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Pharmacognostic Study of a Plant Seed Extract

Maxwell Osaronowen Egua

Abstract

Most research work on plant source for medicines end up without the researcher reaching a conclusive indication of the implicated chemical name/structure for the cure claimed. A large majority stop at just authenticating the claimed folkloric use of the crude extract of the said plant. A thorough authentication experimental process from plant identification, literature, and methodology to bioassay-guided pursuance of the active compound is carefully penned down. In addition, a vivid descriptive literature of the separation process, difficulty encountered, financial implication of the process, and joy in achievement of results is discussed in a friendly read. Furthermore, a close of the chapter with a plea to researches to endeavor to provide answers in their quest, rather than unending questions.

Keywords: *Corchorus olitorius*, crude extract, separation, liquid-liquid partitioning, fractionation, column chromatography

1. Introduction

Most research work on plant source for medicines end up without the researcher reaching a conclusive indication of the implicated chemical name/structure for the cure claimed. A large majority stops at just authenticating the claimed folkloric use of the crude extract of the said plant. Another group of researchers search for the bioactivity even when there is not a history or folkloric evidence giving lead to their quest. In the hunger of researchers' quest and bid not to "perish" and the unavailability of fund, we are satisfied with the mere authentication of crude plant extract. This is seen in the richness of the literature that abounds on the Internet with multiple claims of almost every disease cure and plant use. The "publish or perish" syndrome which is not matched with the provision of research funds or available research grants and the stiff promotion requirements seem to be counterproductive in terms of paucity of robust quality research especially in the developing world.

The researcher, in most times is like a child who wants to know the reason for everything around him. The child does not just stop at the first answer but persist to the exact utmost reason for his curiosity. The difficulties encountered in a conclusive end of a research quest are numerous though. That notwithstanding there should be some effort at our very best. In so doing, our goal would be met with genuine success and mind-blowing inventions. There could be some difficulty in the knowledge of how to go about the research work, and such the available becomes the only option. And being that the majority seen in the source (internet) is inconclusive, we satisfy our minds at the ease of a publishable authentication of the crude

extract. This chapter is met to follow up from the identification of a choice of plant part particularly seed, through to the process of isolation of the active ingredient and structural characterization.

Our ancient fathers knew the natural source of medicines which was once the only option known to man. They used this source to cure every disease that came their way. Over the centuries several options of drug source and discovery were uncovered. The era of synthetic substances (synthesized drugs) relegated our once popular natural gift of nature. The history of drug discovery has cited several drugs from natural sources with plant source earning a reasonable portion [1]. The notion that is still held high about natural products is its likelihood of being accepted by the body than synthetic substances [2]. Herbs due to their high chemical diversity and broad biological functionality have consistently been considered the leading source of pharmaceuticals, employed in the treatment of various human diseases [3]. An important drug used in treatment of diabetes, metformin, is a derivative of plant-derived compound guanidine from *Galega officinalis* [4]. It is now commonplace to include herbal or botanical extracts as a part of medical treatment (as an adjunct to hypoglycemia agents) [5]. It is obvious there still lay in nature's bank a lot of uncovered chemicals useful for the treatment of the numerous diseases that inflict man. These await researchers dogged enough to uncover them. They may remain hidden (researches) if we satisfy ourselves with just the authentication of herbal use. There is need therefore to conduct pharmacognostic and pharmacological studies conclusively, to ascertain chemicals with therapeutic values.

In the bid to guide researchers through a thorough authentication experimental process from plant identification, literature, methodology to bioassay-guided pursuance of the active compound, a bias toward diabetes and a seed plant part research would be described with some effort at generalizing the process.

2. Research study justification

In Africa, hundreds of plants are used traditionally for the management of diabetes mellitus; however, only a few of these African medicinal plants have received scientific scrutiny, even though the World Health Organization has recommended medical and scientific examinations of these plants that are undertaken [6]. Diabetes mellitus, a serious endocrine syndrome, is a group of multiple disorders with different etiologies and characterized by derangement of carbohydrate, protein, and fat metabolism caused by a complete or relative insufficiency in insulin secretion and/or insulin action [7]. Approximately 140 million people worldwide are estimated to suffer from diabetes mellitus [8]. The side effects of taking insulin and oral hypoglycemic agents have brought about a growing interest among patients for using natural products having antidiabetic activity [9]. Pharmacology and toxicological evaluations of medicinal plants are essential for drug discovery, and not to forget, there lay in nature's bank a lot of uncovered chemicals useful for the treatment of numerous diseases that inflict man.

In the guide of the above paragraph, and with a lead to its use, such as a traditional medicine practitioner use whose patient was patronizing an orthodox medical practitioner that was noticed a once poorly controlled diabetic patient having an almost normal control by laboratory test, the choice of the plant seed was drawn from the concoction implicated. Several lead modest stories abound literatures for numerous plants as with the choice of a seed plant part, *Corchorus olitorius* explored for antidiabetic property [10]. Worldwide, a number of plants with acclaimed antidiabetic properties are being studied, among these are *Treculia africana* and *Bryophyllum pinnatum* [11], *Gynostemma pentaphyllum* tea [12], *Ganoderma lucidum*

[13] ginger and garlic [14], *Phyllanthus niruri* [15] *Ficus religiosa* [16], *Boerhaavia diffusa* and *Ocimum sanctum* [17], and *Fumaria parviflora* [18]. Other plants reported study the world over, of hypoglycemic effect are *Fructus Mume* formula [19], Diabetan tablet (a blend of *Salvia officinalis, Trigonella foenum*, and *ginseng*) [20], flower heads of *Artemisia maritima* [21], *Eriobotrya japonica* seeds [22], and fenugreek (*Trigonella-foenum graecum*) seed [23] to mention few.

After this lead choice of the plant (seed), the plant identity is sort, with the help of a botanist, following purchase from traders of seeds of farm produce. The plant and part (seed) of the plant of interest are deposited in the researchers' institution with a herbarium voucher specimen number obtained. The ecology of the choice plant is a necessary literature to be included in the researcher's write-up. *Corchorus olitorius* plant grows in grassland and does well on abandoned fields, often close to marshes, rivers, and lakes, ranging from warm temperate through tropical desert to wet forest life zones, at up to 1250(−1750) m altitude [24]. It thrives best under hot and humid conditions [24]. The geographical origin of *Corchorus olitorius* is often disputed; it is rather pantropical in distribution [24]. In the savanna and Sahel zone, it grows best during the hot rainy season [24]. It is cultivated where annual rainfall averages 600–2000 mm [24]. The optimal temperature is 25–32°C, and growth stops below 15°C [24]. *Corchorus olitorius* is a short-day species. In Nigeria a day length of 12.5 hours caused a much stronger vegetative growth expressed in weight of roots, stems, and leaves than a day length of 11.5 hours, but the fruit and seed production was higher at a photoperiod of 11.5 hours [24]. The plant grows best in sandy loam soils rich in organic matter and grows poorly on heavy clay [24]. It is a leading leaf vegetable in Côte d'Ivoire, Benin, Nigeria, Cameroon, Sudan, Kenya, Uganda, and Zimbabwe [24]. It is also cultivated as a leaf vegetable in the Caribbean, Brazil, India, Bangladesh, China, Japan, Egypt, and the Middle East [24]. *Corchorus* is genus of about 40–100 species of flowering plants in the family *Malvaceae* [24]. The specie of interest is *Corchorus olitorius*, others are *Corchorus capsularis*, *Corchorus tridens*, *Corchorus walcottii*, etc. [24].

The following description of the process of seed preparation, extraction, fractionation, and bioassay is guided by works of Egua et al. [10, 25–27]. Care is taken here at the generalization of the procedure for all seeds and plant parts and advice for an educated modification in choice of solvents for intending users.

3. Preparation of seed extract

The dried seeds are grinded to powder using a blending machine. Soxhlet extractor is used for extraction of the dried powdered seed using ethanol as the solvent. A 10 g of the powdered seed extract is placed inside the thimble made from thick filter paper, which is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor allows for several cycles to repeat many times, over hours and days with the desired compound dissolved in the warm ethanol solvent. During each cycle, a portion of the nonvolatile compound (powdered extract) dissolved in the solvent. After many cycles the desired compound is concentrated in the distillation flask. The advantage of this system is that instead of many portions of warm solvent passing through the sample, just one batch of solvent is recycled. After extraction, the solvent is removed by means of a rotary evaporator, yielding the extracted compound (which is weighed). The non-soluble portion of the extract remains in the thimble and is weighed also before it is discarded. These values would help in the calculation of the yield of crude extract from the seed.

The crude extract is first tested for the acclaimed bioactivity before the fractionation procedure is carried out. At this stage, the entire model met for sufficient

scientific authentication of the disease is used as it is documented later. And the crude extract is also tested for its phytochemical properties.

4. Extraction and fractionation procedure

Extraction and fractionation were according to Gandhi et al. [28] and Leila et al. [29] with some modification in the choice of primary solvent (water) and partitioning solvents (hexane, chloroform, ethyl acetate, and butanol) [26]. The solvents are chosen in order of polarity. The advantage is to allow for different chemical compounds in the plant part (seed) to selectively dissolve in solvents they are accommodated in (as with the chemical axiom "like dissolves like"). The extracted compound is collected from the rotary evaporator under vacuum at 45°C and is ready for use.

The ethanol extract residue obtained is dissolved in 100% water (500 ml) and exhaustively extracted by consecutive liquid/liquid partition with hexane (500 ml), chloroform (500 ml), ethyl acetate (500 ml), and saturated butanol (500 ml) using a separating funnel (1000 ml). That is, on your initial fill of the separating funnel with primary solvent (500 ml water) and the extracted compound, shake vigorously to make a solution before adding the next solvent. The content is again shaken vigorously following the addition of the next solvent, before it is left to stand till the obvious separation of both solvents. The separated solvent is let out into a container and labeled the solvent (say hexane) extract. The next solvent (500 ml) of choice (chloroform) is again added to the remaining water extract solution left in the separation funnel and again shaken vigorously. The higher the number of solvent used is, the better the expected result and further ease at subsequent separation. The experiment takes advantages of the immiscibility of the solvents with water (the primary solvent used to dissolve the extracted compound).

The hexane, chloroform, ethyl acetate, saturated butanol, and last remaining aqueous fractions are evaporated to obtain fractions [26]. It should be borne in mind that each of the fractions contains a portion of some of the chemical constituent in the primary water-extract mixture in the initial fill of the funnel. The fractions obtained (hexane, chloroform, ethyl acetate, saturated butanol, and last remaining aqueous) are bioassayed for bioactivity. At this stage, the entire model met for sufficient scientific authentication of the disease (investigated) is used as with the case of the crude extract [25]. That is tested for antidiabetic and phytochemical properties in the case of this writer/researcher.

5. Further fractionation in column chromatography

The most potent fraction is selected and subjected to fractionation in column chromatography using silica gel. The column fractions are eluted using hexane, hexane ethyl acetate, ethyl acetate, ethyl acetate methanol, and methanol (in order of polarity) as mobile phase. The obtained pure compounds are isolated and identified through thin layer chromatography (TLC) and functional group analysis by nuclear magnetic resonance (NMR) spectroscopy [26, 27].

A glass chromatography column is set up. A piece of wool is placed in the bottom of the column and tamped down with a glass rod. The column is attached to a clamp stand and securely fastened in a vertical position. The column is then filled with hexane. Some (about 20) grams (20 g) of silica gel (60 mesh) is poured into a flask containing hexane; the slurry (mixture of hexane and silica gel) is then packed into the column while tapping the glass. After packing, the excess solvent is drained (using the tap at the bottom of the column) until it just reached the top level of

the silica gel. A thin layer of cotton (adsorbent) is placed on top of the column to prevent it from being disturbed when fresh solvent is added. The setup is ready for loading of the choice fraction with the best bioactivity. In the case of this research, the chloroform fraction was chosen. A quantity (say 2 g) of the chosen (chloroform) most bioactive fraction is mixed with little quantity (2 g) of silica gel and left to air dry.

The chloroform fraction and silica gel is loaded dried to the top of the column. A small amount of the eluting solvent (hexane) with which the setup was loaded is added and allowed to drain in until the mixture was a little way into the adsorbent (cotton), and then the column was filled to the top with eluting solvent (hexane). The solvent system, starting with 100% hexane and 0% ethyl acetate, with subsequent increase in the polarity by 1%, is added. The eluent (fluid/solvent mixture) is collected in numbered test tubes of 15 ml each from the tap below. The column fractions are further eluted using hexane ethyl acetate (i.e., 99–1%…1–99%), ethyl acetate, (in order of polarity) as mobile phase. This was done by increasing the polarity by 1% alteration in eluent solvents' ratio (i.e., ethyl acetate 100%; ethyl acetate 99%/methanol 1%; till methanol 100%) [27]. The process is guided by thin layer chromatography (TLC) monitoring, for an effective separation in the eluent (solution containing "pure compound"). The procedure was stopped on eluent of the last pure compound. The solvent level is never allowed to drop below the top of the adsorbent. The eluent with the same bands of compounds are pooled. It should be said that the researcher could at this stage harvest pure compounds, seen in the TLC as single band. The process is discontinued when the compound(s) desired are off the column. The eluents collected in pools are put in the rotary evaporator and run to harvest the compound (pure or in two or three).

6. Separation with preparative TLC plate

Finally the compounds in two or three bands are separated using a preparative TLC plate after collecting compound in the rotary evaporator. There are different types of preparative plates. The difference lies in the capacity of how much compound it can separate and whether it has a concentration zone or not. Though expensive, the relaying researcher advices for the preparative TLC plate with concentration zone and avoidance of locally made ones that may input impurities with poor separation quality. The quantity (concentration) of the compound to be loaded onto the preparative plate at the concentration zone is dependent of the type purchased. It should be stated here that the loading of the compound is the crucial skill for a successful separation. So if the type purchased can only separate 50 mg at a go, make sure you load say 48 mg for a successful uniform separation.

In preparation one would have first found out the solvent system that would best separate the compound through thin layer chromatography (TLC) testing. This solvent system is what is poured into the preparative TLC chamber (made of thick glass) that can accommodate a 20 by 20 cm preparative TLC plate with concentration zone. The procedure starts with first weighing out 50 mg of the compound to be separated and making into a fine dissolved solution ready to be loaded onto the plate at the concentration zone. The loading is done with a pipette such that it is diligently spread uniformly onto the plate from one end of the concentration zone to the other, just avoiding the very edges (right and left). Allow the plate to dry after the loading. Prepare the developing chamber (thick glass preparative TLC chamber) by making sure it's clean and dry before pouring in your solvent system of choice for the separation. The solvent level in the developing chamber should not be higher than the level of the concentration zone (or the point of load of the compound). It

should be just below the loading point when the preparative plate is dropped gently into the developing chamber and allowed to separate for an hour tops. The time is guided by a virtualized capillary movement of the solvent seen on the preparative plate. The plate is removed from the chamber after completion of the capillary movement to almost the tip of the plate and dried at very low temperature say 40°C. The separated compounds are located in the plate by using an ultraviolent light source, and the positions are marked. Since the compound would have traveled the same distance on the plate, it is easy to mark the region and scrape out the position on the plate into a clean beaker. A solvent is then poured into the beaker to dissolve the separated pure compound. The content of the beaker is then filtered to collect the solution (solvent and dissolved separated pure compound) which is allowed to evaporate by low temperature heat of 40°C.

The described procedures are repeated until the researcher has the desired quantity (mg) for both bioassay and structural elucidation. It is advised that one should have secured a research grant because several repeat processes are expected before a reasonable quantity can be gotten that would warrant for a successful bioassay.

7. Bioassay

At this point of the bioassay with the most active pure compound, the methodology chosen should be definitive and not the whole array of test for the particular disease of interest.

7.1. The experimental model of a disease

The experimental model of a disease aids not only the understanding of the pathophysiology of the disease but also the development of drugs for its treatment [30]. In the bias of the relaying researcher, the several animal models existing for studying diabetes mellitus (DM) are treated below. And with this knowledge, the reader can deduct relevant idea to use in her/his field of interest. It should be noted that certain disease state may have more than one model for scientific study. And the choice of test may be the use of more than one model which would be sufficient to satisfy the general entity of the pathophysiology of the disease state.

7.1.1 Normoglycemic animal model

Normal healthy animals are used for testing potential oral hypoglycemic agents. This method allows for the effect of the drug to be tested in the animal with an intact pancreatic activity [30]. This is a valid screening method often used in addition to diabetic animal models [31]. This means that using this model alone would not be sufficient for the study of this disease of interest.

7.1.2 Oral glucose loading animal model

In this method the animals are fasted overnight, then an oral glucose load (1.0–2.5 g/kg body weight) is given, and blood glucose level is monitored. This method is often referred to as physiological induction of DM because there is no damage to the pancreas even with raised blood glucose level. And in the clinical setting, it is referred to as oral glucose tolerance test (OGTT), used for diagnosis of borderline DM [30].

7.1.3 Chemical induction of DM

The most frequently used drugs are streptozotocin and alloxan. Both drugs exert their diabetogenic action on parenteral administration (intravenously, intraperitoneally, or subcutaneously) [30]. The dose required for DM induction depends on the animal species, route of administration, and nutritional status of the animal [32].

7.1.3.1 Alloxan model of DM

Alloxan is a well-known diabetogenic agent widely used to induce type 2 DM in animals [33]. The animals are administered with a single dose of alloxan 140–180 mg/kg (usually 150 mg/kg) as a 5% w/v in distilled water after overnight fast intraperitoneally in the case of rats and mice. Alloxan causes selective necrosis of pancreatic islet β cells producing different grades of the severity of DM by varying dose used. These may be classified by measuring the animals' fasting blood sugar (FBS) level. Moderate DM is defined as FBS level of 180–250 mg/dl and severe DM as FBS level above 250 mg/dl in rabbits [34]. The simplistic argument made against the use of alloxan to induce type 2 DM is that alloxan produces β cell damage, thus leading to type 1 rather than type 2 DM. But studies showed that there are no differential responses to hypoglycemic agents by alloxan and glucose loading hyperglycemic (with intact pancreatic cells) rats [30]. The best known drug-induced DM is the alloxan-induced DM, capable of inducing both type 1 and type 2 DM with proper dosage selection [30].

7.1.3.2 Streptozotocin model of DM

Streptozotocin prevents DNA synthesis in mammalian cells (and bacteria cells) resulting in mammalian cell death. The induction of DM with streptozotocin takes some time. Diabetes develops gradually and may be assessed after a few days, usually 4 days in mice and 7 days in rats. Single dose of streptozotocin in sterile citrate buffer may be used: rats 80 mg/kg; mice 150 mg/kg administered intraperitoneally. This may produce a serum glucose level of about 180–500 mg/dl as DM induction. Although it is the most commonly used model, problems involved in its use include spontaneous recovery from high blood glucose levels by development of functioning insulinoma [35] and high incidence of kidney and liver tumors, due to the oncogenic action of streptozotocin [36].

7.1.3.3 Other chemical methods

Other chemical methods are ferric nitrilotriacetate, ditizona, and anti-insulin serum [30].

7.1.4 Surgical model of DM

This model employs more recently partial pancreatectomy with large resection (over 80% of the pancreas in rats) required to obtain mild to moderate hyperglycemia. Another technique is complete removal of the pancreas (total pancreatectomy). Few researchers have employed this model due to the limitations of the technique which include high technical expertise and adequate surgical room environment, major surgery and high risk of animal infection, adequate postoperative

analgesia and antibiotic administration, supplementation, and loss of pancreatic counter regulatory response to hyperglycemia [30].

7.1.5. Genetic model of DM

7.1.5.1. Two types exist

7.1.5.1.1. Spontaneously developed diabetic rats

An example is the diabetic Gato-Kakizaki rat which is a genetic lean model of type 2 diabetes originating from selective breeding over many generations of glucose-intolerant nondiabetic wistar rats [37]. One great advantage of these models is that they can be employed as model of atherosclerosis which represents the long-term complication of diabetes mellitus and tested against several natural products and is without the interference of side effects induced by chemical drugs [38]. Mutant strains obese diabetic mice are available such as the C57BL/Ksj-db/db. With this model it is possible to test for effects of plant extracts on blood sugar, body weight, insulin production, and insulin resistance [38].

7.1.6. Genetically engineered diabetic mice

In this case, rodents may be produced to over- (transgenic) or under (knockout)-expressed proteins thought to play a key part in glucose metabolism [39]. Certainly, the high cost restricts their study in sophisticated protocols which explore mechanism of potential therapeutic agents that stimulate pancreatic β-cell death [40]. Insulin-dependent diabetes mellitus (IDDM) can be developed by inserting into the unique viral protein of mice which is then expressed as a self-antigen in the pancreatic islets of Langerhans. Another is lymphocytic chorio-meningitis virus (LCMV)-induced IDDM mice [41]. This procedure is relatively new and rarely used because of the sophisticated techniques, cost, and equipment required.

It suffices here to know that in the choice of assessing a compound, not all the available methods are chosen. Like in the case of the relaying researcher, the following were used to screen for an active compound especially at the stage of crude extract and the liquid-liquid partitioning fractions: normoglycemic animal model, oral glucose loading animal model, and alloxan model of DM. The choice for these three models was influenced by fund, availability, technical skill, and satisfaction of study requirement. At the stage of bioassaying (testing) the pure compound, the only model used was the definitive alloxan-induced DM [27].

After a successful bioassay of the pure compounds, that (pure compound) with the best activity is sent for structural elucidation through functional group analysis by nuclear magnetic resonance (NMR) spectroscopy and confirmed with gas chromatography and mass spectroscopy (GC MS) [27]. The readings are then read out by an experienced chemist. At this point the researcher would be fulfilled for such hard work to be graced by a chemical structure implicated for the bioactivity claimed.

It suffices here to note that there are some quests undertaken by the relaying researcher, not earlier mentioned in this chapter, such as lethal dose estimation (LD50) test of the extract; subacute toxicity; chronic toxicity study [10]; phytochemical analysis [25]; and peroxidatation test of the extract, that were necessary for a successful, question answering research (by providing parameters that aided the meaning/answers of the whole research quest).

8. Conclusion

The best research tends to answer and satisfy the questions asked before the quest, in such a manner that little or no questions are being asked by the research work itself. Though it is impossible for a research work not to leave some unsolved curiosity, after its conclusion, we should have solved or answered to a point that subsequent questions/quests would be too minute to matter. I therefore indulge every intending researcher to engage in research with such resort to have a conclusive end that would be truly conclusive. A thorough authentication experimental process from plant identification, literature, methodology to bioassay guided pursue of the active compound and chemical structural identification of the implicated active compound.

Acknowledgements

Conflict of interest

There is no conflict of interest, as I here declare.

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