**PROPOSAL**

**Project Title:**
**Phytochemical and Pharmacological Investigations of Potentially Active Antidiabetic West African Herb: *Picralima nitida***

**Aim:**
Diabetes mellitus is a complex metabolic disorder characterized by persistent high blood glucose level. It is a disorder in which the use of carbohydrates by the body is disturbed along with proteins and fat. Globally, the incidence of diabetes mellitus is increasing. The World Health Organization (WHO) documented that millions of people are suffering from diabetes and the number is expected to increase rapidly over time. The increased incidence of diabetes is due to variables such as sedentary behavior, obesity, and inadequate eating practices. Diabetes causes significant morbidity and mortality, and has been linked to severe sequelae, such as heart disease, kidney failure, and neurological disorders, making it one of the most significant public health challenges worldwide. The management of diabetes mellitus often encompasses lifestyle modifications and pharmacological therapies. Despite the success of the current therapies in managing diabetes, there are still issues with their use such as restricted effectiveness and harmful side effects. Consequently, there is an increasing demand for innovative therapeutic strategies that can proficiently regulate blood glucose levels with minimal adverse consequences. This has prompted the search for more effective treatments among researchers. The phyto-ingredients of plants are promising alternative drugs to manage diabetes mellitus. While traditional medicine systems have acknowledged the therapeutic benefits of plants, contemporary pharmaceuticals have not yet commercialized herbal remedies with antidiabetic activity. Several plants have been documented for their anti-diabetic efficacy. *Picralima nitida* commonly called picralima is an important plant of the Apocyanaceae family. Picralima is highly medicinal and popular in many West African countries where it is prepared in different forms and used in the treatment of malaria, gastrointestinal disorder, worm infestation, and diabetes. Picralima contains a lot of phyto-constituents notably alkaloids, and phenolic compounds. Numerous studies have investigated the antimalarial activity of this plant, only a limited information is available on its antidiabetic activity. For this reason, the present study aims to carryout in-depth study on the antidiabetic activity of Picralima by asking the following questions;

- Does Picralima extract have antidiabetic activity?
- What is the most active fraction of the extract with antidiabetic activity?
- What are the phytoconstituents with antidiabetic activity?
- Can the active fraction or phytoconstituents be formulated into herbal remedy for diabetes management?

To address these questions, the study is designed to meet the following objectives:

1. To determine the antidiabetic activity of picralima leaves and stem bark extracts in vitro, in vivo, and in silico
2. To fractionate the extracts into different organic solvents, and determine the antidiabetic activity of each solvent
3. To determine the effective dose that cause significant reduction in blood glucose.
4. To determine the phytoconstituents and proximate composition of the powdered picralima leaves and stem bark.
5. To evaluate the acute and chronic toxicity of picralima extracts in experimental animal model.

6. To determine some functional properties such as Antioxidant Capacity, Glucose Adsorption Capacity, and mineral composition of picralima extracts.
7. To isolate and characterize potentially bioactive constituent(s) from picralima using different chromatographic techniques and instrumental methods.
8. To formulate the active fraction or phyto0ingredients into antidiabetic nutraceuticals.

The potential overall impact of the study will be the discovery and development of antidiabetic agent(s) from indigenous medicinal plant for the management of diabetes mellitus. In addition, the study will enhance the knowledge on the nutritional and medicinal use of historic medicinal plants in line with the mission of the Herb Society of America (HAS).

**Background and Significance:**

Diabetes mellitus is a group of metabolic disorders arising from insufficient production of insulin by the pancreas, inability of the body to utilize the insulin produced or both (American Diabetes Association., 2011). It is characterized by increase in the blood sugar level which leads to the manifestation of symptoms like; polyphagia, polyuria and polydipsia (Saravanan *et al.*, 2011). Improper management of diabetes can lead to different complications such as; microvascular complications characterized by neuropathy, nephropathy and reticulopathy and also macrovascular complications leading to the damage of several organs in the body (Shobana *et al*., 2010). According to the International Diabetic Federation 2015, there are 1 in 11 adults living with diabetes worldwide and by 2040, there will be 1 in 10 adults living with diabetes in the world. These statistics are scary and calls for concern. It also shows that there is an urgent need for researchers to find effective means to prevent and manage the disease. Newer and more efficacious therapies with minimal side effects are highly needed to ensure that the disease is effectively managed. Current methods used in the management of diabetes include; diet change, regular exercises, use of oral hypoglycaemic agents which are not only expensive but have numerous side effects, and insulin therapy (Saravanan *et al.*, 2013). Different medicinal plants are currently being used in different cultures around the world in the management of diabetes (Donga *et al.*, 2011). In fact, more than 800 medicinal plants are currently being used in the management of diabetes (Juarez *et al.*, 2014). Some of these plants include; *Picralima nitida* (Inya-Agha *et al*., 2006; Okonta and Aguwa, 2007), *Acacia arabica*, *Hibiscus rosasinensis*, *Eleusine coracana*, *Cassia auriculata* (Elavarasi *et al.*,2012), *Abroma augusta*, *Ocimum sanctum*, *Eclipta alba*, *Piper longum*, *Magnifera indica,* etc (Sharavanan *et al.*,2011).

*Picralima nitida* (Stapf.) T.A. Durand & H. Durand is the only species of the genus Picralima and is commonly called Picralima, Akuamma or Pile plant. It belongs to the hunterieae tribe of the apocynaceae family. The plant is widely distributed in high deciduous forest of WestCentral Africa (Ajanohoun *et al*., 1996; NNMDA, 2008). Picralima has widely varied applications in West Africa folk medicine. Various parts of the plant; the leaves, seeds, stem bark and roots are used by herbalists for the treatment of fever, hypertension, jaundice, gastrointestinal disorders, malaria, and diabetes (Iwu, 1993; Etukudo, 2003; Inya-Agha et al., 2006; Okonta and Aguwa, 2007; Kouitcheu *et al*., 2008). The extract from different parts of the plant have been found to exhibit a broad range of pharmacological activities which lends credence to its ethnomedicinal uses.

The antidiabetic potential of this plant has only partially been investigated; available studies focused mainly on the crude extracts. Therefore, more research is required to completely explore the antidiabetic potential of picralima. The current study aims to carry out a detailed investigation into the antidiabetic activity of picralima using a combination of *in vitro*, *in vivo*,

and *in silico* approaches, to identify the potentially active phyto-ingredients, and formulation into herbal nutraceuticals.

**Preliminary Studies:**
Studies related to the antidiabetic activity of some medicinal plants have been conducted by the investigators of this proposed study. The investigators have also reported some pharmacological activities of *Picralima nitida*. Some of the preliminary work done include;

1. The Hypoglycaemic Potentials of the Leaf Extracts of *Stachytarphyta jamaicensis* have been investigated in vivo using streptozotocin-induced diabetes rat model (Egharevba *et al*., 2019).
2. The Antidiabetic Potentials of Extracts of the Stem Bark of *Cylicodiscus gabunensis* have been evaluated in an vitro method using α-Amylase inhibition assay and glucose uptake in yeast cells (Imieje *et al*., 2022).
3. The antioxidant activity of Picralima nitida has been evaluated using in vitro free radical scavenging assay (Erharuyi and Falodun, 2012; Erharuyi et al., 2023)
4. In addition to its antioxidant activity, *Picralima nitida* has been demonstrated to have antimalarial activity in vivo using Plasmodium berghei-induced malaria in mice (Erharuyi et al., 2023)
5. *Picralima nitida* extract has been shown to possess antiproliferative and apoptotic effect against breast cancer cell line in an in vitro assay (Erharuyi *et al*., 2011).

**Research Design and Methods:**
The research work will involve two (2) major stages; Phytochemical and biological. The study will involve the participation of postgraduate students to assist in their training and for capacity building.

**1. Phytochemical**

**1.1 Sample collection and authentication**
*Picralima nitida* leaves and stem bark will be collected from a forest in Edo State, Nigeria. The sample will be identified and authenticated in the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Nigeria.
The plant parts will be air-dried and pulverized using a mechanical grinder.

**1.2 Proximate and mineral/nutritional analysis**
Proximate analysis of the powdered plant samples will be carried out, parameters such as moisture, total ash, crude protein, crude fat and crude fibre contents will be determined. Mineral/nutritional composition including mineral elements, sugar, and amino acids contents will be carried out following standard methods (African Pharmacopoeia 1986; AOAC, 1984).

**1.3 Extraction and fractionation of powdered plant materials**
About 2 kg each of the powdered plant parts will be extracted by maceration in methanol at room temperature. The filtered extracts will be evaporated at 40$^{o}$C in a rotary evaporator. The methanol extracts will be fractionated by solvent-solvent partitioning in n-hexane, dichloromethane, ethyl acetate. The fractions will be concentrated in a rotary evaporator to obtain dried n-hexane, dichloromethane, and ethyl acetate fractions.

### 1.4 Phytochemical screening

The extracts and fractions will be subjected to simple chemical tests for the presence of alkaloids, tannins, saponins, carbohydrates, anthraquinones, flavonoids and other phytoconstituents according to standard methods (Stahl, 1973; Sofowora, 1982; Evans, 2002). The phytochemicals present will be quantified using quantitative phytochemical screening methods.

### 1.5 Isolation and identification of secondary metabolites

The Thin-layer chromatographic (TLC) profile of the fractions will be determined using a commercially available precoated F254 silica gel plates. Selected fractions based on their biological activity, yield and TLC profile will be separated by silica gel column chromatography using appropriate eluting solvents. The column fractions will be monitored by TLC. The plates will be visualised under UV lamp (254 nm and 366 nm), and by spraying with appropriate spray reagents.

Semi-purified column fractions will be purified by repeated silica gel column chromatography, and where necessary, Sephadex LH-20 column chromatography, preparative TLC (PTLC) and recycling preparative high-performance liquid chromatography (RP-HPLC). Some semipurified fractions will also be subjected to gas chromatographic-mass spectrometric (GC-MS) analysis to identify metabolites present.

Characterization of the isolated compounds will be done using various spectroscopic techniques including one and two-dimensional nuclear magnetic resonance spectroscopy, highresolution mass spectrometry, ultraviolet and infra-red spectrophotometry.

## 2. Biological

### 2.1 Toxicity Studies

#### *2.1.1 Animals*

Twenty-four adult Swiss albino mice and forty adult Wistar rats of either sexes will be obtained from the Animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin. The animals will be kept under 12-hour light/dark cycle in clean and well-maintained cages for two weeks to acclimatize to the laboratory environment. The animals will be fed with standard rodent pellets, and allowed access to water *ad libitum*.

#### *2.1.2 Ethical consideration*

The animals will be maintained and cared for in accordance with the international guidelines for the use and maintenance of experimental animal (OECD, 2001). Ethical approval will be obtained from the Ethical Committee for the Use of Experimental Animals, Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

#### *2.1.3 Acute Toxicity Screening*

The study will be carried out according to method previously described (Lorke, 1983). Nine adult mice of either sex will be divided into three groups of three animals per group. Extracts will be administered orally at doses of 10, 100, and 1000 mg/kg to groups I, II, and III, respectively. Another three mice will be divided into three groups of one mouse per group. The extract will be administered at doses of 1600, 2900, and 5000 mg/kg. General symptoms of toxicity and mortality in each group will be observed within 24 hours.

#### *2.1.4 Sub-Chronic Toxicity Screening*

Animals (rats) will be randomly allotted into 4 groups of five animals per group.

**Group 1:** Animals will receive distilled water
**Groups II:** Animals will receive 100 mg/kg of the extract.
**Groups III:** Animals will receive 200 mg/kg of the extract.
**Groups IV:** Animals will receive 400 mg/kg of the extract.
The extracts will be administered once daily to the animals for 28 days. The rats will be monitored for general and behavioural signs of toxicity, body weight changes and mortality for the duration of the experiment. After 28-day treatment period, animals will be sacrificed, blood samples will be collected, and internal organs (liver, kidney, heart, spleen and lungs) harvested, and stored in 20 mL sample bottles containing 10% formal saline. The organs will be processed for histopathological analysis.
The following haematological parameters will be determined: red cell count, packed cell volume, haemoglobin concentration, and platelet count, total white blood cell counts and its differentials.
The following biochemical parameters will also be determined: alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, creatinine, urea, bicarbonate, total proteins, albumin, total and conjugated bilirubin.

**2.2 Preliminary Antioxidant Screening**
Antioxidant capacity of the extracts and fractions of Picralima leaves and stem bark will be carried out *in vitro* using various antioxidant screening methods including DPPH Radical Scavenging Assay, ABTS radical scavenging assay, Ferric Reducing Antioxidant Power, Chelating ability on ferrous ions, and Total Antioxidant Capacity.

**2.3 Antidiabetic Screening**
***2.3.1 In Vitro Antidiabetic Assay***
***2.3.1.1 α-Amylase inhibitory activity***
The *α*-amylase inhibition assay will be performed using previously described method (Kwon *et al*., 2007). A total of 0.5 mL of various concentrations of the extracts and fractions (20-2000 µg/mL) and 0.5 mL of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing porcine pancreatic α-amylase (0.5 mL) will be mixed at room temperature for 10 min, then 0.5 mL of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) will be added to each test tube at timed intervals. The reaction mixtures will be incubated at room temperature for 10 min. The reaction will be stopped with 1.0 mL of dinitrosalicylic acid colour reagent. The test tubes will be incubated in a boiling water bath for 5 min and cooled. The mixture will be diluted with 10 mL distilled water and absorbance measured at 540 nm. The negative control experiment will be carried out using 1% dimethylsulphoxide which will be used as solvent in place of extract. Acarbose (20-200 µg/mL) will be used as the positive control. The above experiment will be done in triplicate. The αamylase inhibitory activity will be calculated using the formula:

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***2.3.1.2 α-Glucosidase inhibitory activity***
The inhibition of α-glucosidase activity will be determined using the modified method of Dewi *et al*., 2007. One milligram (1 mg) of α-glucosidase will be dissolved in 100 mL of phosphate buffer (pH 6.8) containing 200 mg of bovine serum albumin. The reaction mixture consisting

10 µL of sample at varying concentrations (0.52 to 33 µg/mL) will be premixed with 490 µL phosphate buffer pH 6.8 and 250 µL of 5 mM p-nitrophenyl α-D-glucopyranoside. After preincubating at 37ºC for 5 min, 250 µL α-glucosidase (0.15 unit/mL) will be added and incubated at 37ºC for 15 min. The reaction will be terminated by the addition of 2000 µL $Na_2CO_3$ (200 mM). α-glucosidase activity will be determined spectrophotometrically at 400 nm by measuring the quantity of p-nitrophenol released from p-NPG. Acarbose will be used as positive control.

### *2.3.2 In vivo* antidiabetic screenin

#### *2.3.2.1 Animals*

Fifty adults male Wistar rats will be obtained from the Animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Benin. The animals will be kept under 12-hour light/dark cycle in clean and well-maintained cages for two weeks to acclimatize to the laboratory environment. The animals will be fed with standard rodent pellets, and allowed access to water *ad libitum*.

#### *2.3.2.2 Induction of Diabetes Mellitus*

Wistar rats to be used for the experiment will be fasted overnight, weighed and a single dose of 40 mg/kg of streptozotocin in freshly prepared 0.1 M citrate buffer (pH 4.5) will be administered intraperitoneally to each rat. Their blood glucose level will be determined after 48 hours of induction and animals with blood glucose level of ≥ 200 mg/dL will be recruited for the study.

#### *2.3.2.3 Experimental Design*

The animals will be grouped into six groups of five rats each and treated for 1 week as follows:

**Group 1:** Healthy animals with no drug treatment

**Group 2:** Diabetic animals and will be treated with 200 mg/kg/day body weight dose of the extract and fractions.

**Group 3:** Diabetic animals and will be treated with 400 mg/kg/day body weight dose of the extract and fractions.

**Group 4:** Diabetic animals and will be treated with 800 mg/kg/day body weight dose of the extract and fractions.

**Group 5:** Diabetic animals will be treated with 5 mg/kg/day body weight dose of glibenclamide.

**Group 6:** Diabetic animals and will not be treated.

#### *2.3.2.4 Determination of blood glucose*

The lateral tail vein of the animals will be pricked with a sterile lancet and blood glucose level will be determined using the Accu-check® active glucometer.

### 2.4 *In silico* antidiabetic screening

The compounds identified in the leaves and stem bark of picralima will be subjected to virtual screening using molecular docking and dynamic simulation tools. The molecular docking modelling will be used to predict and describe the interaction of the compounds (ligands) with selected target proteins that play critical role in diabetes mellitus.

### *2.4.1 Selection of proteins targets*

The three-dimensional X-ray crystallographic structures of 3 proteins known to play crucial role in diabetes mellitus will be retrieved from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (http://www.pdb.org) and saved in.pdb format. Proteins with PDB ID: 1IR3 (Phosphorylated insulin receptor tyrosine kinase in complex with peptide substrate and ATP analog), PDB ID: 2HWQ (Peroxisome proliferator-activated receptor agonists), and PDB ID: 3C45 (Human dipeptidyl peptidase IV/CD26 in complex with a fluoroolefin inhibitor) will be used as target receptor proteins for molecular docking experiments.

### *2.4.2 Ligand preparation*

Anonical SMILES of ligands (identified compounds from Picralima) will be retrieved from PubChem compound database (https://pubchem.ncbi.nlm.nih.gov/) and the three dimensional structures of the compounds will be simulated using online server CORINA (http://www.mnam.com/online_demos/corina_demo) and saved in.pdb format. The compounds; metformin (PubChem CID:4091), repaglinide (PubChem CID:65981), and sitagliptin (PubChem CID: 4369359) will be used as positive control ligands for comparative analysis. The 2D structures of test ligands and positive control ligands will be generated using ChemDraw Professional Suite 23.1.1.3.

### *2.4.3 Molecular docking analysis*

Molecular docking between the ligands and the selected protein targets will be carried out using AutoDock VINA algorithm PyRx algorithm. Each ligand will be docked to the 3 target proteins. Bond energies, such as, van der walls (VDW) interaction, hydrogen bond (H-Bond) and electrostatic energy will be investigated. Docked poses will be visualized using PyMOL and the interactions will be ranked according to their docked energy. Docking scores of the test ligands will be compared with the positive control ligands.

### *2.4.4 Drug likeness prediction*

MolSoft (MolSoft, 2007) software (https://molsoft.com/mprop/) will be used to predict the drug likeness of the ligands. Overall score will be based on molecular weight, total number of hydrogen bond acceptors, total number of hydrogen bond donors and logP values. Canonical SMILES data from PubChem server will be used as an input data for Molsoft prediction.

### *2.4.5 Absorption, distribution, metabolism, excretion, and toxicity (ADMET) analysis*

AdmetSAR, an online server will be used to predict the toxicity upon consumption of the compounds and determine whether the compounds follow the Lipinski Rule. Human intestinal absorption (hia), blood–brain barrier (bbb) penetration, caco-2 permeability, carcinogens toxicity and other factors will be predicted. The SMILES of the ligands will be submitted to the AdmetSAR program for their pharmacokinetics and toxicity properties.

### Statistical analysis

Data will be expressed as Mean ± SD. The graph pad in-stat statistical package will be used for the analysis of data making use of student t-test, one-way analysis of variance (ANOVA) and Turkey's post hoc test. P values $< 0.05$ will be regarded as significant.

**Budgets**

| S/N | Description of item | Parameters | No. of items | Rates (US$) | Total amount (US$) |
|---|---|---|---|---|---|
| **1.0** | **Personnel Compensation/Allowance** | | | | |
| 1.1 | Principal Investigator | Stipend | 1 | 400.00 | 400.00 |
| 1.2 | Co-Investigators | Stipend | 4 | 800.00 | 800.00 |
| 1.3 | Technical Support | Stipend for plant collector and Technical staff | 3 | 300.00 | 300.00 |
| **2.0** | **Consumables - Solvents and chemicals for extraction, fractionation and isolation of plants metabolites** | | | | |
| 2.1 | Methanol | Analytical grade | 2.5 L × 24 | 30.00 | 720.00 |
| 2.2 | n-Hexane | Analytical grade | 2.5 L × 24 | 30.00 | 720.00 |
| 2.3 | Ethyl acetate | Analytical grade | 2.5 L × 20 | 32.00 | 640.00 |
| 2.4 | Acetone | Analytical grade | 2.5 L × 12 | 32.00 | 384.00 |
| 2.5 | Dichloromethane | Analytical grade | 2.5 L × 16 | 35.00 | 560.00 |
| 2.6 | Silica gel 200-400 mesh | Analytical grade | 500 g x 20 | 15.00 | 300.00 |
| 2.7 | Sephadex LH-20 | Sigma Aldrich | 10 g x 2 | 100.00 | 200.00 |
| 2.8 | Pre coated TLC Plates | Merck | 6 Packs | 30.00 | 180.00 |
| **3.0** | **Bioassay Cost (Rats, Mice, Animal feeds, toxicity screening and antidiabetic activity screening of extracts, fractions and isolated compounds** | | | | |
| 3.1 | Purchase of mice for acute toxicity screening | Albino mice | 24 mice | 1.00 | 24.00 |
| 3.2 | Purchase of Rats for subchronic toxicity and in vivo antidiabetic screening | Wistar rats | 90 rats | 2.00 | 180.00 |
| 3.3 | Rodent feeds for duration of experiment | | 3 bag | 10.00 | 30.00 |

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|---|---|---|---|---|---|
| 3.4 | Alpha amylase assay kit | Sigma Chemical Company | 1 kit | 150.00 | 150.00 |
| 3.5 | Alpha glucosidase assay kit | Sigma Aldrich | 1 kit | 587.00 | 587.00 |
| 3.6 | Acarbose | Sigma Aldrich | 1 g | 265.00 | 265.00 |
| 3.7 | Streptozotocin for inducing diabetes | Sigma Aldrich | 1 g | 377.00 | 377.00 |
| 3.8 | Syringes and needles | 10 mL, 5 mL, 2 mL, 1 mL | 4 packs | 4.00 per pack | 16.00 |
| 3.9 | Sodium citrate | | 500 g | 10.00 | 10.00 |
| 3.10 | Citric acid | | 500 g | 9.70 | 9.70 |
| 3.11 | Glibenclamide | (Daonil®) | 1 satchet of 5 mg/tablet | 1.50 | 1.50 |
| 3.12 | Glucose test strips | Accu Check | 20 packs | 7.00 | 140.00 |
| **4.0** | **Bioanalysis and Spectroscopic analysis** | | | | |
| 4.1 | Haematological analysis | RBC, PCV, WBC, Hb, | 35 samples | 2.00/sample | 70.00 |
| 4.2 | Biochemical analysis | ALT, AST, Creatinine, urea | 35 samples | 2.00/sample | 70.00 |
| 4.3 | Histological analysis | | 35 samples | 4.5/sample | 157.50 |
| 4.4 | Spectroscopic Analyses of isolated compounds | MS, IR and NMR- 1D and 2D experiments ($^{1}H$ and $^{13}C$) including GC-MS | | 400.00 | 400.00 |
| | **Total** | | | | **7,691.70** |

**Project Timeline**

| S/N | Description of activity | Duration |
|---|---|---|
| 1. | Collection and authentication of plant samples | 1 month |
| | Proximate and mineral/nutritional analysis | 1 month |
| 2. | Preparation of plant extracts and fractions | 1 month |
| | Phytochemical screening | 1 month |
| | *In vitro* and *in vivo* antidiabetic screening | 2 months |

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|---|---|---|
| | Mid-term Report | 1 month |
| 3 | Isolation and identification of metabolites | 3 months |
| 4. | Acute and sub-chronic toxicity studies | 2 months |
| 5. | *In silico* antidiabetic screening | 1 months |
| 6. | Final Report/Manuscript writing | 1 month |

**Dissemination of Results:**

On completion of the study, findings will be **Disseminated in the following ways;**

- report to the academic community directly, by speaking at meetings, seminars, conferences or symposia.
- publication in peer-reviewed National and International scientific journals.
- Highlights of the research will be reported and published in HAS Website, and HSA will be acknowledged in all the publications that would emanate from this work.

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