Development of sustainable method for the Quality Control of traditional medicines on the basis of phytoequivalence and chemical fingerprinting

A dissertation Submitted to Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka in partial fulfillment of the requirements for the degree Doctor of Philosophy in Pharmaceutical Chemistry

by

Sabiha Ferdowsy Koly

Session: 2020-2021; Registration no-20/2020-2021 (New)

Department of Pharmaceutical Chemistry

Faculty of Pharmacy

University of Dhaka-1000, Bangladesh

DECLARATION

I hereby certify that the dissertation that I have submitted for Doctor of Philosophy entitled as **"Development of sustainable method for the Quality Control of traditional medicines on the basis of phytoequivalence and chemical fingerprinting"** is my original work and has not been submitted before to be considered for any degree.

I further declare that the thesis has properly acknowledged the use of any material from outside sources. If any plagiarism or other abnormalities are found in the thesis, I will be held completely responsible for this.

Signature of the Research Scholar: Date:

Name of Researcher: **Sabiha Ferdowsy Koly**

CERTIFICATE

I attest that the candidate worked under my supervision. The research work entitled as **"Development of sustainable method for the Quality Control of traditional medicines on the basis of phytoequivalence and chemical fingerprinting"** is submitted by **Sabiha Ferdowsy Koly**. According to my knowledge: (i) The submitted thesis is a record of the Research Scholar's original work conducted during the period of study under my supervision, (ii) The thesis represents independent research work on the part of the Research Scholar and (iii) The candidate has not submitted the same research work to any other institution for any degree/diploma, associate ship, fellowship or other similar titles.

Supervisor

Professor Dr. Md. Shah Amran
Department of Pharmaceutical Chemistry, University of Dhaka-1000, Bangladesh
and
Co-supervisor

Professor Dr. Abu Asad Chowdhury
Department of Pharmaceutical Chemistry, University of Dhaka-1000, Bangladesh

ABSTRACT

Background: Herbal drugs are composed of single or several types of medicinal plants with additives. This type of preparation is gaining a wide range of popularity among a large a number of people throughout the world. With the increase of usage, it is necessary to maintain the quality of these drug preparations. Therefore, analytical approaches for their intended use in drug quality evaluation need to be validated. This study was planned to develop simple, rapid, selective, precise and economical method for the quality control of herbal preparations.

Methods and Materials: Infrared (IR) spectroscopy was used to determine phytoequivalence among the samples. This work outlined a method for identifying herbal drugs on the basis of phytoequivalence. However, as this is a time-consuming and expensive process, the current analysis did not attempt to identify the specific chemical compounds found in the herbs. The marked bands served as a comparative tool for herbal plants and the medications made from them. The quantitative method of comparison was applied for the comparison of crude herbal standard with herbal sample containing formulations.

Thin Layer Chromatography (TLC) was used as another analytical method for identification and showing phytoequivalence. It has mostly been used for the qualitative analysis of herbal medicines and to characterize and track the components visually or as an initial separation technique.

Single or multiple herbs containing preparation can be analyzed using ultraviolet (UV) spectroscopy. In this technique, it involves the measurement of ultra violet radiation absorption by the substance dissolved in solution. Both qualitative and quantitative analysis can be done through this technique. In the present study, it was aimed at development and validation of UV-spectroscopic technique according to International Conference on Harmonization guidelines which is known as ICH (Q2) guidelines for the analysis of herbs containing polyherbal formulation. The method validation parameters like specificity, precision, accuracy, linearity, range, repeatability and robustness were studied according to ICH (Q2) guidelines.

Results and Discussion: Amlaki (*Phyllanthus emblica*) showed the presence of prominent peak and maximum absorption at 303 nm. The detector response for the Amlaki was linear over the selected concentration range of 1 to 5 μg/mL with a correlation coefficient of 0.998. The absorbance values for intraday precision, found

for 1 µg/mL, 3 µg/mL and 5 µg/mL were 0.0082, 0.234 and 0.396 having %RSD of 0.998%, 0.0080% and 0.0058%, respectively. The absorbance values for intermediate precision, found for 1 µg/mL, 3 µg/mL and 5 µg/mL were 0.0084, 0.234 and 0.398 having %RSD 0.6846%, 0.0137% and 0.0038%, respectively. The absorbance value for repeatability was 0.084 having %RSD 0.6901. The accuracy was between 99.348% and 101.478%. Robustness of the method was studied. The %RSD for analyst to analyst variation was 0.4851% and instrument to instrument variation was 0.9726%. The assay results of Amlaki were about 86.588%, 82.150% and 90.828% for three market preparations A, B and C, respectively, indicating insignificant interference from the other ingredients in the formulation.

Black plum (*Syzygium cumini*) showed the presence of prominent peak and maximum absorption at 279 nm. The detector response for the *S. cumini* was linear over the selected concentration range of 0.1-0.5 µg/mL with a correlation coefficient of 0.9914. The absorbance values for intraday precision found for 0.1 µg/mL, 0.3 µg/mL and 0.5 µg/mL were 0.234, 0.432 and 0.735 having %RSD 0.9245%, 0.5751% and 0.4668%, respectively. The absorbance values for intermediate precision found for 0.1 µg/mL, 0.3 µg/mL and 0.5 µg/mL were 0.233, 0.432 and 0.736 having %RSD 0.8921%, 0.8346% and 0.3421%, respectively. The absorbance value for repeatability was 0.225 having %RSD 0.7950%. The accuracy was between 99.647% and 101.943%. Robustness of the method was studied. The %RSD for analyst to analyst variation was 0.8251% and instrument to instrument variation was 0.3609%. The assay results of *S. cumini* were about 83.152%, 86.821%, 90.082% and 80.579% for four market preparations A, B, C and D, respectively, indicating insignificant interference from the other ingredients in the formulation.

During the method development phase, a number of solvents were used. Among them, methanol was selected in analysis of Amlaki and ethanol was selected in analysis of black plum. These solvents were selected as these solvents satisfied all the conditions relative to peak quality and non-interference at the specified wavelength. The wavelength of maximum absorption (λ_{max}) was found to be 303 nm and 279 nm in Amlaki and Black plum, respectively. As the values of %RSD is <2%, the method is validated according to ICH (Q2) guidelines.

Conclusion: It can be capitulated that this method can be conveniently employed for routine quality control analysis of herbal drugs in bulk drug and other formulations.

Table of contents

List of Tables

List of Figures

List of Abbreviations

Conc	Concentration
AR	Analytical Reagent
FDA	Food and Drug Administration
BP	British Pharmacopoeia
QC	Quality Control
HCl	Hydrochloric Acid
HPTLC	High Performance Thin Layer Chromatography
ICH	International Conference on Harmonization
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantitation
mg	Milligram
µg	Microgram
ml	Milliliter
ng	Nanogram
SD	Standard Deviation
RSD	Relative Standard Deviation
WHO	World Health Organization

CHAPTER ONE: INTRODUCTION

1.0 Introduction

Nature has adorned herself with various ingredients which are beneficial for humans. Nature has provided humans with a vast array of various herbs, each with a wealth of medicinal characteristics that can be used to cure and prevent a variety of maladies. Plants are considered a principal source of different drugs. A large number of plants contain different compounds which are responsible for showing different types of pharmacological activities [1].

1.1 Traditional medicine and Herbal drugs

Traditional medicines are being used from ancient times to treat diseases. Traditional medicines (TMs) employ the usage of natural products and many of them are plant derived. The custom of employing plant derived material for medical purposes is known as herbal medicine or "phyto-medicine". Herbal medicine is a crucial component of alternative medicine. Different parts of the plant are used in herbal medicine. Herbal medicine is an oldest form of medicine. The Latin word "herba" and the old French word "herbe" are the origins of the word "herb". A flowering plant that often dies back at the conclusion of each growing season and whose stem doesn't produce woody tissue is referred to as "herb". Different parts of herbs like leaves, stem, flowers, fruits, seeds and roots have therapeutic potential. Whole plant, plant parts or plant materials that have been processed or are still in their raw state can be utilized as excipients in herbal remedies [2-3]. These compounds actively defend plants from harm and pathogens while also enhancing their flavor, color and scent. In the scientific community, they are referred to as phytochemicals and comprise saponins, flavonoids, glycosides, tannins, alkaloids and terpenoids [4]. Over time, it has become scientifically established that phytochemicals have advantages for human health [5]. Herbal sedatives and stomachic mixes, for example, mostly contain different compounds having different therapeutic properties. They can show antibacterial, antispasmodic, stomach-soothing and many other pharmacological activities. Plants with a rich tannin containing content are employed in combinations for treating of diarrhea and stomach ulcers, with antibacterial properties.

Outside of traditional medicine, herbal therapy has a long history of use from ancient times. Traditional use of this medicine is documented in written books for some cultures, while traditional knowledge and applications have been verbally passed on from generation to generation to the next for others. Herbal medicines are also playing

a growing role in worldwide healthcare, where they are finding new and growing markets for nutritional products and preventative drugs. Wild gathered and grown medicinal plants are considered as the primary sources of supply and there is growing demand for a consistent repository of high-quality material. Herbal goods have a global market of around $60 billion per year. The global “functional food” and dietary supplement sectors are expanding rapidly, necessitating greater volumes of high-quality botanical components.

1.2 Benefits of Herbal medicine

As herbal medicine is a natural entity and it aims at restoring body’s natural balance it shows less side effects. The current popularity of herbal treatment is based on real data and testimonies. But, the rate at which herbal therapy has grown in popularity is worrying. It’s also worth noting that benefits of herbal treatment include:

(I) The value and accessibility of herbal goods: Medical care and pharmaceutical treatments are growing increasingly expensive and the average person can no longer afford them. Herbal medicine has become a viable alternative to modern treatment as more and more people testify to its rising effectiveness and reduced incidence of adverse effects.

(II) Availability: Supplements, herbal teas, extracts, essential oils and other herbal goods are widely available. They can be found in health food stores, which are cropping up all over the place, as well as on the internet and in pharmacies. Furthermore, as these products are derived from plants it is not needed doctor’s prescription to buy them.

(III) Efficacy: The effectiveness of these plants in treating various disorders has been established. Several researches have been conducted to verify their effectiveness. There are herbs that can be used to cure common colds and others that can be used to treat more serious ailments like cardiovascular disease, diabetes, and cancer.

(IV) Increasing the body's defenses: Herbal products don't obstruct the body's natural physiological functions they on the contrary, encourage such processes. Herbal products, in particular, enhance the immune system due to their chemical components. They interact with every aspect of the system to improve its performance. As an example, we can say that the antioxidant enhances our immune system by providing adequate antioxidants containing chemicals present in plants.

1.3 Historical perspective of herbal medicine

According to ancient Babylonian records, the usage of plants as medicines stretches back 60,000 years. Written data on herbal treatment extends back roughly 5000 years in Egypt and China. It is recorded that about 2500 years ago, the people of Asia Minor and Greece started practicing herbal treatment [6]. The techniques and philosophy of diverse herbal therapeutic systems are affected by the place in which they initially originated [7]. Traditional Chinese medicine is a system that has been practiced in China throughout history [8]. About 2000 years ago, "The Devine Farmer's Classic of Herbalism" was compiled in China. It is considered as the oldest known herbal book in the world. A number of herbal pharmacopeias and different monographs of using specific herbs certify the usage of herbal medicine from ancient times [9]. One form of ancient Indian medicine is Ayurveda.

1.3.1 African traditional medicine

Traditional African medicine system is one of the oldest and most diverse medical systems in the world. Africa is regarded as the birthplace having a diverse biological and cultural heritage and distinct regional healing methods. Regrettably, medicine systems are still poorly documented today. Due to the increasing loss of these plants' native habitats caused by human activity, recording of medical uses of African plants is becoming more significant. According to reports, one of the greatest rates of deforestation worldwide is seen in Africa. The high endemism rate of the continent, with Madagascar at the top of the list with 82 percent, makes this loss more pronounced [10]. All kinds of traditional African medicine are holistic and address both the physical body and the psychological self. The healer typically examines and treats the both physical and psychological cause of the illness before to providing medications to alleviate the symptoms.

1.3.2 Traditional medicine in America (North, Central and South)

1.3.2.1 North America

The indigenous healer or Shaman in the United States, like in many other cultures, addressed both the medical and spiritual components of sickness. Chanting, dancing and other rituals are used in these Shamanistic rites to expel bad energies and heal either the sufferer or the entire community. Early immigrants learned from indigenous methods and subsequently embraced many of the herbal treatments that became the foundation of the early United States Pharmacopoeia. Goldenseal (*Hydrastis*

canadensis) and Echinacea (*Echinacea purpurea*) are two well-known medicinal plants in the United States.

Herbs were viewed with mistrust for the most of the twentieth century, and herbal medicine practice declined. Plants were primarily seen as a possible source of pure chemical substances for medical research. Herbs and botanicals have been increasingly popular in the United States and Canada in recent years, although they are still regarded as nutritional supplements rather than standalone medications [11-13].

1.3.2.2 Central and South America

Countries in Central and South America, like Africa, possess a wealth of various healing traditions that are poorly understood and documented. In the coming years, they will undoubtedly be a source of innovative herbal treatments. The utilization of conventional American Indian herbal remedies is common, however, the impact of Spanish, European, East Asian and African system of medicine is clear [14].

1.3.3 Southeast Asian and Australian medicine

Traditional medicine has regained popularity in this region, and several governments now promote study of pharmacological activity of plants as a possible source of novel treatments. Although the Aborigines possessed a sophisticated healing process, before it could be carefully recorded, Australia's most of traditional knowledge was lost. Many healing techniques in Malaysia, New Zealand, Thailand, Vietnam, Borneo and the Polynesian Islands, on the other hand, have remained intact and are being recorded and developed. In most countries, there is a considerable Chinese impact [15-17].

1.3.4 Ayurvedic medicine (Indian traditional medicine)

Ayurveda is considered as one of the oldest world health care systems in the world. It is thought to be the beginning of systemized medicine and is likely older than traditional Chinese medicine. It is, in fact, a realistic and holistic set of principles for maintaining system balance and harmony. Ayurveda is the science of life, derived mainly from the Indian terms “Ayur” and “Veda” where Ayur signifies life and Veda signifies (knowledge or science). This system of medicine treats spiritual, mental and physical condition. Through following this system, it helps a person to live a long life, which is seen to be the key to gaining righteousness (dharma), riches (artha), and happiness (sukha). Ayurvedic treatment also relies heavily on herbs [18-19].

1.3.5 Chinese traditional medicine

China and India had flourishing civilizations while Europe was producing just somewhat advanced cultures. Articles about medicinal plants say the beauty of greenery were plentiful. This ancient medical system, which is thought to be written over 5000 years ago, is based on two different beliefs regarding the natural principles that govern health. According to this medicinal system it has five elements and yin and yang are the keys to long life and health [20-21].

Chinese herbs are frequently prescribed in fixed combinations or formulas containing up to 20 herbs, meticulously assembled according to old traditions, just like Western and African traditional medicines. Hundreds of such recipes are utilized in conjunction with Western medicine. Traditional recipes, as in other healing systems, are preferred for chronic ailments, whereas Western drugs are utilized to treat acute or serious illnesses. With the dissemination of traditional Chinese medicine across the majority of the global continents has boosted the acceptability of herbal medicines today.

1.3.6 European medicine

The Greeks made important contributions to the logical evolution of herbal medication use in the ancient Western civilization. Hippocrates and Aristotle are credited with developing the European healing system, which was based on ancient Indian and Egyptian beliefs. In his History of Plants, the great philosopher and scientist Theophrastus (300 BC) discussed the therapeutic properties of herbs and observed the capacity to substitute their traits through culture. At the time of his travels with Roman armies, the gathering, storage and application of medicinal herbs were documented by a Greek physician, Dioscoridesin the year 100 AD, and Galen, a Roman physician and pharmacist who worked and lectured in Rome from 130 to 200 AD, wrote at least 30 books on these topics and is considered the father of modern pharmacy [22].

According to Roman and Greek medicine, the world is made up of earth, wind, fire and water are the four elements. The four humors-blood, phlegm, black bile and yellow bile, respectively sanguine, phlegmatic, melancholy, and choleric-determine both health and character.

Extreme measures were used to restore equilibrium, including bloodletting to remove extra blood and purging to get rid of extra black bile. Each of the four humours had a matching variety of cold, hot, damp, or dry plants that were thought to be able to

correct imbalances. Cold, heat, wetness and dryness were likewise connected to the four humours. Both European tradition and local folk practices and traditions were influenced by a variety of regional factors.

Many ancient herbal remedies have gained popularity in Europe owing to commercialization. From medicinal plants, various active chemicals have been extracted and they are currently employed as different chemical entities.

1.3.7 Traditional North African medicine and classical Arabic medicine

The oldest recorded information in Arabic traditions comes from Mesopotamia's Sumerians and Akkadians and hence originates from the same locations as Shanidar IV's archeological records. The earliest known record of medicinal plants was discovered in the grave of a Neanderthal man from Shanidar IV, an archeological site in Iraq, 60 000 years before the common era (BCE). The Middle East is known as the "cradle of civilization" and many of the plants grown today originated there. Herbal cures were written in cuneiform writing on countless clay tablets by the Babylonians, Assyrians, and Sumerians. The Code of Hammurabi (about 1700 BC) is a complete system of civil laws etched in stone and commissioned by a king.

1.4 Pharmacological Activity of Herbal drugs

Medicinal plants act as a repository of numerous types of bioactive compounds which possess varied therapeutic properties. Different plant species contain a diverse range of primary and secondary metabolites which are responsible for showing different types of pharmacological activities. The vast array of therapeutic effects associated with medicinal plants includes anti-inflammatory, antiviral, antitumor, antimalarial, analgesic and many other properties.

1.4.1 Anti-inflammatory activity

Inflammation is an essential part of body's healing process. It is a step in the immune system's defense against dangerous substances like germs and viruses by generating a biological reaction. Inflammation may be initiated by radiation, environment-related substances, injury-causing pathogens (germs) such as bacteria, viruses, or fungus, insect stings, scrapes. Immune cells, blood arteries and chemical mediators all participate in the body's defensive response. Different substances are produced from tissues and migratory cells. Prostaglandins (PGs), leukotrienes (LTs), histamine, bradykinin and, more recently, platelet-activating factor (PAF) and interleukin-1 are those most significantly implicated. It fights against infection and injury. It is

beneficial to health but it is damaging when it occurs in healthy tissues or lasts too long. Inflammation can cause gastrointestinal problems including inflammatory bowel disease, autoimmune conditions like rheumatoid arthritis, cardiovascular conditions such high blood pressure and heart disease, lung conditions such as asthma, conditions such as depression, disorders of the metabolism, such as type 2 diabetes, diseases of the nervous system, such as Parkinson's disease. Inflammation can lead to certain cancers, such as colon cancer. Different species of medicinal plants can play important role to stop this inflammation and can show anti-inflammatory activity [23].

1.4.2 Analgesic activity

In medicine and dentistry, one of the most dreaded sensations is pain. Pain occurs due to different disorders in the body. Endorphins are produced to help relieve pain, reduce stress and improve mood. It is the body's natural painkillers. Endorphins are released by the hypothalamus and pituitary gland in response to pain or stress. But every time, only body's natural defense mechanism is not capable to reduce pain alone. In that case we need analgesics for pain management. Analgesics are substances that, without altering awareness, selectively reduce pain by acting on peripheral pain mediators and the central nervous system. There are two broad categories of analgesic medications: nonopioid analgesics, which is known as nonsteroidal anti-inflammatory drugs (NSAIDs) and opioid analgesics. Both classes of drugs have side effects like as NSAIDs act through blocking COX pathways it stops prostaglandin release it can cause ulcer to gastric mucosa and opioid analgesics can create narcosis. Herbal drugs can show lesser side effects. Moreover, in search of new drugs, studies are being done continuously. *Justicia gendarussa, Sansevieriatri fasciata, Calliandra surinamensis, Musaenda philippica, Steudneravirosa, Trewia polycarpa* plants showed significant analgesic activities [24].

1.4.3 Antimicrobial activity

Microorganisms create different diseases in our body. Different types of drugs are used to treat this. But these drugs can create antibiotic resistance. To avoid this resistance, development of alternative drugs has become a major concern now days. Plants are rich in a wide variety of secondary metabolites, such as tannins, terpenoids, alkaloids and flavonoids. These compounds have antimicrobial properties. Plants rich in these compounds can be a major source of antibiotics. Studies found that *Punica granatum, Cuminum cyminum, Hymus vulgaris, Zingiber officinales, Syzygium aromaticum* and a number of plants show antimicrobial properties [25].

1.4.4 Antidiarrheal activity

Diarrhea is a state of condition where typical bowel movement is altered by an increase in the water content, volume, or frequency of stools. *Myrtus communis* L, *Acacia leucophloea, Allium cepa* L., *Citrus limon* L., *Glycyrrhiza glabra* L. , *Helicteres isora* L., *Plumbago zeylanica* L., *Plumbago zeylanica*L. show antidiarrheal activity [26].

1.4.5 Antidiabetic Activity

One of the most prevalent endocrine metabolic disorders is diabetes mellitus. It is defined by hyperglycemia coupled with impaired insulin secretion as well as abnormalities in the metabolism of carbohydrates, proteins and lipids during the intermediate phase. The number of diabetic patients is increasing day by day. People with diabetes have elevated blood glucose levels as a result of an insulin insufficiency. Diabetes mellitus can cause long-term damage, dysfunction and failure of various organs of our body. It can cause cardiovascular, peripheral vascular and cerebrovascular diseases. Plants containing steroids, carbohydrates, glycopeptides, terpenoids, galactomannan, polysaccharides, peptidoglycans, hypoglycans, guanidine and amino acids can show antidiabetic activity. *Allium cepa L., Cinnamomum tamala, and Clerodendron phlomoides Linn (Buch.-Ham.) Cannabis indica (Lam.), Syzygium cumini L., Casearia esculenta Roxb., Cephalandra indica* plants are reported to show antidiabetic effects [27-29].

1.4.6 Antioxidant Activity

Many communicable and non-communicable diseases occur due to oxidative stress. Oxidative stress results in different types of diseases like CKD, hypertension, endothelial dysfunction, dementia, cancer, stroke, various neurological disorders and other chronic diseases. Potent antioxidants are necessary to treat these diseases. Herbal drugs rich in compounds containing phenolics, flavonoids, anthocyanins, lignans and stilbenes and tannins can act as antioxidants. *Allium sativum, Capsicum annuum, Curcuma longa, Eugenia caryophyllus, Geranium sanguineum* and many other plants contain antioxidants [30].

1.4.7 Antihypertensive Activity

Hypertension is a condition where blood pressure increases than normal level having a systolic blood pressure (SBP) of ≥140 mmHg and a diastolic blood pressure (DBP) of ≥90 mmHg. It can cause different types of heart diseases and sometimes death. Different types of antihypertensive drugs are used to treat hypertension. *Hibiscus*

sabdariffa, Andrographis paniculata, Graveolens apium, Coptis chinensis, Camellia sinensis, Crocus sativus, Coriandrum sativum, Salvia miltiorrhizae, Zingiber officinale, Nigella sativa, Rauwolfia serpentina have antihypertensive activity [31].

1.4.8 Anti-asthmatic activity

Asthma is a chronic inflammatory respiratory disease which is characterized by an obstruction, eosinophilia and bronchial hyperresponsiveness [65]. Asthma can be control by the use of anti-asthmatic compounds. *Aerva lanta, Ageratum conyzoides, Argemone Mexicana, Asystasia gangetica, Bacopa monnieri, Cassia sophera, Casuarina equisetifolia, Clerodendrum Serratum,Crinum glaucum, Cnidium monnieri* are reported to show anti asthmatic activity [32].

1.4.9 Antimalarial activity

Malaria is a mosquito borne disease. It is spread through the larvae of *Plasmodium* genus containing species like as *Plasmodium faliciparum, Plasmodium vivax*. It has become one of the major concerns in developing countries. It can be fatal for children and pregnant woman. It may be controlled by early detection and taking immediate actions. Quininine, choroquinine, artemesin are commonly used drug to treat malaria. *Alstonia boonei, Picralima nitida* and *Rauvolfia vomitoria, Annona muricata, ernonia amygdalina, Bidens pilosa, Microglossa pyrifolia, Conyza sumatrensis* are found to have antimalarial activity [33].

1.4.10 Anti-cancer activity

Cancer is a state of condition where when the body's normal control mechanism stops working and results in uncontrolled growth of abnormal cells in the body. It can grow abnormal number of cell in the body, sometimes mass of tissue. As they spread, cancerous cells have the potential to harm good organ function. This could lead to a decrease in oxygen delivery and an accumulation of waste products. If a vital organ's function is disrupted, death could follow. The body's normal cells divide and grow normally for a while before ceasing to do so. After then, they only multiply themselves when it's required to replace damaged or dead cells. When this cellular reproduction process spirals out of control, cancer develops. When any damage to the cells' DNA occurs it causes abnormal division and growth of cell. Some phytochemicals are rich sources of anticancerous components which can be used in cancer treatment. Flavonoids, tannins, curcumin, resveratrol and gallacatechins all are considered to be anticancer compounds. Epipodophyllotoxin obtained from

Podophyllum peltatum L, Vincristine, Vinblastine, Vindesine, Vinorelbine, Vinflunine obtained from *Catharanthus roseus* have anticancerous property [34].

1.5 Herbal medication safety and toxicological concerns

With the increasing usage of herbal medicine products, the evaluation of the safety and effectiveness of herbal medicine has become an increasing topic in recent years. It is necessary to ensure the quality of the medicinal herbal products by using different methods and appropriate standards. Standardization is used to validate the identity, quality and purity of herbs and herbal products. The purity of herbs is influenced by preliminary identification, physical properties, chemical properties and biological properties. The quality and freshness of the herbal items are determined by their purity. A single plant's herbal medicine may include hundreds of ingredients, and combination formulations may contain many more. It would take a long time to extract every single active component from every herb. Furthermore, many countries lack the necessary operational infrastructure to legitimize manufacturing quality norms and practices. As a result, sometimes herbal products may become more dangerous without proper quality control. In order to demonstrate why herbal medicines and their formulations are acceptable in the current medical system, quality control is crucial. So it has become a prior concern to develop new methods and validate the methods for the quality control of herbal drugs [35].

1.6 Difficulties with quality control

As acceptability of herbal formulations is in increase in modern system of medicine it’s the importance of quality control is also increasing. The lack of a reliable source for herbal substances and their formulations, as well as their poor quality is a significant issue for the herbal medication market [36]. Temperature, the use of fresh plants, light exposure, nutrients, water accessibility, the period and time of harvest, the method of harvesting, drying, packing, storing and transporting raw herbal material, among other factors, can have a significant impact on the beneficial value and quality of medicinal plants. Low temperatures are required for drying plants containing specific heat-sensitive plant components.

Analytical techniques are used to identify pharmaceuticals and determine their identification, purity, physical features and potency. Drug testing against standards is supported by methods that are established for production, quality release and long-term stability research. Methods may also be used to help drug characterization and

safety research. An integral part of analysis is development of new method and validation of that method. A well-developed analytical method can contribute to the overall development and save time and cost of an industry. The process of developing new methods continues along with the expansion of the drug production.

To show that an analytical method is appropriate for its intended function, it must be validated before it may be utilized in medication development or production. Additionally, due to the nature of its products, the pharmaceutical business is subject to strict laws worldwide.

We can identify some reasons why analytical method development is critical for any drug company developing new drug candidates.

First off, the quality of a drug is undoubtedly at the heart of a pharmaceutical development program's chances for success, so pharmaceutical companies creating novel compounds must take the development of analytical methods extremely seriously.

Second, regulatory organizations from all over the world require analytical technique validation for both clinical trial applications and marketing authorizations.

To assure patient safety and perhaps, observe potential efficacy in novel treatments, it is crucial to design and manufacture excellent medicines.

In several working groups of national and international bodies, the parameters for method validation have been established and are published in the literature. Regrettably, certain definitions fluctuate amongst the various organizations. Through the ICH, representatives from the industry and regulatory organizations from the United States, Europe and Japan defined the parameters, requirements and to some extent, methodology for the validation of analytical methods. This was an attempt at harmonization for pharmaceutical applications [37].

1.7 The various parameters for method development and validation

1. Selectivity/Specificity
2. Precision and Reproducibility
3. Accuracy and Recovery
4. Stability
5. Range
6. Limit of Detection
7. Limit of Quantitation

8. Repeatability
9. Reproducibility
11. Sensitivity
12. Ruggedness

These features are in accordance with Validation of Analytical Procedures: Text and Methodology ICH Q2(R1).

1.7.1 Specificity

The ability to clearly evaluate the analyte in the presence of components that would be anticipated to be present defines the specificity of an analytical method. In the presence of large amounts of other chemicals that are similar but not identical to the target substance, a highly specific approach will still be able to detect it. Specificity is the capacity to evaluate the analyte categorically in the presence of components that may be expected to be present, such as impurities, degradation products and matrix. Specific criteria must be followed while evaluating specificity throughout the projected range of values.

1.7.2 Linearity

Linearity is an analytical method's capacity to provide test outcomes that are proportional to the concentration of the analyte in the sample or by a clearly established mathematical transformation. A non-linear model can be employed if linearity cannot be achieved. Any quantitative technique must show a clear quantitative link between the quantity of the item of interest in the tested sample and the assay measurement.

1.7.3 Accuracy

The degree to which test findings produced by a procedure are in close agreement with the true value, which is either acknowledged as a standard true value or reference value is called accuracy. The computation of the percentage of recovery of a known additional amount of analyte in a sample or the use of reference standards are frequently used in the evaluation of accuracy. The accuracy should be evaluated over the expected range of values for the analytical method because it is intended to be correct over its entire range.

1.7.4 Precision

The degree to which multiple measurements of the same homogeneous sample taken under a specified set of experimental conditions agreed is called precision. It should

reflect the reliability and repeatability of the procedure. It must be assessed across the anticipated range of measurements, much like in the case of accuracy determination.

1.7.5 Range

The range of an analytical method is the space between the higher and lower analyte concentrations in the sample (including these concentrations), for which it has been shown that the method exhibits the necessary linearity, precision and accuracy.

1.7.6 Limit of detection

The lowest concentration of analyte in a sample that, under specific experimental conditions, can be detected but not always quantitated as an accurate value is known as the detection limit of an analytical procedure. It is the smallest amount of tested material that is present in the sample under analysis and yields a signal above noise.

1.7.7 Limit of Quantitation

The smallest possible concentration of analyte in a sample is quantitatively identified with enough precision and accuracy under specific experimental conditions is known as the quantitation limit of an analytical method. The lowest point in the method's range will be determined by the quantitation limit that has been defined.

1.7.8 Robustness

A technique's robustness, which assesses how unaffected it can be by modest but intentional changes in method parameters, gives a clue as to how reliable it will be under typical conditions.

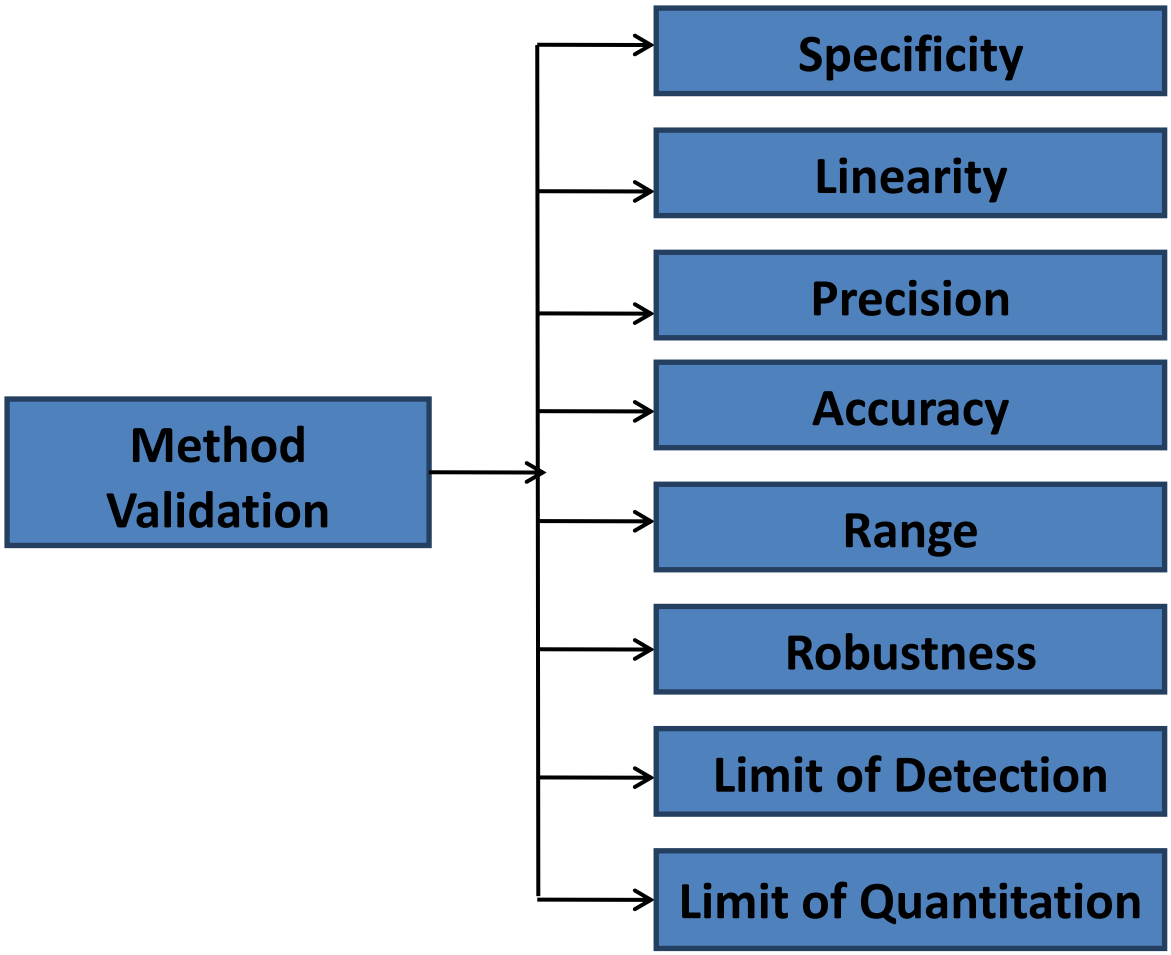


Figure 1: Parameters for method validation.

Amlaki containing drug formulation is prepared from the whole fruit of *Phyllanthus emblica* also known as Indian gooseberry, Malacca tree, or amla of the family Phyllanthaceae.

1.8 Taxonomy of *Phyllanthus emblica*

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Malpighiales

Family: Phyllanthaceae

Genus: *Phyllanthus*

Species: *Phyllanthus emblica*

Amalaki fruit has six vertical stripes or furrows, each holding typically two seeds, and is fleshy, spherical, light greenish yellow, smooth, and firm in appearance. The seeds are 4-5 mm long and 2-3 mm wide, and each weighs between 572 and 590 mg. The

numerous types of amalaki fruit were discovered to differ in size, shape and weight. Distinct types of Amalaki have different compositions. Amalaki fruits contain about moisture 81.2%, protein 0.5%, fat 0.1%, carbs 14.1%, mineral matter 0.7%, fiber 3.4%, Ca 0.05%, K 0.02%, Fe 1.2 mg/100g, nicotinic acid 0.2 mg/g, quercetin, hydroxymethyl furfural, and ellagic acid.

(a) *Phyllanthus emblica* fruits

(b) Powder of *Phyllanthus emblica* fruits

Figure 2: Amlaki (*Phyllanthus emblica*) fruits (a) and its powder (b).

Fixed oil, phosphatides and a minor amount of essential oil are all present in different amounts in amalaki seeds. Physical and chemical characteristics of the fixed oil yield

(16%) include the following: Saturated fatty acids 7%, acetyl value 2.03, acetyl value 12.7, acetyl value 185, iodine value 139.5, unsaponifiable matter 3.81%, sterol 2.70%, linolenic acid (8.78%), linoleic acid (44.0%), oleic acid (28.40%), stearic acid (2.15%), palm oil 5. Oleic acid and linoleic acid (18:2n-6), two unsaturated fatty acid are abundant in the seed oil [38-40]. Amalaki leaves have the appearance of pinnate leaves because they are subsessile, tightly spaced along the branchlets, distichous, narrowly linear and obtuse. It includes the Gallotannins Amlic acid, Gallic acid, Ellagic acid, Chebulic acid, Chebulagic acid, Chebulinic acid, Alkaloids and Phyllatidine.

This mixture is healthy for the skin, hair, liver, eyes, brain, heart and eyes. It enhances these organs' capabilities and wellbeing [41]. According to ayurvedic literature, amlaki plays a protective role against conditions including cancer, diabetes, liver and heart disease, gastric ulcers and a host of other illnesses. Antioxidant, immunomodulatory, antipyretic, analgesic, cytoprotective, antitussive and gastroprotective effects are all present in amlaki-containing preparations. Additionally, it improves memory and lowers blood cholesterol levels. According to reports from numerous scientific researches, consuming it may help shield tissues from radiation harm [42-45].

Syzygium cumini or Black plum is a member of Myrtaceae family and native to Indian subcontinent. It is widely distributed in tropical and subtropical region. It is found in India, Pakistan, Bangladesh, Myanmar, China, Indonesia, and Ceylon. It is also found in Asia, South Africa and Nepal. It is also cultivated in different parts of the world like as United States and Australia due to its great economic importance. It is grown as a fruit producer and as a source of timber.

1.9 Taxonomy of *Syzygium cumini*

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Subclass: Rosidae

Order: Myrtales

Family: Myrtaceae

Genus: *Syzygium*

Species: *Syzygium cumini*

(a) *Syzygium cumini* fruit

(b) Powder of *Syzygium cumini* seed

Figure 3: Black plum (*Syzygium cumini*) fruits (a) and its powder of seed (b).

S. cumini is rich in compounds containing phenolics, flavonoids, glucoside derivative. The leaf extracts contain flavonoids like as quercetin, kaempferol, myricetin, myricitrin and gallic acid, ellagic acid, ferulic acid, chlorogenic acid as phenol derivatives [46]. The stem bark of *S. cumini* contains gallic acid, ellagic acid, β-sitosterol, betulinic acid, myricetin, quercetin, friedelin, epi-friedelanol, eugenin, tyannin and flavonoids [47-52]. The most widely used parts of *S. cumini* is its seed. The seed contains hydrolysable tannins, phenolic contents, eugenol, terpenes. The fruits also contain glucose, gallic acid, citric acid, raffinose, anthocyanins, 7-hydroxycalamenene, oleanolic acid, β-sitosterol, methyl- β-orsellinate [53-54].

Various disorders are treated using *S. cumini* seed. It is also reported to have its usage in the treatment of diarrhea, stomach-ache, piles, dysentery, digestive problems [45-46]. Different literature studies prove the usage of its seed has antidiabetic effects. Various parts of the *Syzygium cumini* plant act as liver tonic, purifies blood, strengthen teeth and gums has a great role in the treatment of ringworm infection of head [55].

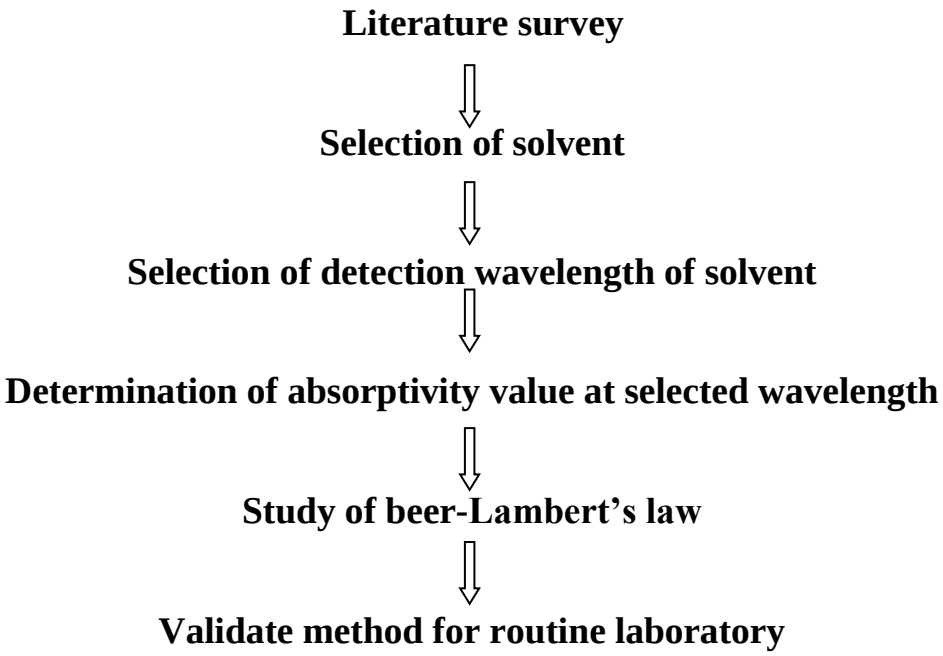
Spectroscopic analysis is a widely used technique in the analysis of herbal drugs. Some studies describe the uses of HPLC, HPTLC method in the analysis of different polyherbal formulations. IR spectroscopy was used to determine phytoequivalence among the sample. Present work outlines a method for identifying herbal drugs on the basis of phytoequivalence. The mid-infrared (IR), or so-called fingerprint region of the spectra (400-2000 cm1), region of the herbal sample contained many primary bands and those were marked. However, as this is a time-consuming and expensive process, the current analysis did not attempt to identify the specific chemical compounds found in the herbs. These marked bands served as a comparative tool for herbal plants and the medications made from them. The quantitative method of comparison proposed by Zou et al. was applied for the comparison of crude herbal samples and their formulations. [56-58].

TLC was used as another analytical method for identification and showing phytoequivalence. TLC is a popular tool for analysis because by examining the chemical elements of extracts, it is simpler to utilize than HPLC to identify plants [59-60].It has been mostly used for the qualitative analysis of herbal medicines and to help characterize and track components visually or as an initial separation technique.

Single or multiple herbs containing preparation like as Ayurvedic, Unani or polyherbal formulations can be analysed using UV-spectroscopy. In this technique, it

involves the measurement of ultra violet radiation absorption by the substance in solution. Both qualitative and quantitative analysis can be done through this technique. It is a simple, accurate, inexpensive technique for the analysis of small amount of sample. This technique is based on Beer-Lambert's law. In the present study, it was aimed at development of a guideline for the analysis of *Phyllanthus emblica* and *S. cumini* seed in polyherbal formulation [61-62].

The stages involved in developing a UV absorption spectroscopy method [63] is shown through flow-chart.



CHAPTER TWO: LITERATURE REVIEW

2.0 Literature Review

The findings from different relevant literatures have been shown in tabular form.

Serial no.	Plants/plants containing formulation	Method	Description	Ref. No.
01	Herbal medicines	IR	Different components are compared through IR spectrum	64
02	Herbal products	IR	Describes IR can be an important tool for the analysis of presence of different chemicals and their secondary metabolites	65
03	Herbal drugs	IR	Use of IR in assessment of herbal drugs	66
04	Herbal drugs	IR	Explains fingerprinting methods published up to now that can be used to standardize and regulate HM quality	67
05	Herbs	IR	Describes authentication of some species of herbs	68
06	Medicinal plants	IR	Describes identification and assessment of compounds by IR	69
07	Fenugreek	IR	Provides information about application of IR in identification of compounds	70
08	*Curcuma caesia L*	IR	Describes identification of compounds by IR	71
09	Lignin	IR	Describes identification of lignin	72
10	Herbal drugs	IR	Reviews different methods for quality control of drugs	73
11	Lignin	IR	Describes characterization of	74

			acetylated lignin by IR	
12	Traditional Chinese medicine	IR	Reviewed application of different methods including IR in analysis	75
13	Tobacco	IR	Describes identification of counterfeit tobacco products from pure tobacco using IR	76
14	*Phyllanthus* species	IR	Describes identification of different species of *Phyllanthus*	77
15	Herbal drugs	IR	Describes application process of IT in compound identification	78
16	*Aerva lanata*	IR	Identifies *Aerva lanata* by IR	79
17	Herbal drugs	IR	Reviewed the use of IR in analysis	80
18	Ginseng	IR	Identification	81
19	Pomegranate	IR	Identification	82
20	*Radix astragali*	IR	Identification	83
21	Ginseng	IR	Identification	84
22	*Crocus sativus*	IR	Identification	85
23	Jatropha spp	IR	Identification	86
24	Herbal preparations	TLC	Describes the usage of TLC as an identification tool	87
25	Herbal medicines	TLC	Describes TLC as an analytical tool for herbal drugs	88
26	For analysis of alkaloids: essential oils, anthraquinone, glycosides, coumarins, flavonoids, triterpene, saponins, tannin containing plant parts	TLC	Compounds are detected using different solvent systems	89

27	Different herbal materials	TLC	Describes detail analytical guidelines about herbal drugs	90
28	A review on quality control of herbal drugs	TLC	It describes TLC may provide valuable additional information to establish the identity of the plant material	91
29	Different plant species	TLC	By using TLC, the semipurified extracts of the ten species were analysed comparing with reference materials	92
30	Herbal samples	TLC	The chromatograms were used to verify 81 samples of more than 50 herbal medicines	93
31	Different ayurvedic formulation	TLC	Describes identification of different compounds using TLC	94
32	Different herbal supplements	TLC	Describes TLC as a tool for determining and comparing the compositions of different plants containing supplements	95
33	Different herbal products	TLC	Reviewed identification and separation of different herbal products using different chromatographic system	96
34	Chinese medicine	TLC	Identification	97
35	*Phyllanthus niruri*	TLC	Identification	98
36	Herbal drugs	TLC	Describes different analytical techniques that are used for standardization of herbal drugs	99
37	Herbal drugs	TLC	Describes TLC as an analytical tool	100

			for identification of herbal compounds	
38	Herbal drugs and their preparations	TLC	Describes different methods for analysis of herbal drugs	**101**
39	Herbal formulations containing steroid	TLC	TLC was used for identification of different steroids in formulation	102
40	Herbal products	TLC	This review work describes TLC as a tool for identification of counterfeit drugs	103
41	Herbal preparation	TLC	Compounds are characterised by TLC	104
42	Quercetin containing herbal drugs	TLC	Quercetin is identified through TLC	105
43	Herbal medicinal products	TLC	Describes different techniques including TLC for quality control of medicinal products	106
44	*Buteamonosperma*	TLC	Identification	107
45	Herbal drugs	TLC	Identification	108
46	Ayurvedic polyherbal formulation, Nyagrodhadi churna.	TLC	Identification by TLC	109
47	Four species of Curcuma rhizomes	TLC	Identification	110
48	Different herbal drugs preparation	TLC, IR	Chemical fingerprints obtained through these techniques are used to show and compare phytoequivalence	111

49	*Upodika- Basella alb*a L	TLC	Identification	112
50	Herbal drugs	TLC, IR	Identification	113
51	Eugenol containing herbal preparation	UV visible spectroscopy	Describes presence and determination of eugenol in herbal formulation	114
52	Ellagic acid	UV visible spectroscopy	Identification and estimation	115
53	*Curcuma species*	UV spectroscopy	Identification and estimation	116
54	*Entada Africana*	UV spectroscopy	Identification and estimation	117
55	*Eucalyptus globules*	UV,IR	Identification and estimation of phenolic OH group by FTIR and UV spectroscopy	118
56	Herbal medicinal product	UV spectroscopy	Detection of expired antimalarial herbal medicinal product	119
57	Herbal medicinal product	UV spectroscopy	Reviewed the application of UV	120
58	*Curcuma longa*	UV spectroscopy	Describe detection of turmeric using uv	121
59	Ellagic Acid	UV spectroscopy	Identificaton and quantification using uv	122
60	Herbal medicine	UV spectroscopy	Review use of UV with other techniques	123
61	Phytotherapeutic agents	UV spectroscopy	Describe identification by UV	124
62	*Curcumae rhizome*	UV spectroscopy	Identificaton and quantification	125
63	Herbal drugs	UV spectroscopy	Describe identification of plants	126
64	Tartaric and malic	UV	Identification by UV	127

	acid	Spectroscopy		
65	Honey	UV spectroscopy	Identification	128
66	Herbal drugs	UV spectroscopy	Identification	129
67	Food and dietary supplements	UV spectroscopy	Identification	130
68	Honey	UV spectroscopy	Identification	131
69	Honey	UV Spectroscopy	Identification	132

CHAPTER THREE: OBJECTIVE

3.0 Aim and Objective

3.1 Aim of the presented work

The development and validation of analytical methods is an ongoing task in pharmaceutical sector. The departments of research and development, quality control, and quality assurance are responsible for the ongoing creation of new methods, which is a very significant duty. In the assessment and management of equivalence and risk, analytical approaches play a significant role. It aids in the development of results stability and product-specific acceptability criteria. Validation should show that the analytical method is effective for the desired outcome.

The use of traditional medicine is very popular and its popularity is increasing. So quality control of the medicine is very important. Due to presence of different components quality control method is critical. The aim of this study was to develop and validate a simple and sensitive method for the identification and estimation of ingredients in standard and market formulation as per ICH guidelines.

This method can successfully be used for the routine analysis of traditional medicine in laboratory.

3.2 Objective and plan of work

- Identification and comparison of compounds from standard and market preparation by IR spectroscopy
- Identification and comparison of compounds from standard and market preparation by Thin Layer Chromatography
- Determination of absorbance by UV-spectrophotometric method for estimation of Amlaki and Black plum seed as standard
- Determination of absorbance by UV-spectrophotometric method for estimation of Amlaki and Black plum seed from dosage form
- Development and validation of the method according to ICH guideline which can be used for routine analysis in the laboratory

CHAPTER FOUR: DETERMINATION OF PHYTOEQUIVALENCE ON THE BASIS OF CHEMICAL FINGERPRINTING AND METHOD DEVELOPMENT ALONG WITH VALIDATION OF A UV-SPECTROMETRIC METHOD FOR THE ASSAY OF MARKET PRODUCT AMLAKI IN METHANOL

4.1 Identification of Amlaki and Amlaki containing dosage form by IR spectroscopy and TLC

4.1.1 Instruments

4.1.1.1 IR spectrophotometer

Shimadzu FTIR-8400S spectrophotometer was used to collect IR spectrum.

4.1.1.2 Instruments for TLC

TLC was performed on 20 cm × 20 cm aluminium foil plates coated with 0.2 mm layers of silica gel 60 F254 (Machery-Nagel, Germany). Plates were developed at room temperature (25 ± 2°C) in 20 cm × 10 cm and 20 cm × 20 cm glass chambers. TLC UV lamp having model-ENF-260CFE was used for identification of compounds. All the other chemicals, kits and reagents employed were of laboratory grade.

4.1.2 Preparation of Amlaki extract

Amlaki fruits were washed properly to remove the dirt attached to it and dried properly. Then the fruit was identified in the national herbarium of Bangladesh having accession no. 50,443. Then it was ground to powder. 350 gm of powdered fruit materials were placed in an amber-colored reagent container and soaked in 1.5 liter methanol. The bottle's contents were sealed and maintained for 14 days, occasionally being shaken and stirred. The entire mixture was then condensed using a rotary evaporator to obtain the crude extract after being filtered using cotton and what man No. 1 filters paper.

4.1.3 Preparation of Amlaki standard for IR analysis

5 mg of Amlaki extract was accurately weighed and mixed well with 250 mg KBr. Pellets were prepared using this mixture.

4.1.4 Amlaki sample preparation from marketed formulation for IR analysis

8.25 mL of Amlaki preparation which is equivalent to 5 mg of Amlaki in the formulation was taken and then it was poured in a separating funnel. 10 mL water is added in the separating funnel and extracted with 25 mL Methanol The aliquot was collected in a container through Whatman No. 1 filter paper. The same process was carried out three times. The combine filtrate was evaporated on water bath to dryness and the residue was collected. The solid extract was mixed properly with 250 mg KBr and pellet was prepared from this mixture.

Both the standard and sample extracts were subjected to IR spectrophotometer for analysis.

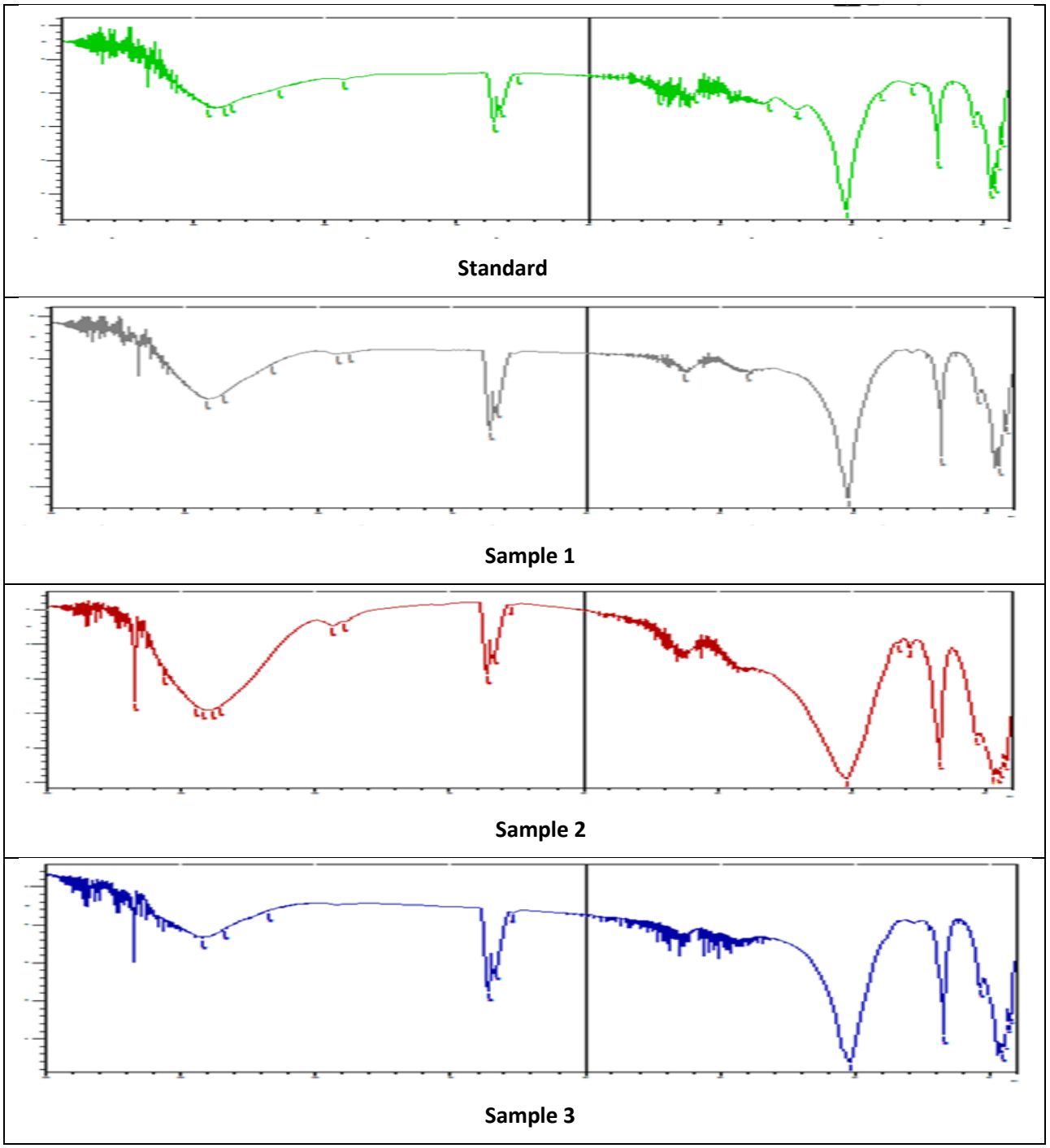


Figure 4: IR spectrum of standard and market preparation of Amlaki.

4.1.5 Preparation of Amlaki standard for TLC

5 mg of Amlaki extract was accurately weighed. Then it was dissolved in methanol. Samples were applied to the plates. Plates were developed at room temperature (25 ± 2°C) in 20 cm × 10 cm and 20 cm × 20 cm glass chambers previously saturated for 1 h**.** The mobile phase used depended on polarity. Three solvent systems were trialed during the analysis. Different study suggests the usage of alkaline system for nonpolar compounds having composition Benzene: Ethanol: Ammonia (9:1:0.1), acidic system for intermediate polar compounds having composition Choloroform: Ethylacetate: Formic Acid (5:4:1) and Ethylacetate: Methanol: Water (10:1.35:1) system for polar compounds. Among these solvent system, Benzene: Ethanol: Ammonia (9:1:0.1) system showed better results.

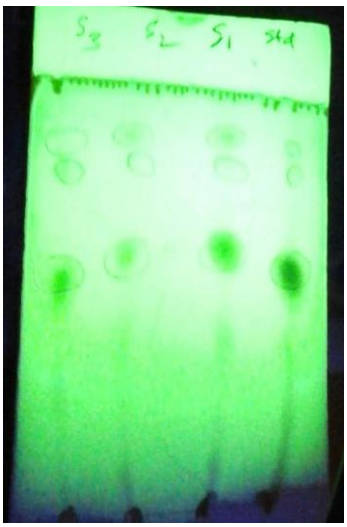


Figure 5: TLC of standard and market preparation of Amlaki.

4.2 Development and Validation of a UV-Spectrometric Method for the Assay of market product containing Amlaki in methanol

4.2.1 Instruments

4.2.1.1 UV spectrophotometer

Shimadzu double-beam UV-Vis spectrophotometer (UV-1800 Model) having 1.5 nm spectral bandwidths with 10 mm quartz cuvette cell was employed in the assay purpose. UV-probe software version 2.43 was used for data acquisition for the studied sample.

4.2.1.2 Analytical Balance

For weighing the standard and sample, analytical balance (Model- Ht224R, Shinkodens-hi Co. Ltd, Japan) was used.

4.2.1.3 Chemical and reagents

All the chemicals, kits and reagents employed were of laboratory grade.

4.2.2 Methods

4.2.2.1 Preparation of standard solutions

100 µg/mL of a standard stock solution were prepared. To prepare this stock solution, 10 mg of Amlaki extract was precisely weighed, transferred into a 100 mL volumetric flask, and diluted to the proper volume with methanol. Standard solutions were then made ready, with concentrations ranging from 1 to 5 µg/mL by further dilution with methanol.

4.2.2.2 Determination of wavelength of maximum absorbance (λ_{max}) of Amlaki

A UV-visible double bean spectrophotometer was used to scan a 1 µg/mL solution of Amlaki from 200 to 800 nm while using Methanol as a blank to establish the maximum absorption wavelength (λ_{max}).

4.2.2.3 Preparation of the standard calibration curve

The absorbance valus of the standard methanol solutions in the 1-5 µg/mL range were quantified at 303 nm. Plotting maximum absorbance (max) against concentration over an average (n=5) value gave rise to the standard calibration curve. To study linearity, a regression equation was used.

4.2.2.4 Method validation

Claimed by ICH Q2(R1) standards for the validation of analytical techniques, specificity, linearity, range, precision, accuracy and robustness were taken into

consideration when evaluating validation qualities. These parameters were viewed as being the most important for validating assay-type analytical procedures.

Specificity:

By using methanol as a blank and UV-spectrophotometric scanning of each Amlaki standard solution (1-5 g/mL) in the 200-800 nm range, the specificity was verified.

Linearity:

By measuring the absorbance vales of the Amlaki standard concentrations (1-5 µg/mL) at 303 nm against methanol as a blank, the linearity was verified. A calibration curve plotted using absorbance verses concentration. For Amlaki standard concentrations (1-5 µg/mL), a regression equation and correlation coefficient were found.

Precision:

Intermediate precision, intraday precision and repeatability were used to assess precision. Using Amlaki (1 µg/mL) six times on the same day, repeatability was examined (intra-day) with three different Amlaki concentrations (1, 3 and 5 µg/mL) three times on same day and on three consecutive days, intra-day precision and the intermediate precision (inter-day) were examined, respectively.

A**ccuracy:**

The accuracy was evaluated by calculating the percentage recovery of known added Amlaki standard concentrations (1, 2 and 3 µg/mL) to the previously tested known solutions (2 µg/mL). For each concentration, the procedure was carried out three times.

% Recovery = $[C_t/C_a]$ x100

Where

C_a is the total Amlaki concentration after standard addition;

C_t is the Amlaki concentration in the test (specimen) sample;

Range:

The range of the approach was assessed using the findings of the linearity and accuracy studies.

Robustness:

Several analysts and tools were used to assess robustness for Amlaki standard solution (5 µg/mL). Calculations were made of the percentage relative standard deviation (%RSD) values for several analysts (analysts 1 and 2) and instruments (UV-1800 and UV-1240V).

4.2.2.5 Estimation of Amlaki in market preparation

Preparation of Amlaki solution from market preparation

To prepare a stock solution (100 µg/mL), 16.50 mL of Amlaki preparation which is equivalent to 10 mg of Amlaki in the formulation was taken. Then it was taken in a separating funnel, 20 mL water is added in the separating funnel and extracted with 50 ml methanol. The aliquot was collected in a container through Whatman filter paper containing sodium phosphate. The same procedure was repeated three times. The combined filtrate was evaporated on a water bath to dryness. The residue was dissolved in 10 mL methanol and transfer to a 100 mL volumetric flask quantitatively. From the transparent stock solution, a 5 µg/mL test solution was created by further diluting it with methanol.

4.2.2.6 Estimation of the content of Amlaki in market preparation

The absorbance ratio method was used to estimate the amount of Amlaki in the test solution (5 µg/mL) of market product. Maximum absorbance at 303 nm was determined with methanol used as the reference.

4.3 Results

Statistical analysis

All the observed results have been shown as Mean ± Standard deviation and %RSD.

4.3.1 Method development

Solvents selection plays a great impact on the quality and shape of the obtained peak. Different solvents like methanol, ethanol, ethyl acetate are trialed during the method development stage. Among them, methanol was selected because using this solvent a good quality peak is obtained at the selected wavelength. It is one of the most widely employed solvents in chromatographic separation. The selected solvent for the analysis of Amlaki in poly-herbal preparations was methanol and the wavelength of maximum absorption (λ_{max}) of Amlaki appeared at 303 nm Figure 6. The developed method has been found to be a specific and selective spectrophotometric method for the analysis of Amlaki preparation.

The analytical method was created, verified, and represented in **Table 1** in compliance with ICH Q2(R1) standards. Amlaki shows maximum absorption at 303 nm wavelength (**Figure 6**).

4.3.2 Method validation

All the validation parameters are summarized in **Table 1**.

Table1 1: Obtained results of validation parameters by developed UV method

Parameters	**Results**
Absorption maxima(λmax)	303 nm
Beer's Law limit (µg/mL)	1-5
Regression equation (y=mx +c)	y = 0.076x + 0.003 R^2 = 0.998
Slope	0.076
Intercept	0.003
Coefficient of correlation	0.998
Repeatability (%RSD), (n=6)	0.6901
Accuracy	99.348-101.478%
Precision (%RSD)	Intra-day = 0.9998; 0.0080; 0.0058 Inter-day= 06846; 0.0137; 0.0038
Robustness (%RSD)	0.485111; 0.97259
LOD;LOQ	0.0386;0.0107

Specificity:

According to the ICH Q2(R1) criteria, specificity is the ability to correctly identify the analyte in the presence of components that could be expected to be present. **Figure 6** displays the specificity results.

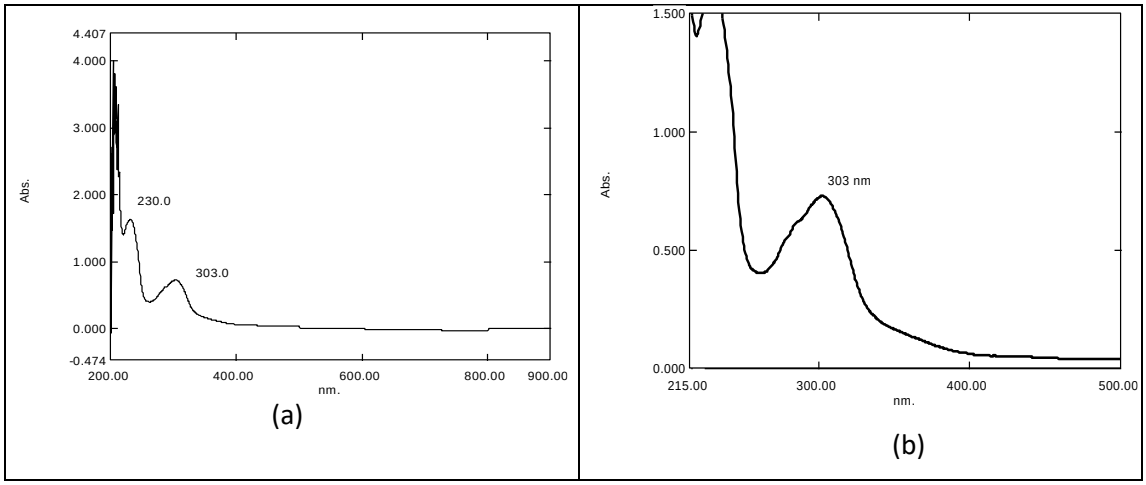


Figure 6: UV spectra of Amlaki in methanol (a) Original spectrum of Amlaki (b) Extended spectrum of Amlaki.

Specificity confirms the identity and measurement of the analyte of interest. The specificity of the selected method was specified by comparing five UV-scans of Amlaki standard at a concentration of 1-5 µg/mL. All the obtained spectrum at different concentration showed the presence of a prominent peak at 303 nm. So, 303 nm wavelength was selected as the average wavelength of maximum absorbance which is denoted as λ_{max}. **Figure 6** shows the results of specificity.

Linearity:

According to ICH Q2(R1) standards, an analytical procedure's linearity is its capacity (within a certain range) to produce test findings that are directly proportional to the concentration (amount) of analyte in the sample. **Table 2** and **Figure 7** display the linearity's findings.

Table 2: Linearity and range study of the developed UV method

Concentration of Amlaki (µg/mL)	Absorbance at 303 nm (Mean±SD)	%RSD
1	0.084±0.0012	1.3801
2	0.153±0.00153	0.9962
3	0.229±0.00208	0.9103
4	0.304±0.00351	1.1540
5	0.389±0.00252	0.6475

The linearity of a method signifies the ability to find out the test results which is directly proportional to analyte concentration present in the sample. **Table 2** and **Figure 7** show the results of these examinations.

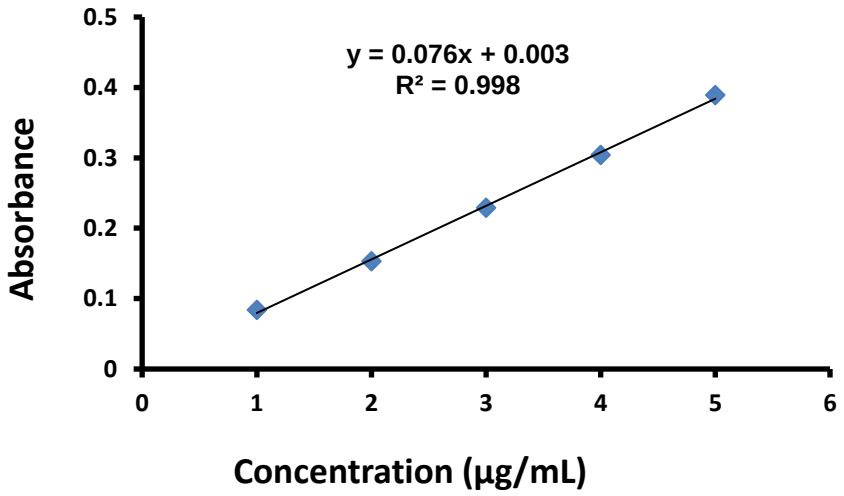


Figure 7: Linearity and range study of the developed UV method.

The value of correlation coefficient (r) is 0.998 which signifies good linearity between measured absorbance and concentration at the selected concentration range of 1-5 µg/mL, which is shown in **Table 2** and **Figure 7**.

Range:

According to ICH Q2(R1) guidelines, the range is between the highest and lowest concentrations of the analyte in the sample (including these values), for which it has been illustrated that the analytical technique has a sufficient level of precision, accuracy and linearity. **Table 2** and **Figure 7** display the results of the range computation.

Precision:

The ICH Q2(R1) recommendations state that the precision of an analytical technique is determined by the degree of agreement between a series of measurements acquired from multiple sampling of the same homogenous sample under the necessary conditions. There are three types of precision: intermediate precision, reproducibility, and repeatability. **Table 3** and **Table 4** display the precision results (intraday precision and inter-day precision).

Table 3: Results of Intra-day precision of the developed method

Intra-day precision (n=6)		
Amlaki (µg/ml)	**Absorbance at 303 nm**	**%RSD**
1	0.082±0.0008	0.9998
3	0.234±0.0019	0.0080
5	0.396±0.0023	0.0058

The precision of an analytical method is signified as the degree of agreement between a series of test results which are performed from multiple samples from the same homogenous sample under a prescribed condition. Repeatability, intermediate precision and reproducibility are three important levels of precision. Results of precision (intraday precision and inter-day precision) are represented in **Table 3 and Table 4**.

Table 4: Results of intermediate precision (inter-day precision) of Amlaki by the developed method

Inter-day precision (n=3)		
Amlaki (µg/ml)	**Absorbance at 303 nm**	**%RSD**
1	0.084±0.0006	0.6846
3	0.234±0.0032	0.0137
5	0.398±0.0015	0.0038

The intraday precision of this method was studied for three independent samples six times which was exhibited in **Table 3**. The %RSD for intermediate precision was calculated from the analysis of three independent sets of samples. The value for %RSD of intraday precision were 0.998%, 0.0080% and 0.0058% and %RSD values of the intermediate precision for studied samples in three consecutive days were found to be 0.0.6846%, 0.0137%, 0.0038% using methanol samples as the solvent. The obtained result showed the low values of %RSD in both intra-day and inter-day analysis and the difference them found to be less than <2%. This value signifies the confirmation of precision.

Repeatability:

The result for repeatability study is shown in **Table 5**.

Table 5: Repeatability study of Amlaki by the developed method

Repeatibility (n=3)		
Amlaki (µg/ml)	**Absorbance at 303 nm**	**%RSD**
1	0.084±0.0006	0.6901

Repeatability indicates the ability of the method to generate a similar type of result repeatedly. The %RSD value for repeatability was obtained is 0.06901% which is represented in **Table 5**.

Accuracy:

According to established ICH Q2(R1) principles, the correctness of an analytical technique is defined by the degree of agreement between the value that is accepted as either a conventional true value or an acceptable reference value and the value discovered. The results of accuracy are shown in **Table 6**.

Table 6: Accuracy study data of Amlaki by the developed UV method

Initial amount of Amlaki (µg/mL)	**Added amount of Amlaki (µg/mL)**	**Predicted concentration (µg/mL)**	**Observed concentration (µg/mL)**	**Residual concentration (µg/mL)**	**% mean recovery**	**%RSD**
2	1	3	2.9870	0.013	99.565	0.8734
2	2	4	3.9739	0.026	99.348	1.1527
2	3	5	5.0739	-0.074	101.478	0.5141

Accuracy of analytical method often termed as trueness is defined, “as the closeness of agreement between the test results obtained by that method to the true value”. The accuracy of the proposed method was proved by the standard addition method and the obtained result was represented in **Table 6**. To estimate the accuracy of the method, three different concentrations were spiked at a pre-analyzed sample. The calculated data showed the closeness of the observed value to the true value for the analyte. The percentage of recovery of the sample was found 99.348% to 101.478% which confirms the developed method as an accurate method for the assay of Amlaki containing preparation and the results are exhibited in **Table 6.**

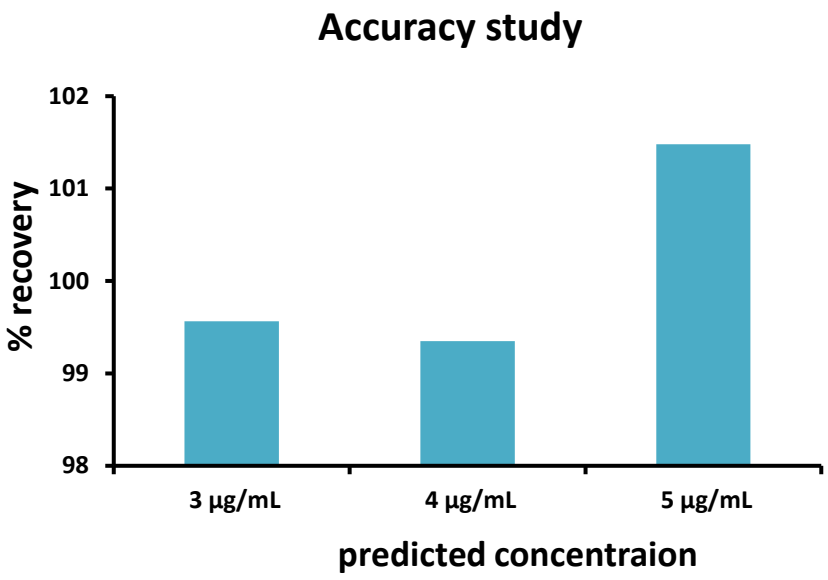


Figure 8: Accuracy study data of the developed UV method.

Robustness:

According to ICH Q2(R1) guidelines, the robustness of an analytical technique is a measurement of its ability to remain unaffected by little but purposeful changes in method parameters and is a sign of its dependability under typical conditions. **Table 7 and Table 8** display the robustness results.

Table 7: Robustness study of Amlaki using developed UV method by analyst

Variable parameters	Absorbance at 303 nm (Mean±SD)	%RSD between sample	% Mean Recovery	%RSD between two analyst
Analyst 1	0.390±0.00153	0.3920	101.652	0.4851
Analyst 2	0.387±0.0020	0.5168	100.957	

The same sample was analyzed in this section at a selected concentration (5 µg/mL) in triplicate by two different analysts (first and second analysts) and in two different instruments (UV-Jasco V-630 and UV-Secom am XTD6). The values of %RSD in both the parameters appeared to be <2%. So, the robustness of the method was confirmed.

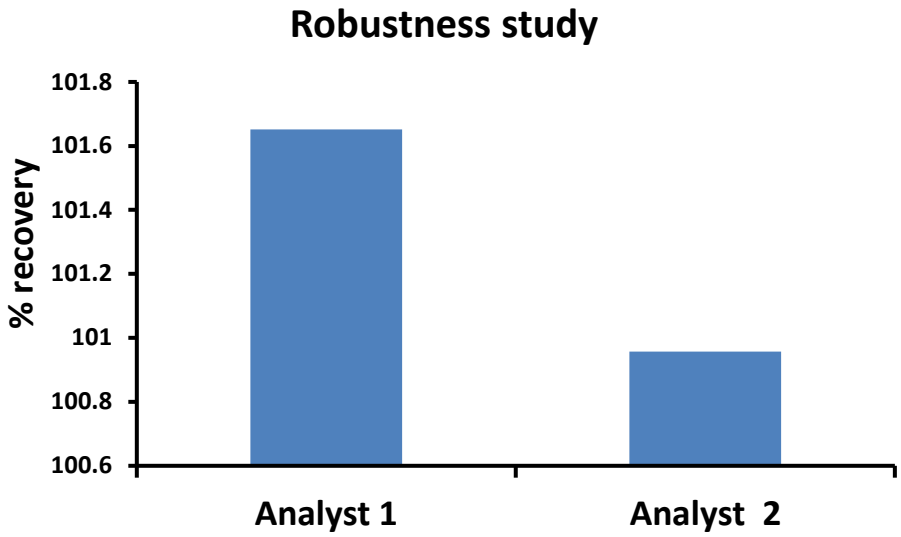


Figure 9: Robustness study of Amlaki using developed UV method by analyst.

Table 8: Robustness study of Amlaki using developed UV method by instrument

Variable parameters	**Absorbance at 303 nm (Mean±SD)**	**%RSD between sample**	**%Mean Recovery**	**%RSD between two analyst**
Instrument 1	0.385±0.00265	0.6872	100.435	0.9726
Instrument 2	0.390±0.00153	0.3913	101.826	

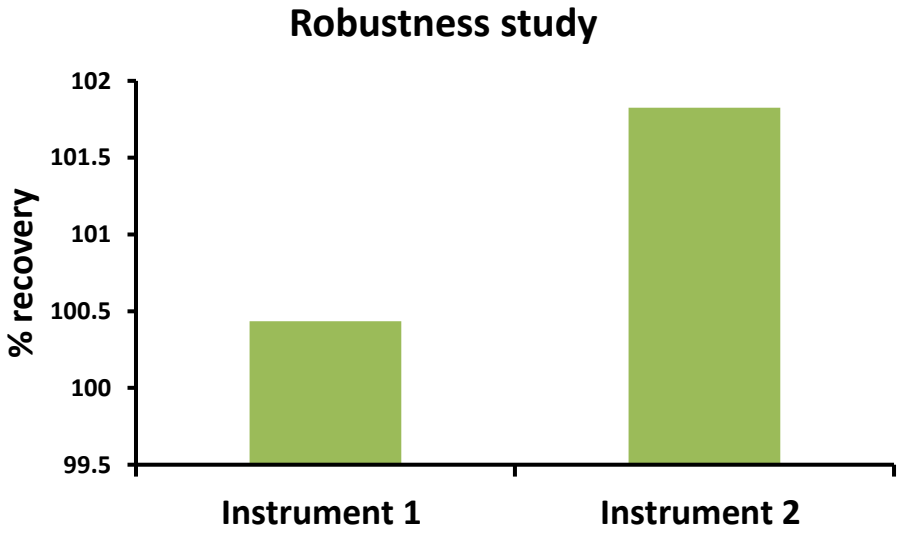


Figure 10: Robustness study of Amlaki using developed UV method by instrument.

Estimation of Amlaki in market preparation

The result of the assay of Amlaki in market preparation is shown in **Table 9 and Figure 11.**

Table 9: Assay results for Amlaki estimation in Amlaki market preparation

Formulation	Amount of formulation tested	Absorbance at 303 nm (Mean±SD)	%RSD	Amount of Amlaki found	Mean %Recovery
A	5 (µg/mL)	0.293±0.0015	0.5219	4.3294	86.588
B	5 (µg/mL)	0.278±0.0015	0.5501	4.1075	82.150
C	5 (µg/mL)	0.307±0.0026	0.8618	4.5414	90.828

The developed method has been successfully used to estimate Amlaki in market preparations. A, B, C three market preparations were collected from the market and the percentage of Amlaki was determined. It was found to be 86.588%, 82.150% and 90.828% with %RSD value 0.5219%, 0.5501% and 0.8618%, respectively as shown in **Table 9**.

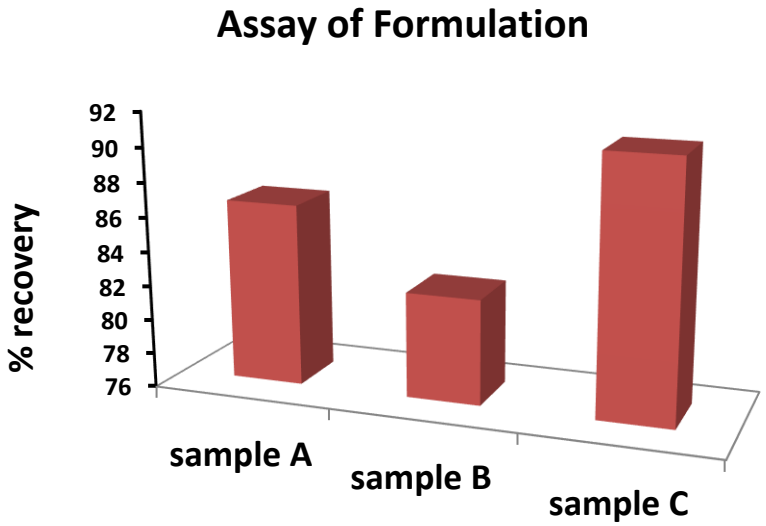


Figure 11: Assay results for Amlaki estimation in Amlaki market preparation.

4.4 Discussion

Solvents have a profound influence on the quality and shape of the peak. During the method development phase, different solvents are used. Among them, methanol was selected as it satisfied all the conditions relative to peak quality and non-interference at the specified wavelength. It is one of the most commonly used solvents in chromatographic separation. The wavelength of maximum absorption (λ_{max}) of Amlaki was found to be 303 nm, and the proposed UV-spectrophotometric approach was found to be specific and selective for measuring Amlaki in poly-herbal formulations which was shown in **Figure 6.**

In accordance with ICH guidelines, the procedure was validated which is summarized in **Table 1**. The UV-scans from the Amlaki standard concentrations (1-5 µg/mL) were compared to determine the specificity. All runs showed a distinct peak at 303 nm, which was determined to be the average wavelength of maximum absorbance (λ_{max}). Therefore, it was selected for linearity study which is shown in **Table 2**. With a correlation coefficient (r) of 0.998 demonstrating high linearity between absorbance and concentration, the regression graph demonstrated conformity to Beer Lambert's law in the concentration range of 1 to 5 µg/mL which is shown in **Figure 7**. The results where UV absorbance was directly proportional to the genuine concentration of the analyte was predefined by the goodness-of-fit test method at 303 nm shown in **Figure 7** confirmed the range (working range). The suggested approach can directly measure analyte concentrations in the (1-5 µg/mL) range and accuracy experiments have shown that this ranges from 50 to 150% of the test concentration. The intraday precision was studied for three independent samples six times which was shown in **Table 3**. The intermediate precision (%RSD) was observed for the analysis of three independent sets of samples. The %RSD value for intraday precision found were 0.998%, 0.0080% and 0.0058% **Table 3** and the intermediate precision %RSD values for selected samples in three consecutive different days were found to be 0.0.6846%, 0.0137%, 0.0038 % in methanol **Table 4**, respectively. The low values of %RSD in both intra-day and an inter-day analysis were found to be less than <2%. Hence, the precision was confirmed. The repeatability %RSD value was found to be 0.06901% in methanol shown in **Table 5**. By using the traditional addition method, the proposed approach's accuracy was determined. **Table 6**, calculated from the analysis of samples spiked at three distinct concentrations (low, medium, high) that cover the operating

range (1-5 µg/mL). The results demonstrated how closely the observed value for the sample matched its true value. The recovery percentage of the additional Amlaki concentrations was used to measure accuracy. To maintain accuracy and avoid interaction with formulation excipients, recovery values for the standard addition method used for the Amlaki analysis ranged from 99.348 to 101.478% shown in **Table 6**. **Table 7** and **Table 8** show that the method was found to be reliable and consistent despite changes to the analysts (analysts 1 and 2) and the instruments (UV-Jasco V-630 and UV-Secom am XTD6). The triplicate determination of the Amlaki at a chosen concentration (5 µg/mL) was carried out, and the %RSD values in both the parameters appeared to be 2%. Thus, robustness was determined. As the %RSD is less than 2%, the method is validated according to ICH guidelines.

Estimation of Amlaki in market preparation

The method was successfully employed for the estimation of Amlaki in market preparations. Three market preparations were collected from the market and the percentage of Amlaki was determined. The results were found to be 86.588%, 82.150% and 90.828% with %RSD value 0.5219%, 0.5501% and 0.8618%, respectively as shown in **Table 9**.

4.5 Conclusion

In conclusion, a simple, reliable, accurate and reproducible method has been developed and validated for analysis of Amlaki containing formulation. The developed method needs low cost, have faster speed with satisfactory precision. The method also has good specificity. The developed method was successfully validated following the guidelines of ICH and it could be employed for quality control analysis of Amlaki containing market preparation.

Statistical analysis:

All the results were expressed as Mean±SD and %RSD.

CHAPTER FIVE: DETERMINATION OF PHYTOEQUIVALENCE ON THE BASIS OF CHEMICAL FINGERPRINTING AND METHOD DEVELOPMENT ALONG WITH VALIDATION OF A UV-SPECTROMETRIC METHOD FOR THE ASSAY OF MARKET PRODUCT BLACK PLUM IN ETHANOL

5.1 Identification of *S. cumini* and *S. cumini* containing dosage form by IR spectroscopy and TLC

5.1.1 Instruments

4.1.1.1 IR spectrophotometer

SHIMADZU-IRSpirit spectrophotometer was used to collect IR spectrum.

5.1.1.2 Instruments for TLC

TLC was performed on 20 cm × 20 cm aluminium foil plates coated with 0.2 mm layers of silica gel 60 F254 (Machery-Nagel, Germany). Plates were developed at room temperature (25 ± 2°C) in 20 cm × 10 cm and 20 cm × 20 cm glass chambers. TLC UV lamp having model ENF-260CFE was used for identification of compounds. All the other chemicals, kits and reagents employed were of laboratory grade.

5.1.2 Preparation of *S. cumini* fruits extract

S. cumini fruits were purchased from local market and seeds were collected from these. Then the seed was identified in the national herbarium of Bangladesh having accession no. 50,442. Laboratory grade ethanol was used in analysis and it was bought from Merck Chemicals, India. Three formulations containing *S. cumini* seed were collected available in local market.

Through proper washing of *S. cumini* seeds, the dust attached to it was eliminated properly and was air dried until it is crispy. Then it was ground to powder. About 350 gm of powdered fruit materials were taken in an amber-colored reagent bottle and kept for soaking in 1.5 litres of ethanol. The soaked materials-containing container was properly sealed and held for a period of about 14 days with occasional shaking and stirring. The whole mixture was filtered using cotton and then Whatman No. 1 filter paper successively to get the clear filtrate. Then concentrated crude seed extract was collected using rotary evaporator through evaporation of solvent. Next the concentrated extract was further taken in water bath and air dried for complete drying. The obtained extract was preserved for further analysis.

5.1.3 Preparation of Black plum seed standard for IR analysis

5 mg of black plum seed extract was accurately weighed and subjected to IR analysis

5.1.4 Black plum (*S. cumini*) sample preparation from market formulation for IR analysis

0.263 mL of black plum preparation which is equivalent to 5 mg of black plum in the formulation was taken. Then it was poured in a separating funnel, 10 mL water is

added in the separating funnel and extracted with 25 mL Ethanol. The aliquot was collected in a container through Whatman No. 1 filter paper. The same procedure was repeated for 3 times. The combine filtrate was evaporated on water bath to dryness and the residue was collected. The solid extract was subjected to IR analysis.

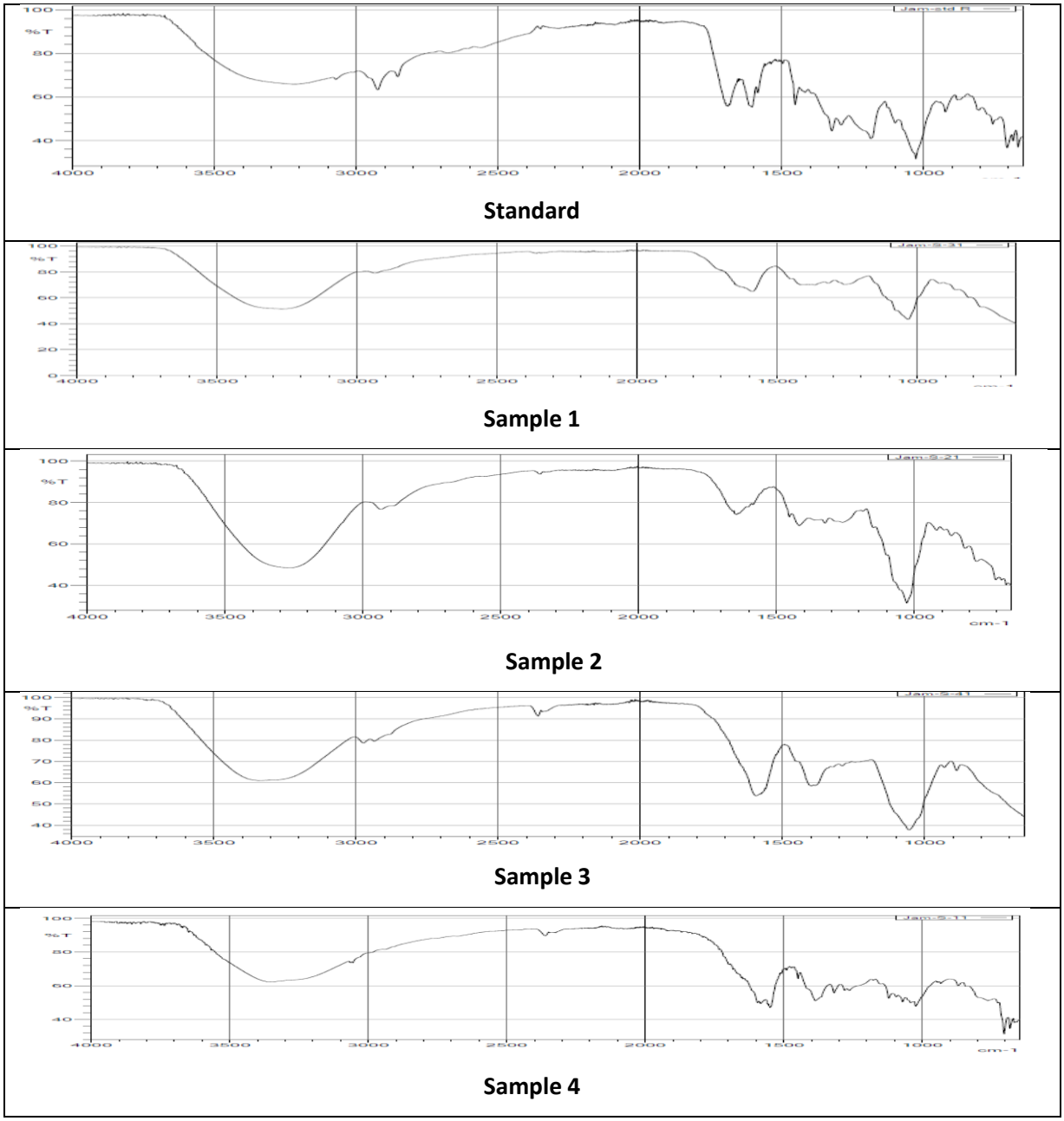


Figure 12: IR spectrum of standard and market preparation of Black plum seed.

5.1.5 Preparation of Black plum standard for TLC

5 mg of Black plum seed extract was accurately weighed. Then it was dissolved in methanol. Samples were applied to the plates. Plates were developed at room temperature (25 ± 2°C) in 20 cm × 10 cm and 20 cm × 20 cm glass chambers previously saturated for 1 h. The mobile phase used depended on polarity. Three solvent systems were trialed during the analysis. Different study suggests the usage of alkaline system for nonpolar compounds having composition Benzene: Ethanol: Ammonia (9:1:0.1), acidic system for intermediate polar compounds having composition Choloroform: Ethylacetate: Formic Acid (5:4:1) and Ethylacetate: Methanol: Water (10:1.35:1) system for polar compounds. Among these solvent system, Choloroform: Ethylacetate: Formic Acid (5:4:1) system showed better results.

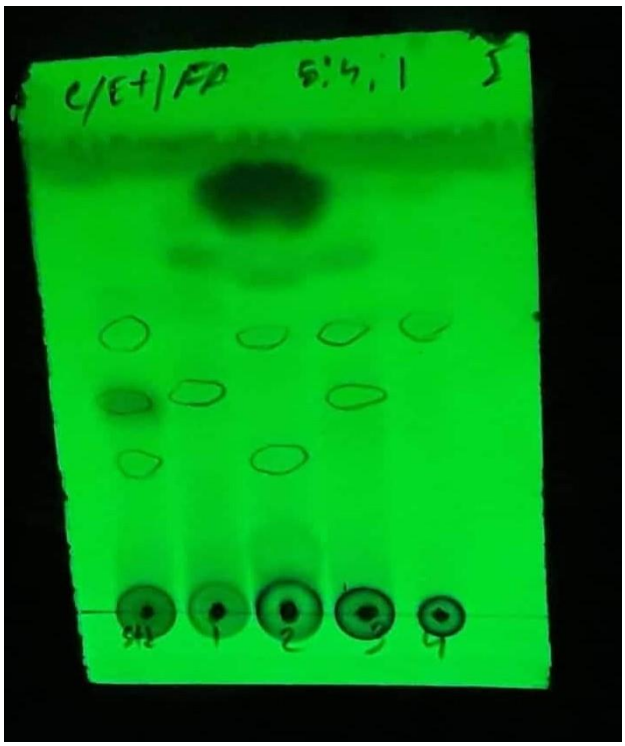


Figure 13: TLC of standard and market preparation of Black plum.

5.2 Development and Validation of a UV-Spectrometric Method for the Assay of market product containing Black plum seed extract in ethanol

5.2.1 Instruments

5.2.1.1 UV spectrophotometer

Shimadzu double-beam UV-Vis spectrophotometer (UV-1800 Model) having 1.5 nm spectral bandwidths with 10 mm quartz cuvette cell was employed in the assay purpose. UV-probe software version 2.43 was used for data acquisition for the studied sample.

5.2.1.2 Analytical Balance

For weighing the standard and sample, analytical balance (Model- Ht224R, Shinkodens-hi Co. Ltd, Japan) was used.

5.2.1.3 Chemical and reagents

All the chemicals, kits and reagents employed were of laboratory grade.

5.2.2 Methods

Solvent selection

The solubility of *S. cumini* seeds was checked using methanol, ethanol, acetone and ethyl acetate. The extract showed better solubility in ethanol in compare to other solvents.

5.2.2.1 Preparation of standard solution

10 mg *S. cumini* seeds extract was accurately weighed and taken in a 100 mL volumetric flask, volume was adjusted up to the mark with ethanol. A stock solution having concentration of 100 µg/mL was found. From the stock solution, standard solution of different concentration (0.1-0.5 µg/mL) was prepared by dilution with ethanol.

5.2.2.2 Selection of the wavelength

From these prepared solutions, 0.03 µg/mL solution was scanned in 200-800 nm uv-visible range in 1.0 cm cell using ethanol as blank. The maximum absorbance for this solution was found at 279 nm.

5.2.2.3 Standard calibration curve preparation

At 279 nm wavelength, the absorbance of *S. cumini* seeds extract was measured at different concentrations (0.1-0.5 µg/mL). A calibration curve was plotted between

concentrations versus absorbance of the extracts and a regression equation was found. From the calibration curve, linearity was observed utilizing a regression equation.

5.2.2.4 Validation of method

Validation is an integral part of quality assurance. A good analytical practice cannot be achieved without validation. Method validation is defined as the process that is used to ensure that the analytical technique used for a particular test is sufficient for its intended use. Method validation results can be used to assess the efficiency, reliability and accuracy of analytical findings. Validation of the developed analytical method was carried out following the guidelines set by International Conference on Harmonization (ICH) which is known as ICH Q2(R1) guidelines. The major validation parameters described in the guidelines like as specificity, linearity, range, precision, accuracy and robustness were studied for validation of the method.

Specificity

Standard solution of *S. cumini* seeds extract at the concentration of 0.03 µg/mL was analyzed by the proposed method and specificity of the method was studied.

Linearity

Linearity of the method was studied through measuring the absorbance of five standard concentrations of *S. cumini* seeds extract (0.1-0.5 µg/mL) at 279 nm using ethanol as blank. A calibration curve was plotted between concentrations versus measured absorbance of the extracts and regression analysis was done from the calibration curve.

Precision

Precision was established by evaluation of repeatability, intra-day precision and intermediate precision. To evaluate precision, the absorbance of standard which is *S. cumini* seeds extract was determined at a concentration of 0.1 µg/mL for 6 (six) times in the same day known as repeatability. The absorbance of 3 (three) concentrations of *S. cumini* seeds extract standard at a concentration of 0.1 µg/mL, 0.3 µg/mL and 0.5 µg/mL were measured in six-let at same day and later on three consecutive days which are known as intra-day precision and inter-day precision, respectively.

Accuracy

The accuracy of the proposed method was established by recovery investigation with the addition of known amounts to a pre-analyzed sample solution. For this purpose, known concentrations of *S. cumini* fruits extract standard solution at a concentration of 0.1, 0.2 and 0.3 µg/mL were added to three pre-analyzed sample solutions,

respectively which concentration was 0.2 µg/mL. The observed result was used to assess the percentage recovery of the standard. Same process is repeated for three times for each concentration.

The percent recovery was calculated using following equation.

$$\% \text{ Recovery} = [C_t/C_a] \times 100$$

Where,

C_a = Conc. of the *S. cumini* after standard addition;

C_t = Conc. *S. cumini* in the test sample

Range

Five different concentration of *S. cumini* seeds (0.1 µg/mL, 0.2 µg/mL, 0.3 µg/mL, 0.4 µg/mL and 0.5 µg/mL) were used for the assessment of the range of the method.

Robustness

Robustness of the method was studied by analyst to analyst variation and instrument to instrument variation studies. For this purpose, the value of absorbance 0.5 µg/mL standard *S. cumini* seeds was estimated and %RSD (percentage of relative standard deviation) values were calculated between them (first analyst and second analyst; UV-spectrophotometer of model UV-1800 and UV-1240V).

5.2.2.5 Use of proposed method for estimation of *S. cumini* seeds in market preparation

0.263 mL *S. cumini* seed containing market preparation, equivalent to 10 mg *S. cumini* seed extract was measured and transferred in a separating funnel. 10 mL water was added to it and extracted with 15 mL of ethanol. Then the aliquot was filtered using Whatman No. 1 filter paper and the filtrate was collected. For better extraction, addition of water and ethanol was repeated three times. In order to get the dry material, the acquired filtrate was subjected to evaporation in a water bath. The residue was dissolved well in ethanol and volume was adjusted up to 100 ml with ethanol. This solution was used as stock for further analysis. A test solution was prepared having concentration of 0.5 µg/mL using ethanol as diluent. The absorbance of the solution was measured at 279 nm wavelength against ethanol as blank.

Statistical analysis

All the observed results have been shown as Mean±Standard deviation and %RSD.

5.3 Results and Discussion

5.3.1 Method development

Different solvents like methanol, ethanol and ethyl acetate were used to determine the solubility, peak quality and peak shape of the studied drug. Among these, ethanol fulfilled all criteria in showing better solubility and giving better peak quality. The sample showed maximum absorbance at 279 nm which is shown in **Figure 14**.

5.3.2 Method validation

The developed method was validated according to the rules of ICH Q2(R1) guidelines and the found results were summarized in **Table 10**.

Table 10: Obtained results of validation parameters of *S. cumini* by developed UV method

Validation parameters	**Obtained results**
Absorption maxima (λ_{max})	279 nm
Limit of Beer's Law (µg/mL)	0.1-0.5
Equation for Regression (y=mx +c)	y = 1.3273x + 0.0441 R^2 = 0.9914
Slope	1.3273
Intercept	0.0441
Correlation coefficient	0.9914
% RSD of Repeatability, (n=6)	0.7950
Accuracy	99.647-101.767%
Precision (%RSD)	Intra-day = 0.924; 0.571; 0.467 Inter-day= 0.892; 0.835;0.342
Robustness (%RSD)	0.825; 0.361
LOD;LOQ	0.117.;0.0325

Specificity

The ability of an analytical method to reliably measure an analyte in the presence of interferences that may be present in the sample matrix is referred to as specificity. It signifies that the presence of excipients in formulation does not interfere with the drug peak. The result of specificity is represented at **figure 14** and the result indicates the proposed method was found specific and selective for the drug.

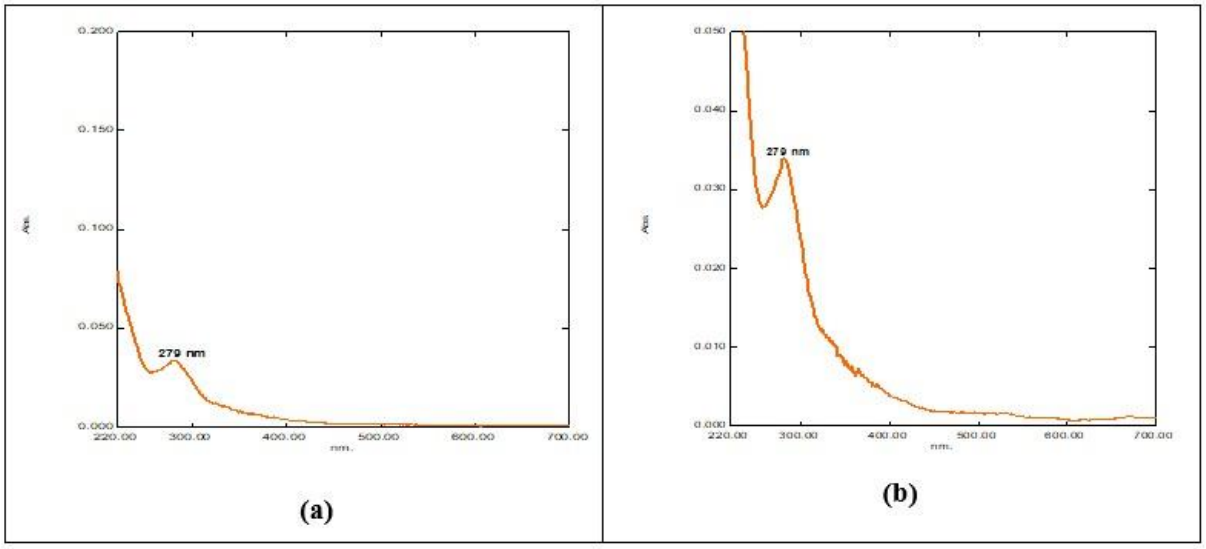


Figure 14: UV spectrum of *S. cumini* seed in ethanol

(a) Whole spectrum of *S. cumini* seed and (b) Zoomed spectrum of *S. cumini* seed.

Linearity and range

Linearity refers to a method's ability to achieve test results that are proportional to the analyte concentration in the sample. The range of an analytical method is the distance between the upper and lower levels that have been demonstrated to be calculated with precision, accuracy, and linearity using the set method.

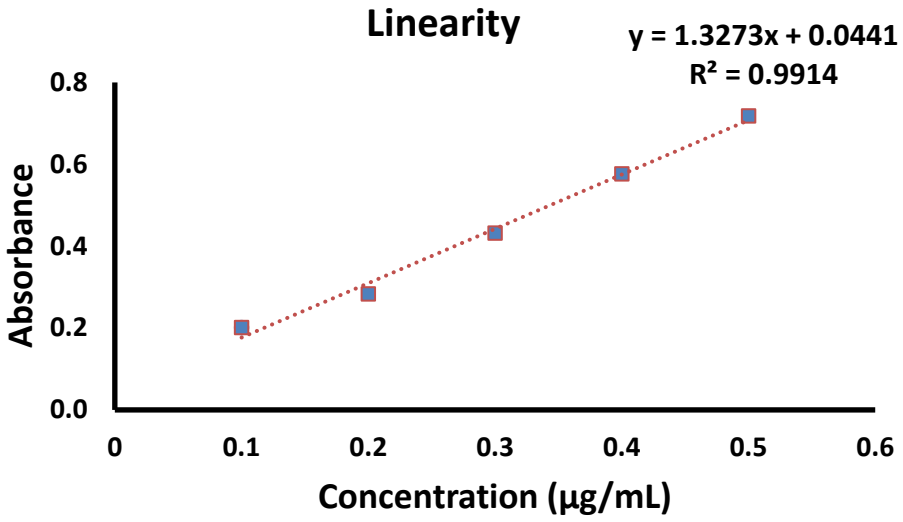


Figure 15: Linearity and range study of *S. cumini* by the developed UV method

A good linear correlation was achieved between absorbance and concentration in the range of 0.1-0.5 µg/ml. A linear regression equation was found to having a slope of 1.327, intercept of 0.0441 and the coefficient of correlation value is 0.9914 (**Figure 15**). The result of linearity is represented in **Table 11**.

Table 11: Linearity and range study of *S. cumini* by the developed UV method

***S. cumini* seed standard conc. (µg/mL)**	**Absorbance (279 nm) (Mean±SD)**	**%RSD**
0.1	0.201±0.002	1.034
0.2	0.283±0.003	0.935
0.3	0.432±0.003	0.694
0.4	0.577±0.005	0.794
0.5	0.718±0.003	0.418

Precision

The degree of agreement among individual test results, when a procedure is replicated on multiple samples of the same homogeneous sample is called precision. Precision is calculated by injecting a set of standards or examining several samples from a homogeneous lot. Precision as relative standard deviation (percent RSD) is determined using the observed standard deviation (SD) and Mean values. Precision can be established through determination of intraday precision, inter-day precision and repeatability. Intraday precision refers to the use of an analytical technique in a laboratory over a short period of time by the same operator with the same equipment, while inter-day precision refers to the calculation of differences in analysis when a process is used in a laboratory on various days by different analysts. A selected concentration 0.1 µg/mL, 0.3 µg/mL and 0.5 µg/mL were analyzed in six-let for intra-day and inter-day precision and 0.1 µg/mL was analyzed for repeatability. The obtained results of precision (intraday precision, inter-day precision and repeatability are represented in **Table 12**, **Table 13** and **Table 14**. The value of relative standard deviation, %RSD for the intra-assay precision, intermediate precision and reproducibility for all the three concentration showed an excellent intraday precision, intermediate precision and reproducibility of the proposed method.

Table 12: Intra-day precision study of *S. cumini* by the developed UV method

Intra-day precision of *S. cumini* seed standard (n=6)		
***S. cumini* seed standard conc. (µg/ml)**	**Absorbance (279 nm)**	**%RSD**
0.1	0.234±0.002	0.9245
0.3	0.432±0.002	0.5751
0.5	0.735±0.003	0.4668

Table 13: Inter-day precision study of *S. cumini* by the developed UV method

Inter-day precision of *S. cumini* seed standard (n=3)		
***S. cumini* seed standard conc. (µg/ml)**	**Absorbance (279 nm)**	**%RSD**
0.1	0.233±0.002	0.8921
0.3	0.432±0.004	0.8346
0.5	0.736±0.002	0.3421

Table 14: Repeatability study of *S. cumini by* the developed UV method

Repeatability (n=6)		
***S. cumini* seed standard conc. (µg/ml)**	**Absorbance (279 nm)**	**%RSD**
0.1	0.2250±0.002	0.7950

Accuracy

The accuracy of an analytical method is defined as the degree of closeness of the test results obtained by that method to the true value. It is also known as trueness. Accuracy was determined by injecting a known concentration of standard to a pre-analysed sample using the "method being validated". The % recovery for the standard addition and reference analysis method for all the three concentration levels found are 101.767% with %RSD 0.6944, 101.943% with %RSD 0.7942 and 99.647% with %RSD 0.4255. From the obtained result, this high degree of confidence interval signifies that any small change in the sample concentration can be accurately determined with high level of accuracy. The results found from the standard addition and reference analysis method were also found signifies the accuracy of the proposed method.

Table 15: Accuracy study of *S. cumini* by the developed UV method

Starting amount of *S. cumini* seed (µg/mL)	**Added amount of *S. cumini* seed (µg/mL)**	**Expected conc. (µg/mL)**	**Obtained conc. (µg/mL)**	**Residual conc. (µg/mL)**	**mean recovery percentage**	**%RSD**
0.2	0.1	0.3	0.305	0.005	101.767	0.6944
0.2	0.2	0.4	0.408	0.008	101.943	0.7942
0.2	0.3	0.5	0.498	0.002	99.647	0.4255

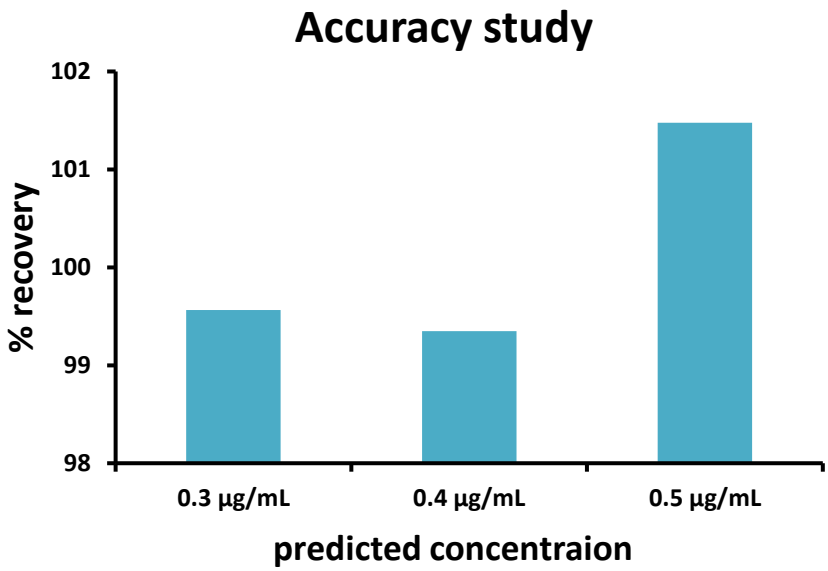


Figure 16: Accuracy study of *S. cumini* using developed UV method.

The term robustness is an analytical method's ability to remain unaffected by minor changes in its parameters. The results of robustness are shown in **Table 16**.

Table 16: Robustness study of *S. cumini* using developed UV method by analyst

Studied parameters	**Absorbance at 279 nm (Mean±SD)**	**%RSD between sample**	**Mean Recovery percentage**	**%RSD between two analysts**
Analyst 1	0.718±0.003	0.425	101.531	0.8251
Analyst 2	0.710±0.002	0.282	100.353	

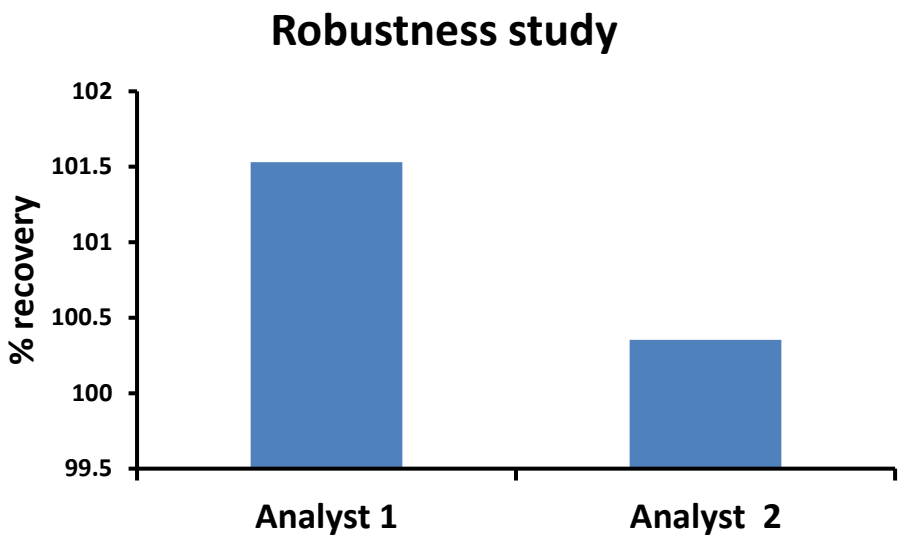


Figure 17: Robustness study of *S. cumini* using developed UV method by analyst.

Table 17: Robustness study of *S. cumini* using developed UV method by instrument

Studied parameters	Absorbance at 279 nm (Mean±SD)	%RSD between sample	Mean Recovery percentage	%RSD between two analysts
Instrument 1	0.717±0.005	0.688	101.296	0.3609
Instrument 2	0.720±0.002	0.289	101.814	

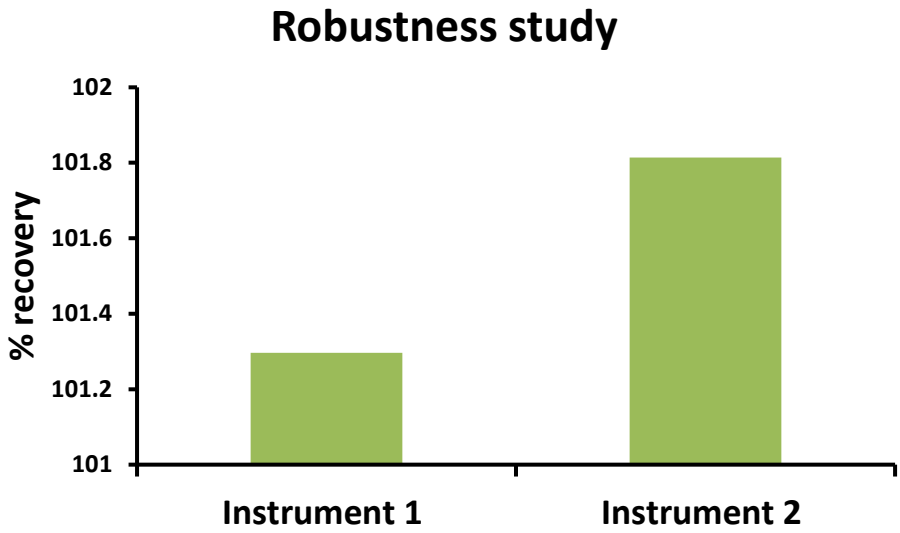


Figure 18: Robustness study of *S. cumini* using developed UV method by instrument.

To study robustness, the same sample at a same concentration (0.5 µg/mL) was analyzed in triplicate by two different analysts (first and second analysts) and in two different instruments (UV-Jasco V-630 and UV-Secom am XTD6). The values of %RSD in both the parameters appeared to be <2%. This result confirms robustness of the method.

Estimation of *S. cumini* seed in market preparation

The result of the assay of *S. cumini* seed in market preparation is shown in **Table 18**.

Table18: Assay results for *S. cumini* seed estimation in market preparation

Formulation	**Tested conc. of *S. cumini* seed in market preparation**	**Absorbance at 279 nm (Mean ± SD)**	**%RSD**	**Observed conc. of *S. cumini* seed in market preparation**	**Mean recovery percentage**
A	0.5 µg/mL	0.614±0.004	0.5735	0.416µg/mL	83.152
B	0.5 µg/mL	0.634±0.0050	0.7935	0.434µg/mL	86.821
C	0.5 µg/mL	0.659±0.0040	0.6070	0.450µg/mL	90.082
D	0.5 µg/mL	0.592±0.0036	0.6090	0.403µg/mL	80.579

The developed method has been successfully used to estimate *S. cumini* seed in market preparations. A, B, C, D four market preparations were collected from the market and the percentage of *S. cumini* seed was determined. It was found to be 83.152%, 86.821%, 90.082% and 80.579% with %RSD value 0.5735, 0.7935, 0.6070 and 0.6090, respectively as shown in **Table 18**.

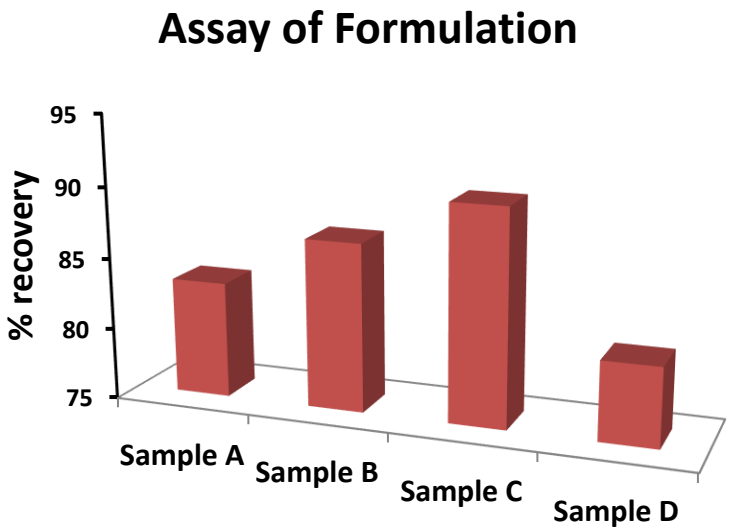


Figure 19: Assay results for *S. cumini* seed estimation in *S. cumini* seed market preparation

5.4 Discussion

Solvents have a profound influence on the quality and shape of the peak. During the method development phase, different solvents are used. Among them, ethanol was selected as it satisfied all the conditions relative to peak quality and non-interference at the specified wavelength. It is one of the most commonly used solvents in chromatographic separation. The wavelength of maximum absorption (λ_{max}) of amlaki was found to be 279 nm, and the proposed UV-spectrophotometric approach was found to be specific and selective for measuring S. *cumini* seed in poly-herbal formulations which is shown in **Figure 14**.

In accordance with ICH guidelines, the procedure was validated which is shown in Table 10. The UV-scans from the *S. cumini* standard concentrations (0.1-0.5 µg/mL) were compared to determine the specificity. All runs showed a distinct peak at 279 nm, which was determined to be the average wavelength of maximum absorbance (λ_{max}). Therefore, it was selected for linearity study which is shown in **Table 11**. With a correlation coefficient (r) of 0.9914 demonstrating high linearity between

absorbance and concentration, the regression graphs demonstrated conformity to Beer Lambert's law in the concentration range of 0.1 to 0.5 µg/mL was shown in Figure 15. The results where UV absorbance was directly proportional to the genuine concentration of the analyte was predefined by the goodness-of-fit test method at 279 nm **Figure 15** confirmed the range (working range). The suggested approach can directly measure analyte concentrations in the (0.1-0.5 µg/mL) range, and accuracy experiments have shown that this ranges from 50 to 150% of the test concentration. The intraday precision was studied for three independent samples six times which was shown in **Table 12**. The intermediate precision (%RSD) was observed for the analysis of three independent sets of samples. The %RSD value for intraday precision found were 0.924%, 0.5751% and 0.4668% represented in **Table 12** and the intermediate precision %RSD values for selected samples in three consecutive different days were found to be 0.8921%, 0.8346%, 0.3421% in ethanol shown in **Table 13**, respectively. The low values of %RSD in both intra-day and an inter-day analysis were found to be less than <2%. Hence, the precision was confirmed. The repeatability %RSD value was found to be 0.7950% in ethanol is shown in **Table 14**. By using the traditional addition method, the proposed approach's accuracy was determined. In **Table 15**, found from the analysis of samples spiked at three distinct concentrations (low, medium, high) that cover the operating range (0.1-0.5 µg/mL). The results demonstrated how closely the observed value for the sample matched its true value. The recovery percentage of the additional *S. cumini* concentrations was used to measure accuracy. To maintain accuracy and avoid interaction with formulation excipients, recovery values for the standard addition method used for the *S. cumini* analysis ranged from 99.647 to 101.943% is shown in **Table15**. **Table 16 and Table 17** show that the method was found to be reliable and consistent despite changes to the analysts (analysts 1 and 2) and the instruments (UV-Jasco V-630 and UV-Secom am XTD6). The triplicate determination of the *S. cumini* at a chosen concentration (0.5 µg/mL) was carried out, and the% RSD values in both the parameters were 0.82551% and 0.3609%, respectively which is less than 2%. Thus, robustness was determined. As the %RSD is less than 2% the method is validated according to ICH guidelines.

Estimation of *S. cumini* in market preparation

The method was successfully employed for the estimation of *S. cumini* in market preparations. Four market preparations were collected from the market and the percentage of *S. cumini* was determined. It was found to be 83.152%, 86.821%, 90.082% and 80.579% with %RSD value 0.5735, 0.7935, 0.6070 and 0.6090, respectively as shown in **Table 18**.

5.5 Conclusion

In conclusion, a simple, reliable, accurate and reproducible method has been developed and validated for analysis of *S. cumini* seed containing formulation. The developed method needs low cost, have faster speed with satisfactory precision. The method also has good specificity. The developed method was successfully validated following the guidelines of ICH and it could be employed for quality control analysis of *S. cumini* seed in market preparation.

Statistical analysis

All the observed results have been shown as Mean±Standard deviation and %RSD.

CHAPTER SIX: REFERENCES

1. Biswas, T.K., 2018. Drug discovery from Ayurveda: mode of approach and applications. *Natural Products and Drug Discovery*, pp. 3-28.
2. Bent, S., 2008. Herbal medicine in the United States: review of efficacy, safety, and regulation: grand rounds at University of California, San Francisco Medical Center. *Journal of General Internal Medicine*, *23*, pp.854-859.
3. Calixto, J.B., 2000. Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). *Brazilian Journal of Medical and Biological Research*, *33*, pp.179-189.
4. Saxena, M., Saxena, J., Nema, R., Singh, D. and Gupta, A., 2013. Phytochemistry of medicinal plants. *Journal of Pharmacognosy and Phytochemistry*, *1*(6), pp.168-182.
5. Hasler, C.M. and Blumberg, J.B., 1999. Phytochemicals: biochemistry and physiology. Introduction. *The Journal of Nutrition*, *129*(3), pp.756S-757S.
6. Qazi, M.A. and Molvi, K., 2016. Herbal medicine: a comprehensive review. *International Journal of Pharmaceutical Research*, *8*, pp.1-5.
7. Wnag S, Li Y. Trdaitiional Chinese medicine.,2005. In: Devinsky O, Schachter S, Pacia S, editors. Complementary and Alternative Therapies for Epilepsy. New York: Demos Medical Publishing;. pp. 177-182. ISBN: 1-888799-89-7
8. Goyal, M., Sasmal, D. and Nagori, B.P., 2012. Ayurveda the ancient science of healing: an insight. *Drug Discovery Research in Pharmacognosy. Croatia: InTech*, pp.1-10.
9. Watanabe, K., Matsuura, K., Gao, P., Hottenbacher, L., Tokunaga, H., Nishimura, K., Imazu, Y., Reissenweber, H. and Witt, C.M., 2011. Traditional Japanese Kampo medicine: clinical research between modernity and traditional medicine-the state of research and methodological suggestions for the future. *Evidence Based Complementary and Alternative Medicine*.
10. Green, G.M. and Sussman, R.W., 1990. Deforestation history of the eastern rain forests of Madagascar from satellite images. *Science*, *248*(4952), pp.212-215.
11. Pieroni, A., 2000. Medicinal plants and food medicines in the folk traditions of the upper Lucca Province, Italy. *Journal of Ethnopharmacology*, *70*(3), pp.235-273.

12. Gurib-Fakim, A., 2006. Medicinal plants: traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine*, *27*(1), pp.1-93.
13. Heinrich, M., Barnes, J., Gibbons, S. and Williamson, E.M., 2004. Fundamentls of pharmacognosy and phytotherapy, Churchill Livingstone, Edinbourgh, London, New York. *Churchill Livingstone, Edinbourgh, London, New York*, pp.245-252.
14. Fabricant, D.S. and Farnsworth, N.R., 2001. The value of plants used in traditional medicine for drug discovery. *Environmental Health Perspectives*, *109* (suppl 1), pp.69-75.
15. Kapoor, L.D., 1990. Butea monosperma. In *CRC Handbook of Ayurvedic Medicinal Plants* (p. 86). CRC Press Boca Raton.
16. Maher, P., 1999. A review of 'traditional' Aboriginal health beliefs. *Australian Journal of Rural Health*, *7*(4), pp.229-236.
17. Newman, D.J., Cragg, G.M. and Snader, K.M., 2000. The influence of natural products upon drug discovery. *Natural Product Reports*, *17*(3), pp.215-234.
18. Magner, L.N. and Kim, O.J., 2017. *A history of medicine*. CRC Press.
19. Chopra, A., 2000. Ayurvedic medicine and arthritis. *Rheumatic Disease Clinics of North America*, *26*(1), pp.133-144.
20. Kapoor, L.D., 1990. Butea monosperma. In *CRC handbook of Ayurvedic Medicinal Plants* (p. 86). CRC Press Boca Raton.
21. De Padua, L.S., Bunyapraphatsara, N. and Lemmens, R.H.M.J., 1999. *Plant Resources of South-East Asia* (Vol. 12, No. 1, pp. 210-218). Leiden: Backhuys Publication.
22. Weiher, E., Van Der Werf, A., Thompson, K., Roderick, M., Garnier, E. and Eriksson, O., 1999. Challenging Theophrastus: a common core list of plant traits for functional ecology. *Journal of Vegetation Science*, *10*(5), pp.609-620.
23. Apu, A.S., Bhuyan, S.H., Prova, S.S. and Muhit, M.A., 2012. Anti-inflammatory activity of medicinal plants native to Bangladesh: A review. *Journal of Applied Pharmaceutical Science*, (Issue), pp.07-10.
24. Sikder, M.A.A., Rashid, R.B., Islam, F., Hossian, A.K.M.N., Siddique, A.B., Kabir, S., Haque, M.R., Rahman, M.S. and Rashid, M.A., 2013. Screening of ten medicinal plants of Bangladesh for analgesic activity on Swiss-albino mice. *Oriental Pharmacy and Experimental Medicine*, *13*, pp.327-332.

25. Mostafa, A.A., Al-Askar, A.A., Almaary, K.S., Dawoud, T.M., Sholkamy, E.N. and Bakri, M.M., 2018. Antimicrobial activity of some plant extracts against bacterial strains causing food poisoning diseases. *Saudi Journal of Biological Sciences*, *25*(2), pp.361-366.
26. Panda, S.K., Patra, N., Sahoo, G., Bastia, A.K. and Dutta, S.K., 2012. Anti-diarrheal activities of medicinal plants of Similipal Biosphere Reserve, Odisha, India. *International Journal of Medicinal and Aromatic Plants*, *2*(1), pp.123-134.
27. Bell, D.S., 2008. Diabetes: a cardiac condition manifesting as hyperglycemia. *Endocrine Practice*, *14*(7), pp.924-932.
28. Yoshimura, M., Anzawa, R. and Mochizuki, S., 2008. Cardiac metabolism in diabetes mellitus. *Current Pharmaceutical Design*, *14*(25), pp.2521-2526.
29. Oyagbemi, A.A., Salihu, M., Oguntibeju, O.O., Esterhuyse, A.J. and Farombi, E.O., 2014. Some selected medicinal plants with antidiabetic potentials. In *Antioxidant-Antidiabetic Agents and Human Health*. Intech Open. Submitted: September 16th, 2013 Published: February 5th, 2014 DOI: 10.5772/57230
30. Anwar, H., Hussain, G. and Mustafa, I., 2018. Antioxidants from natural sources. *Antioxidants in Foods and its Applications*, *3*.
31. Verma, T., Sinha, M., Bansal, N., Yadav, S.R., Shah, K. and Chauhan, N.S., 2021. Plants used as antihypertensive. *Natural Products and Bioprospecting*, *11*, pp.155-184.
32. Taur, D.J. and Patil, R.Y., 2011. Some medicinal plants with antiasthmatic potential: a current status. *Asia Pacific Journal of Tropical Biomedicine*, *1*(5), pp.413-418.
33. Titanji, V.P., Zofou, D. and Ngemenya, M.N., 2008. The antimalarial potential of medicinal plants used for the treatment of malaria in Cameroonian folk medicine. *African Journal of Traditional, Complementary and Alternative Medicines*, *5*(3), pp.302.
34. Greenwell, M. and Rahman, P.K.S.M., 2015. Medicinal plants: their use in anticancer treatment. *International journal of Pharmaceutical Sciences and Research*, *6*(10), pp.4103.

35. World Health Organization, 2005. *National Policy on Traditional Medicine and Regulation of Herbal Medicines: Report of a WHO Global Survey*. World Health Organization.
36. Verma, S. and Singh, S.P., 2008. Current and future status of herbal medicines. *Veterinary World*, *1*(11), p.347.
37. Boulanger, B., Chiap, P., Dewé, W., Crommen, J. and Hubert, P., 2003. An analysis of the SFSTP guide on validation of chromatographic bioanalytical methods: progresses and limitations. *Journal of Pharmaceutical and Biomedical Analysis*, *32*(4-5), pp.753-765.
38. Ghosal, S., 1996. Active constituents of *Emblica officinalis*: Part I. The chemistry and antioxidative effects of two new hydrolysable tannins, Emblicanin A and B. *Indian Journal of Chemistry.*, *35*, pp.941-948.
39. Arora, A., Kumar, I., Sen, R. and Singh, J., 2011. *Emblica officinalis* (amla): Physico-chemical and fatty acid analysis from arid zone of Rajasthan. *International Journal of Basic and Applied Chemical Sciences*, *1*(1), pp.89-92.
40. Mandal, A. and Reddy, J.M., 2017. A review on phytochemical, pharmacological and potential therapeutic uses of *Phyllanthus emblica*. *World Journal of Pharmaceutical Research*, *6*, pp.817-830.
41. Sharif, M.T., Jahan, S., Nahar, K., Rahman, M.S., Shajjad, M.M.R., Chowdhury, A.A., Rashid, M.A. and Amran, M.S., 2017. Cardiovascular Activities of an Ayurvedic Preparation Amalaki Rasayan in Rat Model. *Bangladesh Pharmaceutical Journal*, *20*(2), pp.157-164.
42. Al-Rehaily, A.J., Al-Howiriny, T.S., Al-Sohaibani, M.O. and Rafatullah, S., 2002. Gastroprotective effects of 'Amla'Emblica officinalis on in vivo test models in rats. *Phytomedicine*, *9*(6), pp.515-522.
43. Baliga, M.S. and Dsouza, J.J., 2011. Amla (Emblica officinalis Gaertn), a wonder berry in the treatment and prevention of cancer. *European Journal of Cancer Prevention*, *20*(3), pp.225-239.
44. Sabu, M.C. and Kuttan, R., 2002. Anti-diabetic activity of medicinal plants and its relationship with their antioxidant property. *Journal of Ethnopharmacology*, *81*(2), pp.155-160.
45. Ram MS, Neetu D, Yogesh BL, Anju B, Dipti P, Pauline T *et al.* Cyto-protective and immunomodulating properties of Amla (*Emblica officinalis*) on

lymphocytes: an *in-vitro* study. *Journal of Ethnopharmacology*. 2002;81(1), pp. 5-10.

46. Timbola, A.K., Szpoganicz, B., Branco, A., Monache, F.D. and Pizzolatti, M.G., 2002. A new flavonol from leaves of Eugenia jambolana. *Fitoterapia*, *73*(2), pp.174-176.
47. Ruan, Z.P., Zhang, L.L. and Lin, Y.M., 2008. Evaluation of the antioxidant activity of *Syzygium cumini* leaves. *Molecules*, *13*(10), pp.2545-2556.
48. Mahmoud, I.I., Marzouk, M.S., Moharram, F.A., El-Gindi, M.R. and Hassan, A.M., 2001. Acylated flavonol glycosides from *Eugenia jambolana* leaves. *Phytochemistry*, *58*(8), pp.1239-1244.
49. Sengupta, P. and Das, P.B., 1965. Terpenoids and related compunds part IV triterpenoids the stem-bark of *Eugenia jambolana* Lam. *Indian Chem Society*, *42*(4), pp.255-258.
50. Sowjanya, K.M., Swathi, J., Narendra, K. and Satya, A.K., 2013. A review on phytochemical constituents and bioassay of *Syzygium cumini*. *International Journal of Natural Product Science*, *3*(2), pp.1-11.
51. Ayyanar, M. and Subash-Babu, P., 2012. *Syzygium cumini* (L.) Skeels: A review of its phytochemical constituents and traditional uses. *Asian Pacific Journal of Tropical Biomedicine*, *2*(3), pp.240-246.
52. Murali, V.S. and Devi, V.M., 2021. Antioxidant Analysis, FTIR and GC-MS Characterization of *Syzygium Cumini* Leaf Extract. *Annals of the Romanian Society for Cell Biology*, pp.15880-15892.
53. Suradkar, N.G., Pawar, V.S. and Shere, D.M., 2017. Physicochemical, proximate and bioactive composition of jamun (*Syzygium cuminii* L.) fruit. *International Journal of Chemical Studies*, *5*(3), pp.470-472.
54. Sapana, S.K., Jadhav, V.M. and Kadam, V.J., 2009. Development and validation of HPTLC method for determination of 3-hydroxy androstane [16, 17-C](6′ methyl, 2′-1-hydroxy-isopropene-1-yl) 4, 5, 6 H pyran in jambul seed (*Syzygium cumini*). *International Journal of PharmTech Research*, *1*(4), pp.1129-1135.
55. Jagetia, G.C., 2017. Phytochemical composition and pleotropic pharmacological properties of jamun, *Syzygium cumini* skeels. *Journal of Exploratory Research in Pharmacology*, *2*(2), pp.54-66.

56. Woo, Y.A., Kim, H.J., Ze, K.R. and Chung, H., 2005. Near-infrared (NIR) spectroscopy for the non-destructive and fast determination of geographical origin of *Angelicae gigantis* Radix. *Journal of Pharmaceutical and Biomedical Analysis*, *36*(5), pp.955-959.
57. Liu, H.X., Sun, S.Q., Lv, G.H. and Chan, K.K., 2006. Study on Angelica and its different extracts by Fourier transform infrared spectroscopy and two-dimensional correlation IR spectroscopy. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, *64*(2), pp.321-326.
58. Zou, H.B., Yang, G.S., Qin, Z.R., Jiang, W.Q., Du, A.Q. and Aboul-Enein, H.Y., 2005. Progress in quality control of herbal medicine with IR fingerprint spectra. *Analytical Letters*, *38*(9), pp.1457-1475.
59. Birk, C.D., Provensi, G., Gosmann, G., Reginatto, F.H. and Schenkel, E.P., 2005. TLC fingerprint of flavonoids and saponins from Passiflora species. *Journal of Liquid Chromatography & Related Technologies*, *28*(14), pp.2285-2291.
60. Gu, M., Su, Z. and Ouyang, F., 2006. Fingerprinting of Salvia miltiorrhiza Bunge by Thin Layer Chromatography Scan Compared with High Speed Countercurrent Chromatography. *Journal of Liquid Chromatography & Related Technologies*, *29*(10), pp.1503-1514.
61. Singh, M., Kamal, Y.T., Tamboli, E.T., Parveen, R., Siddiqui, K.M., Zaidi, S.M.A. and Ahmad, S., 2012. Simultaneous estimation of gallic acid, ellagic acid, and ascorbic acid in *Emblica officinalis* and in Unani polyherbal formulations by validated HPLC method. *Journal of Liquid Chromatography & Related Technologies*, *35*(17), pp.2493-2502.
62. Shailajan, S., 2015. Quality evaluation and standardization of a traditional Unani formulation Jawarish-e-Amla Sada. *International Journal of Green Pharmacy (IJGP)*, *9*(1), pp.21-25.
63. Guideline ICH. Validation of analytical procedures: text and methodology. Q2 (R1). 2005;1:1-5.
64. Singh, S.K., Jha, S.K., Chaudhary, A., Yadava, R.D.S. and Rai, S.B., 2010. Quality control of herbal medicines by using spectroscopic techniques and multivariate statistical analysis. *Pharmaceutical Biology*, *48*(2), pp.134-141.

65. Balekundri, A. and Mannur, V., 2020. Quality control of the traditional herbs and herbal products: a review. *Future Journal of Pharmaceutical Sciences*, *6*, pp.1-9.
66. Sim, C.O., Hamdan, M.R., Ismail, Z. and Ahmad, M.N., 2004. Assessment of herbal medicines by chemometrics–assisted interpretation of FTIR spectra. *J Analytica Chimica Acta*, *1*, p.14.
67. Noviana, E., Indrayanto, G. and Rohman, A., 2022. Advances in fingerprint analysis for standardization and quality control of herbal medicines. *Frontiers in Pharmacology*, *13*.
68. Abdullah & Kamal, N.,2022. Current authentication methods of herbs and herbal products: a systematic review. *Food Research*.
69. Ahmad, I., Ahmad Khan, M.S. and Cameotra, S.S., 2006. Quality assessment of herbal drugs and medicinal plant products. *Encyclopedia of Analytical Chemistry: Applications, Theory and Instrumentation*, pp.1-17.
70. Kandil, N.H., Ayoub, I.M., El Ahmady, S.H. and El Moghazy, S.A., 2022. Advances in the quality control of fenugreek seeds using chromatographic, spectroscopic and DNA based techniques: A comprehensive review. *Phytochemical Analysis*, *33*(2), pp.155-169.
71. Pakkirisamy, M., Kalakandan, S.K. and Ravichandran, K., 2017. Phytochemical screening, GC-MS, FT-IR analysis of methanolic extract of *Curcuma caesia* Roxb (Black Turmeric). *Pharmacognosy Journal*, *9*(6).
72. Lupoi, J.S., Singh, S., Parthasarathi, R., Simmons, B.A. and Henry, R.J., 2015. Recent innovations in analytical methods for the qualitative and quantitative assessment of lignin. *Renewable and Sustainable Energy Reviews*, *49*, pp.871-906.
73. Tshitenge, D.T., Ioset, K.N., Lami, J.N., Ndelo-di-Phanzu, J., Mufusama, J.P.K.S. and Bringmann, G., 2016. Rational quality assessment procedure for less-investigated herbal medicines: Case of a Congolese antimalarial drug with an analytical report. *Fitoterapia*, *110*, pp.189-195.
74. Lisperguer, J., Perez, P. and Urizar, S., 2009. Structure and thermal properties of lignins: characterization by infrared spectroscopy and differential scanning calorimetry. *Journal of the Chilean Chemical Society*, *54*(4), pp.460-463.

75. Jiang, Y., David, B., Tu, P. and Barbin, Y., 2010. Recent analytical approaches in quality control of traditional Chinese medicines-a review. *Analytica chimica acta*, *657*(1), pp.9-18.
76. Assi, S., Moorey, P., Kouris, N., Kneller, P. and Osselton, D., 2014. Identification of counterfeit tobacco using Fourier transform infrared spectroscopy. In *Meeting of the London Toxicology Group*.
77. Santos, M.S., Pereira-Filho, E.R., Ferreira, A.G., Boffo, E.F. and Figueira, G.M., 2012. Authenticity study of *Phyllanthus* species by NMR and FT-IR Techniques coupled with chemometric methods. *Química Nova*, *35*, pp.2210-2217.
78. Huck, C., 2015. Infrared spectroscopic technologies for the quality control of herbal medicines. In *Evidence Based Validation of Herbal Medicine* (pp. 477-493). Elsevier.
79. Murugan, M. and Mohan, V.R., 2014. Phytochemical, FT-IR and antibacterial activity of whole plant extract of *Aerva lanata* (L.) Juss. Ex. Schult. *Journal of Medicinal Plants Studies*, *4*(3), pp.51-57.
80. Zou, H.B., Yang, G.S., Qin, Z.R., Jiang, W.Q., Du, A.Q. and Aboul Enein, H.Y., 2005. Progress in quality control of herbal medicine with IR fingerprint spectra. *Analytical Letters*, *38*(9), pp.1457-1475.
81. Yap, K.Y.L., Chan, S.Y. and Lim, C.S., 2007. Infrared-based protocol for the identification and categorization of ginseng and its products. *Food Research International*, *40*(5), pp.643-652.
82. Vardin, H., Tay, A., Ozen, B. and Mauer, L., 2008. Authentication of pomegranate juice concentrates using FTIR spectroscopy and chemometrics. *Food Chemistry*, *108*(2), pp.742-748.
83. Zhang, L. and Nie, L., 2010. Discrimination of geographical origin and adulteration of radix astragali using fourier transform infrared spectroscopy and chemometric methods. *Phytochemical Analysis*, *21*(6), pp.609-615.
84. Yap, K.Y.L., Chan, S.Y. and Lim, C.S., 2007. Authentication of traditional Chinese medicine using infrared spectroscopy: distinguishing between ginseng and its morphological fakes. *Journal of Biomedical Science*, *14*, pp.265-273.
85. Cusano, E., Consonni, R., Petrakis, E.A., Astraka, K., Cagliani, L.R. and Polissiou, M.G., 2018. Integrated analytical methodology to investigate

bioactive compounds in Crocus sativus L. flowers. *Phytochemical Analysis*, *29*(5), pp.476-486.

86. Umar, A.H., Syahruni, R., Ranteta'dung, I. and Rafi, M., 2023. FTIR-based fingerprinting combined with chemometrics method for rapid discrimination of Jatropha spp (Euphorbiaceae) from different regions in South Sulawesi. *Journal of Applied Pharmaceutical Science*, *13*(1), pp.139-149.
87. Gao, H., Wang, Z., Li, Y. and Qian, Z., 2011. Overview of the quality standard research of traditional Chinese medicine. *Frontiers of Medicine*, *5*, pp.195-202.
88. Indrayanto, G., 2018. Recent development of quality control methods for herbal derived drug preparations. *Natural Product Communications*, *13*(12), p.1934578X1801301208.
89. Pascual, M.E., Carretero, M.E., Slowing, K.V. and Villar, A., 2002. Simplified screening by TLC of plant drugs. *Pharmaceutical biology*, *40*(2), pp.139-143.
90. World Health Organization, 1998. *Quality Control Methods for Medicinal Plant Materials*. World Health Organization.
91. Gedar, R.C., Sharma, R. and Sharma, S., 2012. A review on quality control of herbal drugs. Pharmatutor, *12* december.
92. Braz, R., Wolf, L.G., Lopes, G.C. and de Mello, J.C., 2012. Quality control and TLC profile data on selected plant species commonly found in the Brazilian market. *Revista Brasileira de Farmacognosia*, *22*, pp.1111-1118.
93. Eloff, J.N., Ntloedibe, D.T. and Van Brummelen, R., 2011. A simplified but effective method for the quality control of medicinal plants by planar chromatography. *African Journal of Traditional, Complementary and Alternative Medicines*, *8*(5S).
94. Das, C., Ghosh, G., Bose, A. and Das, D., 2019. Analytical Methods for Standardization of Ayurvedic Asavas and Aristas; A Review. *Indian Journal of Pharmaceutical Sciences*, *81*(3), pp.396-405.
95. Durón, R.R., Almaguer, L.C., Garza-Juárez, A.D.J., De La Luz, M., Cavazos, S. and Waksman-De-Torres, N., 2009. Development and validation of thin-layer chromatographic methods for quality control of herbal products. *Acta Chromatographica*, *21*(2), pp.203-215.

96. Mohammad, A., Bhawani, S.A. and Sharma, S., 2010. Analysis of herbal products by thin-layer chromatography: a review. *International Journal of Pharma and Bio Sciences*, *1*(2).
97. Leong, F., Hua, X., Wang, M., Chen, T., Song, Y., Tu, P. and Chen, X.J., 2020. Quality standard of traditional Chinese medicines: comparison between European Pharmacopoeia and Chinese Pharmacopoeia and recent advances. *Chinese Medicine*, *15*, pp.1-20.
98. Wahyuni, W.T., Saharah, M., Arif, Z. and Rafi, M., 2020. Thin layer chromatographic fingerprint and chemometrics analysis for identification of *Phyllanthus niruri* from its related species. *Journal of the Indonesian Chemical Society*, *3*(1), pp.47-52.
99. Choudhary, N. and Sekhon, B.S., 2011. An overview of advances in the standardization of herbal drugs. *Journal of Pharmaceutical Education and Research*, *2*(2), p.55.
100. Chanda, S. and Dave, R., 2009. In vitro models for antioxidant activity evaluation and some medicinal plants possessing antioxidant properties: An overview. *African Journal of Microbiology Research*, *3*(13), pp.981-996.
101. Muyumba, N.W., Mutombo, S.C., Sheridan, H., Nachtergael, A. and Duez, P., 2021. Quality control of herbal drugs and preparations: The methods of analysis, their relevance and applications. *Talanta Open*, *4*, p.100070.
102. Mwankuna, C.J., Mariki, E.E., Mabiki, F.P., Malebo, H.M., Styrishave, B. and Mdegela, R.H., 2022. Thin Layer Chromatographic Method for Detection of Conventional Drug Adulterants in Herbal Products. *Separations*, *10*(1), p.23.
103. Deconinck, E., Sacré, P.Y., Courselle, P. and De Beer, J.O., 2013. Chromatography in the detection and characterization of illegal pharmaceutical preparations. *Journal of Chromatographic Science*, *51*(8), pp.791-806.
104. De Souza, J.L., Da Silva, W.V., Bezerra, I.F., Ferreira, M.A. and Soares, L.L., 2018. Chemical profiles by thin-layer chromatography and high-performance liquid chromatography of plant species from Northeast Brazil. *Pharmacognosy Magazine*, *14*(56), pp.437-443.
105. Kagawad, P., Gharge, S., Jivaje, K., Hiremath, S.I. and Suryawanshi, S.S., 2021. Quality control and standardization of Quercetin in herbal medicines by

spectroscopic and chromatographic techniques. *Future Journal of Pharmaceutical Sciences*, *7*(1), p.176.

106. Kaushik, D., Pandey, M.K. and Sharma, A., 2014. Current issues in Authentication and Quality control of Natural Products. *Research in Plant Biology*. 4(5),57-64.
107. Iqbal, D., Pawar, R.K. and Sharma, R.K., 2010. Physico-chemical standardization of Butea monosperma (Lam.) Kuntze (Palasha): An ayurvedic drug. *International Journal of Pharmaceutical Quality Assurance*, *2*(1), pp.49-51.
108. Fan, X.H., Cheng, Y.Y., Ye, Z.L., Lin, R.C. and Qian, Z.Z., 2006. Multiple chromatographic fingerprinting and its application to the quality control of herbal medicines. *Analytica Chimica Acta*, *555*(2), pp.217-224.
109. Simha, K.R. and Laxminarayana, V., 2007. Standardization of Ayurvedic polyherbal formulation, Nyagrodhadi churna. Indian Journal of Traditional Knowledge. 6(4). pp .648-652.
110. Zhang, J.S., Guan, J., Yang, F.Q., Liu, H.G., Cheng, X.J. and Li, S.P., 2008. Qualitative and quantitative analysis of four species of *Curcuma* rhizomes using twice development thin layer chromatography. *Journal of Pharmaceutical and Biomedical Analysis*, *48*(3), pp.1024-1028.
111. Liang, Y.Z., Xie, P. and Chan, K., 2004. Quality control of herbal medicines. *Journal of Chromatography B*, *812*(1-2), pp.53-70.
112. Shantha, T.R., Patchaimal, P., Reddy, M.P., Kumar, R.K., Tewari, D., Bharti, V., Venkateshwarlu, G., Mangal, A.K., Padhi, M.M. and Dhiman, K.S., 2016. Pharmacognostical standardization of *Upodika-Basella alba* L.: an important ayurvedic antidiabetic plant. *Ancient Science of Life*, *36*(1), p.35.
113. Srujana, T.S., Konduri, R.B. and Rao, B.S.S., 2012. Phytochemical investigation and biological activity of leaves extract of plant *Boswellia serrata*. *The Pharma Innovation*, *1*(5, Part A), p.22.
114. Saran, S., Menon, S., Shailajan, S. and Pokharna, P., 2013. Validated RP-HPLC method to estimate eugenol from commercial formulations like Caturjata Churna, Lavangadi Vati, Jatiphaladi Churna, Sitopaladi Churna and clove oil. *Journal of Pharmacy Research*, *6*(1), pp.53-60.
115. Bajpai, R., Chaturvedi, P. and Tiwari, A., 2021. Development and Validation of UV Spectroscopic method for estimation of Ellagic acid in Herbal capsule

used for the treatment of Diabetes. *International Journal of Pharmacy & Life Sciences*, *12*(1).

116. Jannah, R., Rafi, M., Heryanto, R., Kautsar, A. and Septaningsih, D.A., 2018. UV-Vis spectroscopy and chemometrics as a tool for identification and discrimination of four *Curcuma* species. *International Food Research Journal*, *25*(2).
117. Baidoo, M.F., Asante-Kwatia, E., Mensah, A.Y., Sam, G.H. and Amponsah, I.K., 2019. Pharmacognostic characterization and development of standardization parameters for the quality control *of Entada africana Guill.* & Perr. *Journal of Applied Research on Medicinal and Aromatic Plants*, *12*, pp.36-42.
118. Gilarranz, M.A., Rodríguez, F., Oliet, M., García, J. and Alonso, V., 2001. Phenolic OH group estimation by FTIR and UV spectroscopy. Application to organosolv lignins. *Journal of Wood Chemistry and Technology*, *21*(4), pp.387-395.
119. Mensah, J.N., Brobbey, A.A., Addotey, J.N., Ayensu, I., Asare-Nkansah, S., Opuni, K.F. and Adutwum, L.A., 2021. Ultraviolet-visible spectroscopy and chemometric strategy enable the classification and detection of expired antimalarial herbal medicinal product in ghana. *International Journal of Analytical Chemistry*, *2021*.
120. Kucharska-Ambrożej, K. and Karpinska, J., 2020. The application of spectroscopic techniques in combination with chemometrics for detection adulteration of some herbs and spices. *Microchemical Journal, 153*, p.104278.
121. Gad, H.A. and Bouzabata, A., 2017. Application of chemometrics in quality control of Turmeric (*Curcuma longa*) based on Ultra-violet, Fourier transform-infrared and 1H NMR spectroscopy. *Food Chemistry*, *237*, pp.857-864.
122. Kaur, H., Thakkar, A. and Nagpal, K., 2019. UV Method Development and Validation of Ellagic Acid for its Rapid Quantitative Estimation. *Journal of Pharmaceutical Technology, Research and Management*, *7*(1), pp.1-5.
123. Kumari, R. and Kotecha, M., 2016. A review on the Standardization of herbal medicines. *International Journal of Pharma Sciences and Research*, *7*(2), pp.97-106.

124. Calixto, J.B., 2000. Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (phytotherapeutic agents). *Brazilian Journal of Medical and Biological Research*, *33*, pp.179-189.

125. Ren, X., He, T., Wang, J., Wang, L., Wang, Y., Liu, X., Dong, Y., Ma, J., Jia, J., Song, R. and Fan, Q., 2021. UV spectroscopy and HPLC combined with chemometrics for rapid discrimination and quantification of *Curcuma* Rhizome from three botanical origins. *Journal of Pharmaceutical and Biomedical Analysis*, *202*, p.114145.

126. Joshi, D.D., 2012. *Herbal Drugs and Fingerprints: Evidence Based Herbal Drugs*. Springer Science & Business Media.

127. Martelo-Vidal, M.J. and Vazquez, M., 2014. Evaluation of ultraviolet, visible, and near infrared spectroscopy for the analysis of wine compounds. *Czech Journal of Food Sciences*, *32*(1), pp.37-47.

128. Ansari, M.J., Al-Ghamdi, A., Khan, K.A., Adgaba, N., El-Ahmady, S.H., Gad, H.A., Roshan, A., Meo, S.A. and Kolyali, S., 2018. Validation of botanical origins and geographical sources of some Saudi honeys using ultraviolet spectroscopy and chemometric analysis. *Saudi Journal of Biological Sciences*, *25*(2), pp.377-382.

129. Sánchez, A.M., Carmona, M., Zalacain, A., Carot, J.M., Jabaloyes, J.M. and Alonso, G.L., 2008. Rapid determination of crocetin esters and picrocrocin from saffron spice (*Crocus sativus* L.) using UV–visible spectrophotometry for quality control. *Journal of Agricultural and Food Chemistry*, *56*(9), pp.3167-3175.

130. Farag, M.A., Sheashea, M., Zhao, C. and Maamoun, A.A., 2022. UV fingerprinting approaches for quality control analyses of food and functional food coupled to chemometrics: A comprehensive analysis of novel trends and applications. *Foods*, *11*(18), p.2867.

131. Roshan, A.R.A., Gad, H.A., El-Ahmady, S.H., Khanbash, M.S., Abou-Shoer, M.I. and Al-Azizi, M.M., 2013. Authentication of monofloral yemeni sidr honey using ultraviolet spectroscopy and chemometric analysis. *Journal of Agricultural and Food Chemistry*, *61*(32), pp.7722-7729.

132. Suhandy, D. and Yulia, M., 2021. The use of UV spectroscopy and SIMCA for the authentication of Indonesian honeys according to botanical, entomological and geographical origins. *Molecules*, *26*(4), p.915.

133. Guideline ICH. Validation of analytical procedures: text and methodology. Q2 (R1). 2005;1:1-5.
134. Koly, S.F., Chowdhury, A.A., S., Shahriar, S. and Amran, M.S. 2021. Development and validation of ultra violet spectroscopic analytic method for analysis of amlaki in marketed preparation using methanol as a solvent. *International Journal of Pharmaceutical Research*, 13(2), Apr – Jun Issue. Pp. 2535-2542. https://doi.org/10.31838/ijpr/2021.13.02.315.
135. Koly, S.F., Munira, M.S., Zaman, S., Shahriar, S., Amran, M.S. and Chowdhury, A.A., 2022. Development and validation of a UV-spectroscopic method for the analysis of black plum in marketed preparations using ethanol as a solvent. *Biomedical and Pharmacology Journal*, *15*(1), pp.157-164.