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FORMULATION, DEVELOPMENT AND EVALUATION OF PLICOSEPALUS ACACIA EXTRACT CAPSULES DELIVERY SYSTEM AS AN ADVANCED PHYTOTHERAPY APPROACH FOR HEPATOPROTECTIVE

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ABSTRACT

The *Plicosepalus Acacia* is plant widely distributed in Yemen, a large number of medicinal plants have been tested and found to contain active principles with curative properties against a variety of diseases. Liver protective plants contain a variety of chemical constituents like flavonoids, Phenol, Tannins, Steroids, Amino acid and Triterpene glycoside. Therefore, a large number of plants and formulations have been claimed to have hepatoprotective activity so the development of plant based hepatoprotective drugs has been given importance in the global market. Plicosepalus Acacia is commonly used in traditional medicine for a wide range of ailments including hepatoprotective activity. A Plicosepalus Acacia was formulated as capsules and evaluated for organoleptic properties and other evaluation parameters. It was concluded that the formulation of Plicosepalus Acacia extract medicinal herbs capsules delivery system as anadvanced phytotherapy approach for hepatoprotective according to the best results of in-vitro dissolution was found to be 76.8% within 45 minutes in acid medium and were evaluated.

KEYWORDS: *Plicosepalus Acacia*, Extract, Capsules, Medicinal herbs, Hepatoprotective activity, Phytotherapy.

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INTRODUCTION

Plicosepalus Acacia Herbal Medicine^[1-65]

Herbal medicine is the oldest form of health care known to mankind. It is an integral part of the development of modern civilization. The World Health Organization estimates that in some Asian and African countries 80% of the population depend on traditional medicine for primary health care; in many developed countries, 70% to 80% of the population has used some form of alternative or complementary medicine.

The WHO recognizes the value of plant medicines in health care delivery and endorses the use of those which have been scientifically proven to be efficacious, safe for use and of good quality.

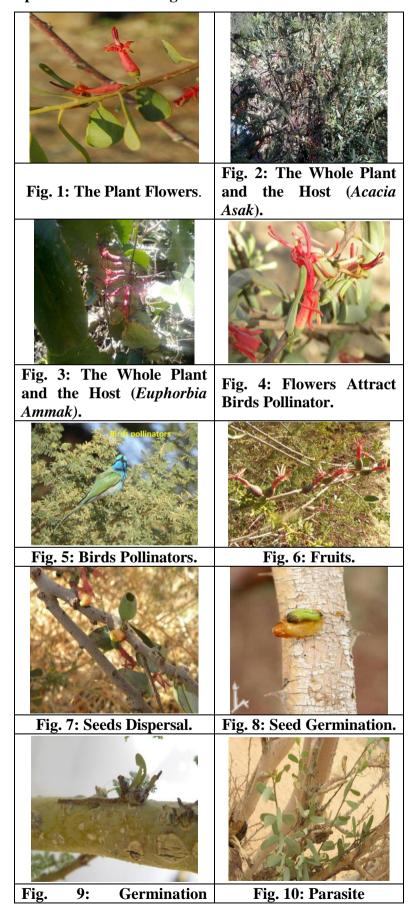
Herbal medicines are in great demand in the developed world for primary health care because of their efficacy, safety and lesser side effects. A detailed investigation and documentation of plants used in health traditions and pharmacological evaluation of these plants and their taxonomical relatives can lead to the development of invaluable plant-based drugs for many dreaded diseases. A large number of plants and purified natural substances have been screened for liver disorders.

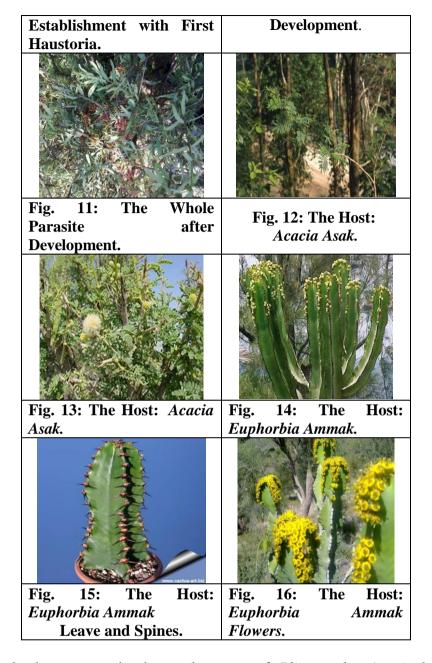
One of these herbal medicine plants is *Plicosepalus Acacia* which widely used in traditional medicine for treatment of variable diseases. *Plicosepalus Acacia* which is generally known as "Enab Ala'mq – kurad "(in Arabic) is a parasitic plant belongs to the family *Loranthaceae*, under the order *Santalales*. And it is distributed through the high mountains of some countries. In Yemen it is widely distributed in Taiz and suhban valley, Ibb city and it is parasiting in different trees which growth in Yemen.

Loranthaceae species (*Plicosepalus Acacia*) play an important and complex rule in the biological system where they live by interacting with insects, birds and mammals.

It is spreading along the host by birds that transfer the seed of these plant from host to another host, the Flowers of *Plicosepalus Acacia* attract birds' pollinators, they swallow the fruits whole and defecated viable seeds that were covered in a viscid pulp, which allowed the seeds to adhere to the substrate when voided seeds. dispersal and germination occur. The germination establishment with First Haustoria follow by Parasite development, this mechanism illustrates in Figures.^[1-16]

Parasitim Development Mechanism Figures





The compounds that present in the total extract of *Plicosepalus Acacia* have the high antioxidant activity such as flavonoids quercetin, ethyl gallate, gallic acid, loranthin and rutin. suggesting that the antioxidant capacity of the plant extract is due to a great extent to its flavonoids content. Hence this plant can be considered as potential sources of bioactive compounds acting as natural antioxidant.

Liver is the most important organ where drugs are structurally altered; resulting biologically inactive or active metabolites and some of these are toxic. Liver diseases are mainly caused by toxic chemicals, excess consumption of alcohol, infections and autoimmune disorder. Most of the hepatotoxic chemicals damage liver cells mainly by lipid peroxidation and other oxidative damages. Liver is also exposed to drugs in higher concentration as whole of the

drug pass through liver to reach systemic circulation. Thus, the liver is a vulnerable target of injury from various chemicals and drugs and disordered hepatic function is an important cause of abnormal drug handling.

If not treated early such injuries can lead to chronic liver disorders. A number of herbal formulations have been claimed to be effective in managing liver disorders.

To enhance the acceptability of the Herbal Medicine by consumers, many of the herbs have been converted into conventional dosage forms such as tablets, capsules, suspensions, solutions and powders.

So it is necessary to formulate the medicinal product into the dosage forms that are practical to use in order to gain the patient compliance and to meet the prescribed medicine delivery requirement while manufacturing is capable. These criteria are the challenge for most researcher and formulation scientist. Capsules are the common dosage form considered for many oral drugs that perceive good patient compliance and more simply to manufacture with less cost compare to the manufacturing of tablet.

There is a considerable need to dry the extracted plant prior to the capsule filling process in order to eliminate the moisture content. Thus, drying of the extracted plant to obtain semisolid powder, a free flowing, non-adherent, crumbly look powder form of semisolid medication, is a challenging option.

In herbal medicine plant-based formulations are used to alleviate the diseases. But the most important challenges faced by these formulations arise because of their lack of complete evaluation. So, evaluation is necessary to ensure quality and purity of the herbal product.

Plicosepalus Acacia is a plant belong to the family Loranthaceae, which is a largest family under the order *Santalales*. Some of the species of the Loranthaceae family are parasites on the roots while the remaining parasites on branches and stems trees like *Plicosepalus Acacia* and known as Mistletoe.

The *Plicosepalus Acacia* is plant widely distributed in Yemen. Plant materials are used through developed and developing countries as home remedies, over-the- counter drug products and raw materials for the pharmaceutical industry, and represent a substantial proportion of the global drug market. Herbal medicines have been used in the treatment of

liver diseases for a long time so the maintenance of a healthy liver is got possible. The main reason for Liver injury is the toxins produced by our body metabolites. Herbal remedies are focused to give safe route for reliving liver disorders.

The use of medicinal herbs and phytonutrients or nutraceuticals continues to expand rapidly across the world with many people now resorting to these products for treatment of various health challenges in different national healthcare settings. This past decade has obviously witnessed a tremendous surge in acceptance and public interest in natural therapies both in developing and developed countries, with these herbal remedies being available not only in drug stores, but now also in food stores and supermarkets. It is estimated that up to four billion people (representing 80% of the world's population) living in the developing world rely on herbal medicinal products as a primary source of healthcare and traditional medical practice which involves the use of herbs is viewed as an integral part of the culture in those community's herbal medicine is still the mainstay of about 75 - 80% of the world population, mainly in the developing countries, for primary health care.

The Indian traditional medicine the herbal drugs have gained importance and popularity in recent years because of their safety, efficacy and cost effectiveness. Several Indian medicinal plants have been extensively used in the Indian traditional system of medicine for the management of liver disorder. The use of natural remedies for the treatment of liver diseases has a long history and medicinal plants and their derivatives are still used all over the world in one form or the other for this purpose. Scientific evaluation of plants has often shown that active principles in these are responsible for therapeutic success.

A large number of medicinal plants have been tested and found to contain active principles with curative properties against a variety of diseases. Liver protective plants contain a variety of chemical constituents like phenols, coumarins, lignans, essential oil, monoterpenes, carotenoids, glycosides, flavonoids, organic acids, lipids, alkaloids and xanthene's. Therefore, a large number of plants and formulations have been claimed to have hepatoprotective activity so the development of plant based hepatoprotective drugs has been given importance in the global market. several phytomedicines (medicinal plants or herbal drugs) are now used for the prevention and treatment of various liver disorders. Although experimental studies have been conducted on a number of these plants and their formulations, however, only some plants have clearly shown the hepatogenic or hepatoprotective effects against liver diseases or hepatotoxicity caused by variety of hepatotoxic agents such as chemicals, drugs, pollutants,

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and infections from parasites, bacteria or viruses (e.g., hepatitis A, B and C), etc. Indeed, to obtain satisfactory herbal drugs for treating severe liver diseases, the medicinal plants must be evaluated systematically for properties like antiviral activity (hepatitis B, hepatitis C, etc.).

Antihepatotoxicity activity (antioxidants and others), stimulation of liver regeneration and choleretic activity. A combination of different herbal extracts / fractions is likely to provide desired activities to cure severe liver diseases. The medicinal plants contain several phytochemicals which possess strong antioxidant property, leading to antihepatotoxic activity. The rising number of patients with liver dysfunction due to overwhelming usage of drugs and alcohol has paved the path for researchers in an interest in herbal medicine. This is because there are only a few universally effective and available options for the treatment of common liver diseases, such as cirrhosis, fatty liver and chronic hepatitis. Herbal treatment has been used to alleviate disorders related to liver and other internal organs for many centuries in the eastern world, and have currently become a favorable therapy internationally for pathological liver conditions.

Several hundred plants have been examined to date, but only a handful has been studied thoroughly. The increasing use of herbal medicines reflects their perceived effectiveness in the treatment and prevention of disease, and the belief that these treatments are safe because they are 'natural'. Previous studies showed that the evaluates the effects of herbal extracts in the treatment of liver diseases, provides a general understanding of the actions of herbal medicines, a background for understanding the hepatoprotective of herbs, and the challenges that are faced by the scientific community in researching thoroughly of each and every compound of the herbs.

$Capsules^{[66-69]}$

Capsules are a common form of dosage for oral administration of pharmaceutical and nutraceutical products. They are produced in various shapes, sizes and materials, each capsule generally containing a single dose of active ingredient. In addition to the active drug ingredient or principal nutrient, other excipients are incorporated into the fill material, including antimicrobial preservatives, fillers, flavoring agents, sweeteners and coloring agent. Branding and dosage information may be printed on the outer surface of the capsule medication or ingredients inside the capsule may be in solid, liquid or paste form, depending on the drug component or, in the case of nutraceuticals, on the form of the main nutrient. The API filled in the capsules may contain solvents or excipients but these do not affect the

integrity of the capsule shell.

Advantages and Disadvantages of Capsules

The Advantages of Capsules: Hard-gelatin capsules suitable for extemporaneous compounding so that the dose and combination of ingredients may vary depending on the patient's needs. More stable than liquid dosage forms. Small-particle size so that the dissolution and absorption in body fluids faster than pills and tablets. Can cover up the taste and smell unpleasant medicine liquid preparations can be made with a certain concentration and can be used for depot capsules and enteric coated capsule.

The Disadvantages of Capsules: Not suitable for very soluble ingredients when the capsule is broken contact with the wall of the stomach, then the solution will be concentrated so that irritate the stomach and the stomach becomes tense. Cannot be used for materials that are very efflorescent or deliquescent. Efflorescent material make capsule become soft while deliquescent material causing the capsule to become brittle and easily broken and the bitter-medicine will cause vomiting and corrosive which are difficult to overcome and it took a relatively long compounding.

Type of Capsules: There are various forms of capsule, including: Based on consistency hard-capsule and soft-capsule. Based on how to use: Per oral, Per-rectal, Per vaginal. Topical Based on purpose of use: For human hard gelatin Capsule: Hard gelatin capsule shell consisting of: Basic ingredients: Gelatin, sugar, water and other ingredients: Dyes, preservatives (eg SO2), blur agent (e.g. TiO2), flavoring agent.

Hard Gelatin Capsule: Hard gelatin capsules contain solid active ingredients. The capsules are formed by dipping finger-shaped pin forms into liquid gelatin solution and then extracting them and allowing the resulting surface film of gelatin to dry out. Once the film dries, each capsule is trimmed and then removed from the pins. The caps and body pieces of the capsule are supplied unlocked to be filled with the appropriate drug or nutraceutical ingredients. More than one type of drug can be encased in a pharmaceutical capsule. In such a case, it is common to have the drugs in different forms, e.g., one as a tablet and one as a smaller capsule. Both drugs can then be encased in the larger capsule.

Soft Gelatin Capsule: Soft gelatin capsules, also called soft gels, are thicker than hard gelatin capsules and are sometimes the gelatin is plasticized by adding glycerin or sorbitol. The

thickness of the gelatin is chosen by the manufacturer according to the requirements of the encased material and the environmental conditions outside the capsule (e.g., air temperature and humidity). The composition of the gelatin used to prepare soft capsules may include preservatives, pigments and dyes. Flavorings and sweeteners may also be added.

Modified Release Capsule: Both hard or soft gel capsules can be chemically modified to control the release of the active ingredient(s). Delivery of the active ingredient is usually affected by dissolution, degradation or disintegration of an excipient in which the active compound is formulated. In the case of capsules, the capsule body may be coated with a material through which the drug diffuses or it may be a slowly dissolving coat that slowly releases the drug over time. A more recent innovation is a system utilizing a semipermeable membrane that blocks the drug from diffusing out through the membrane, but where the water on the exterior of the membrane can diffuse into the formulation, allowing the drug to be released through channels within the membrane.

Enteric Capsule: Enteric capsules are another form of modified release capsule, and again they maybe in the hard or soft form. The encapsulating material is designed to resist the stomach acid until it reaches the intestinal fluid where at a higher pH it breaks down and releases the active ingredients. It is important to observe during the manufacturing, packaging, storing and distribution of capsules that microbial contamination is possible as the capsules made of gelatin are susceptible to microbial attack and growth.

In the present study the *Plicosepalus Acacia* freeze -dried extract powder solid dosage form medicinal herbs capsules delivery system was prepared and evaluated as an advanced phytotherapy approach for hepatoprotective.

MATERIALS AND METHODS

The Freeze-dried dried methanolic extract of *Plicosepalus Acacia* was prepared and gift from (Prof Dr. Amina El-Shaibany, Professor Dr. of Pharmacognosy, Department of Pharmacognosy, Faculty of Pharmacy, Sana'a University, Sana'a, Yemen). Hard Gelatin Capsules (Size 00), Microcrystalline cellulose, starch, Carboxymethylcellulose, colloidal silicon dioxide (Aerosil), Methylparaben, Sodium starch glycolate, Crospovidone, Methanol, water, ethyl acetate, and chloroform. All chemicals used were all of analytical grade and other materials were gift from (Shaphaco Pharmaceutical Industry Company-Yemen).

Equipment: UV spectrophotometry (Jasco, Japan), Electronic balance (Metler, Germany), Disintegrator Erweka, Germany), water bath (Triup international CORP).

Formulation and Evaluation of *Plicosepalus Acacia* Extract^[25-139]

Determination of The Organoleptic Properties of Extract

The following organoleptic properties of the extract were assessed: Physical appearance, odor and taste. For the powder of *Plicosepalus Acacia* extracts were inspected and assessed using the natural senses (e.g. eyes, nose, mouth). As shown in Figure 17.



Fig. 17: Semisolid and Powder of Plicosepalus Acacia Flowers Extract.

Determination of the Solubility of Extract

The solubility of a substance fundamentally depends on the solvent used as well as on temperature and pressure. The extent of solubility of a substance in a specific solvent is measured as the saturation concentration where adding more solute does not increase its concentration in the solution. Oral ingestion is the most convenient and commonly employed route of drug delivery due to its ease of administration, high patient compliance, cost-effectiveness, least sterility constraints, and flexibility in the design of dosage form. As a result, many of the generic drug companies are inclined more to produce bioequivalent oral drug products. So, the solubility application according to standard parameters of solubility as shown in Table 1.

Table 1: Standard Parameters of Solubility.

Description	Part of The Solvent Required Per Part of Solute	
Very soluble	Less than 1	
Freely soluble	From 1 to 10	
Soluble	From 10 to 30	
Sparingly soluble	From 30 to 100	
Slightly soluble	From 100 to 1000	
Very slightly soluble	From 1000 to 10,000	
Practically insoluble	More than 10,000	

Determination of the Density of Extract

Preformulation parameters like bulk density, tap density and carr's index, were obtained for the powders. A known quantity of powder was poured into the measuring cylinder carefully level the powder without compacting, if necessary and read the unsettled apparent volume, Vo, to the nearest graduated unit as shown in Table 2.

Calculate the bulk density, in gm per ml, by the formula.

Bulk density = Bulk Mass/ Bulk Volume

Carr's compressibility index:

Carr's index (%) = (Tapped density – Poured density) / Tapped density

Table 2: Carr's Index Parameters of Powder Flowability.

Carr's Index%	Type of Flow
5 -15	Excellent
12 – 16	Good
18 – 21	Fair to Passable
23 – 35	Poor
33 – 38	Very Poor
>40	Extremely Poor

Determination of the Flowability of Extract

The angle of repose (θ) is another important parameter that can be used to describe the flowability of a powder. In this study a special apparatus was used for the test. The apparatus consisted of a glass cylinder kept in the center of the plate, a plate with scale and a ruler for measuring the height of powder mound. To determine the angle of repose, the glass cylinder was filled with 10g of plant extract (pass 180 sieve), the cylinder smoothly lifted allowing the powder to flow out at the bottom into the plate leaving a conical mound as shown in Table 3. The height and radius of the mound was measured and angle of repose then calculated using the following equation:

Tan $\theta = h/r$

 θ : Angle of repose.

h: height of the conical mound. **r:** radius of the conical mound.

Table 3: The Flow Properties of Powder and Angle of Repose.

Flow Property	Angle of Repose (Degrees)
Excellent	<20
Good	20-30
Passable	30-34
Very poor	>40

Formulation of Plicosepalus Acacia Extract Capsules

A uniform powder is obtained by mixing the semisolid of *Plicosepalus Acacia* extract with the appropriate adsorbent microcrystalline cellulose, sodium starch glycolate and methyl paraben the materials filled into the capsules as shown in Table 4.

Table 4: The Formulation of *Plicosepalus Acacia* Extract Capsules.

Ingredients	Amount (g)
Plicosepalus Acacia	0.5g
Microcrystalline Cellulose	0.8g
Sodium Starch glycolate	0.072g
Methyl Paraben	0.0018g

Evaluation of *Plicosepalus Acacia* **Extract Capsules**

Determination of Uniformity of Weight and the Amount of *Plicosepalus Acacia* Capsules

For the determination of the uniformity of weight, the British Pharmacopoeia method was used. In which Twenty of the *Plicosepalus Acacia* capsules prepared, their contents individually weighed and the average weight (mass) of the content determined. Not more than two of the individual weights (masses) had to deviate from the average weight (mass) by more than 7.5% and none of the deviates by more than twice that percentage. The amount of powder actually filled into the capsules was also compared with the desired quantity and the difference between the desired and actual quantity calculated. According to the formulation, 1.37g *Plicosepalus Acacia* extract was to be filled in one capsule. Twenty capsules were thus randomly chosen, their contents weighed, the percentage difference between this and the desired weight calculated and averaged for the 20 capsules to assess the accuracy of the filling process.

Determination of Moisture Content of Plicosepalus Acacia Extract Capsules

The presence of water plays an important role in the physical and chemical stability of the active pharmaceutical ingredients, and pharmaceutical preparations, because they may lead to their degradation. Water in pharmaceutical substances and preparations, provides a favorable environment for bacterial growth. Once a composition which contains a certain number of bacteria enters the organism, in the gastrointestinal tract may come to the death of bacteria and release of endotoxin. Therefore, the presence of water in the pharmaceutical substances affect; quality of the finished product, commercial reasons, i.e. process ability of the product, storage of the finished product, accuracy of the finished product, analytical indicators on the

dry matter, since it is necessary to know the water content for their calculations. The amount of water contained in the solid substance is not constant and depends on: nature of the substance, degree of its fragmentation, solution in which the substance is formed, ambient humidity and temperature.

Determination of the In-Vitro Dissolution of Plicosepalus Acacia Extract Capsules

The dissolution test measures the rate at which a drug is released into solution from a dosage form and is used as an indication of the bioavailability of a pharmaceutical product and of product quality. In order to simplify testing procedures. In this study the paddle method was used. The quantitation of the amount of extract dissolved was measured based on UV absorbance measured at 437nm, the wavelengths for maximum UV absorbance of solutions of the *Plicosepalus Acacia* extract determined by using a UV- Vis Spectrophotometer. For the dissolution study the following requirements and Procedure were used:

- Apparatus: paddle

- Medium: 0.1N HCL

- Volume of medium: 900ml

- Temperature: 37±0.5°C

- Rotation speed: 50 rpm

- Dissolution time: 10, 20, 30, 40 and 45 minutes.

RESULTS AND DISCUSSION

The Organoleptic Properties of The Freeze -Dried Extract of Plicosepalus Acacia

As shown in Table 5, and Figure 18, the organoleptic properties of the freeze -dried extract.

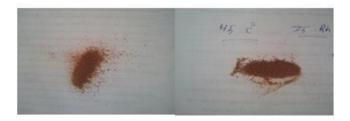


Fig. 18: The Organoleptic Properties of *Plicosepalus Acacia* Extract.

Table 5: The Organoleptic Properties of *Plicosepalus Acacia* Extract.

Properties	Plicosepalus Acacia Extract
Physical Appearance	ng, Small Particulate Powder
Color	Brown
Odor	Characteristic Odor
Taste	Bitter

The bitter taste and unpleasant odors normally result in poor patient acceptance of dosage forms. Hopefully these negative characteristics still present in the extract can be masked when incorporated in capsule form.

Table 6: Solubility Test.

Solvent	Solubility
Water	Freely soluble
Methanol	Partially soluble

The Solubility of the Freeze -Dried of *Plicosepalus Acacia* Extract

For oral solid dosage forms aqueous solubility is a crucial factor influencing the bioavailability of drugs. The results obtained in the solubility testing of the extract *Plicosepalus Acacia* show that the extract is freely soluble in water and partially soluble in methanol as shown in Table 6.

Table 7: The Results of Evaluation Parameters of *Plicosepalus Acacia* Extract.

Testing	Plicosepalus Acacia	
The Solubility of Extract	1gm/10 ml of water	
Particle size	Fine powder	
Carr's Index (%)	13.3%	
Angle of Repose (°)	11.9°	
Moisture Level of The	6 %	
Content		

Yield of Drying Total Extract of *Plicosepalus acacia*

Extracted *Plicosepalus Acacia* in a form of thick semisolid with some moisture content was dried by mixing separately with adsorbent powders the most appropriate adsorbent was microcrystalline cellulose. It used in different percentage start from 20% rach to 80% it is appropriative percentage maxed with total extract to produce powder.

The Particle Size of the Total Extract of *Plicosepalus Acacia* Powder

Particle size and shape are crucial parameter. They are important for the manufacture of the dosage forms, influence dissolution and bioavailability. *Plicosepalus Acacia* extract powder was moderately fine powder based on the British Pharmacopoeia standard and possessed appropriate flowability for the manufacture of the capsule dosage form.

The Densities of the *Plicosepalus Acacia* Total Extract Powders

The results of the Carr's index of Compressibility for *Plicosepalus Acacia* extract is 13.3%. The density study results shows that the *Plicosepalus Acacia* extract powders can be

categorized as having excellent flow properties as shown in Table 7.

The Flowability of the Plicosepalus Acacia Total Extract Powders

The *Plicosepalus Acacia* extract powders have angles of repose of (11.9°) can be categorized as having Excellent flow properties. This implicated that the *Plicosepalus Acacia* extract powders possessed appropriate flow ability for the manufacture of capsule dosage form as shown in Table 7.

Moisture Level of the Content of Plicosepalus Acacia Extract Capsules

The results of these tests are given and indicated that the moisture level of the contents of *Plicosepalus Acacia* capsules when analyzed in the preformulation study, the moisture content for *Plicosepalus Acacia* extract were however 6 %. Thus, appeared to have a slight increase in the moisture level of *Plicosepalus Acacia* material after encapsulation. This suggested that this extract absorbed some moisture during the filling procedure, presumably because it was hygroscopic.

The Uniformity of Weight and The Amount of Plicosepalus Acacia Extract Capsules

The results of the uniformity of weight and content of the *Plicosepalus Acacia* capsules were calculated. The average deviation in weight from average for *Plicosepalus Acacia* capsules were 0.1% and the average total content per capsule 92.72%. According to the British Pharmacopoeia, the limit on the acceptable deviation in weight from average for capsules is $\pm 7.5\%$ and the limits on the amount of content in the capsules 90% to 110%. The aforementioned results thus indicated that the *Plicosepalus Acacia* capsules met the British Pharmacopoeia specifications.

The In-Vitro Dissolution Test of Plicosepalus Acacia Extract Capsules

The result of the dissolution study on the *Plicosepalus Acacia* capsules showed that 76.8% of the *Plicosepalus Acacia* capsule contents dissolved in the dissolution medium within 45 minutes. These results are within the specification set in the British Pharmacopoeia and indicated that *Plicosepalus Acacia* capsules were immediate release solid oral dosage forms with good in vitro bioavailability.

Fig. 19: Dissolution Profile of *Plicosepalus Acacia* Capsules.

CONCLUSION

It was concluded that the formulation of Plicosepalus Acacia extract medicinal herbs

capsules delivery system as an advanced phytotherapy approach for hepatoprotective according to the best results of *in-vitro* dissolution was found to be 76.8% within 45 minutes in acid medium and were evaluated.

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