlled radical polymerization technology**		
• **Solid-phase synthesis technology**		

2.7.1 Indigneous imprinting technology

Molecular imprinting has generated lots of new technological advances, including surface imprinting, nanoimprinting, living/controlled radical polymerization, porous polymer synthesis imprinting, click chemistry cycloaddition reaction and Solid-phase synthesis (Vasapollo et al., 2011). The imprinting method will open up new avenues for fascinating projects. applications. Recently, the development of molecular imprinting nanotechnologies has attracted considerable research interest in that nanostructured MIPs (N-MIPs) because of high area-to-volume ratio, good accessibility to target species and significant binding kinetics and capacity. Moreover, recently atom transfer radical polymerization (ATRP) and reversible addition– fragmentation chain transfer (RAFT) has succeeded in the preparation of surface initiated and emulsion type MIPs, where acidic monomers like methacrylamide have been employed, which is an important development in the MIP field since it considerably widens the range of functional monomers (Zhang, 2013). Further, the solid phase synthesis is also one of the methods that emerged with advantage that all binding sites had the same orientation and were located at the surface of the glass beads, thus improving binding site homogeneity and accessibility. However, this class offered short synthesis/ purification times, thus making it potentially suitable for industrial applications and production.

2.7.2 Special imprinting technology

Aside from Indigneous imprinting technologies, some unique imprinting strategies evolved during the development process, including as multi-template/functional imprinting, dummy imprinting, composite imprinting and segment imprinting strategies (Chen et al., 2016). The introduction of special imprinting