MOLECULAR AND BIOLOGICAL STANDARDISATION OF *Alstonia boonei* DE WILD. AND *Alstonia congensis* ENGL. LEAVES AND STEM-BARKS

BY

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ABSTRACT

Alstonia boonei (AB) and *Alstonia congensis* (AC), belonging to the family Apocynaceae are different but closely related species, indigenous to Africa. The two plants possess varied biological activities including antimalarial, antihypertensive and antidiarrhoeal. However, the apparent similarities between the two species could lead to misidentification and inappropriate medicinal utilisation, necessitating the need to establish diagnostic characters for each species for proper identification. This study, was therefore, aimed at evaluating molecular, pharmacognostic, antispasmodic and antidiarrhoeal profiles of the two plants for their pharmacopoeial standardisation.

Deoxyribonucleic acid was isolated from nine accessions of AB and AC leaves (ABL and ACL) collected from southwestern Nigeria and amplified, using Internal Transcribed Spacer (ITS) region. Micromorphological diagnostic characters of leaves and stem-barks were studied by light microscopy. Physico-chemical and elemental analysis of the powdered samples were determined using incineration method and Atomic Absorption Spectroscopy, respectively. Aqueous extraction of plant samples was done, extracts were concentrated *in vacuo* and freeze dried. The freeze-dried extracts were partitioned successively into dichloromethane (DCM), ethyl acetate and aqueous fractions. Antispasmodic activities of the extracts and fractions were evaluated on high-potassium induced and spontaneous contractions on isolated rat ileum. The AB and AC stem-barks (ABSb, ACSb) extracts and their dichloromethane fractions (DCM-ABSb, DCM-ACSb) showing high antispasmodic activity were evaluated for *in vivo* antidiarrhoeal activities in mice (22-25 g *b.w*). Loperamide (5mg/kg) was used as standard. Compounds were isolated from the most active fraction (DCM-ABSb) using chromatographic techniques (Column, TLC). Structures of the isolated compounds were elucidated using spectroscopic techniques (NMR and MS) and their antispasmodic activities evaluated. Data were analysed using descriptive statistics, unweighted pair group method with arithmetic mean and one-way ANOVA and Tukey's multiple comparison at $\alpha_{0.05}$.

The amplification of the ITS region discriminated between the two *Alstonia* species. The adaxial epidermal cells of both species were polygonal, straight anticlinal walls and leaves were hypostomatic. The vascular bundle of ABL mid-rib was arc-shaped, while that of ACL was V-shaped. Moisture content values of stem-bark of AB (6.4±0.06%) was significantly different from that of AC (7.2±0.4%), while the values were not significantly different for the leaves. Elemental analysis revealed that calcium was the most abundant mineral in the leaves of AB (68.2±3.2 mg/g) and AC (65.7 ±1.0 mg/g). The stem-bark extracts of AB and AC were antispasmodic with IC_{50} of 0.03±0.2, 0.12±0.01 mg/mL and 1.15±0.1, 1.05±0.8 mg/mL on high-potassium induced and spontaneous contractions, respectively. The DCM-ABSb fraction exhibited significant antispasmodic activity (IC_{50}: 0.02±0.05, 0.31±0.02 mg/mL) on high-potassium induced and spontaneous contractions, respectively. The DCM-ABSb (200 mg/kg *b.w*.) showed significant antidiarrhoeal activity (87.3%) comparable to Loperamide (87.5%). The isolated compounds from DCM-ABSb were β-amyrin and boonein with only boonein exhibiting antispasmodic activities on both high-potassium induced (IC_{50}: 0.09±0.01 µg/mL) and spontaneous (0.29±0.05 µg/mL) contractions.

Alstonia boonei and *Alstonia congensis* were identified as two distinct species. The diagnostic indices of the two plants provided pharmacopoeial standards for their identification. The isolated boonein could serve as a template for the development of antidiarrhoeal drugs.

Keywords: *Alstonia boonei*, *Alstonia congensis*, Pharmacopoeial standardisation, Antidiarrhoeal activity, Boonein

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CERTIFICATION

We certify that this research was carried out by Opeyemi Josephine Omitola, under our supervision, in the Department of Pharmacognosy, Faculty of Pharmacy, University of Ibadan, Ibadan, Nigeria.

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DEDICATION

This thesis is dedicated to my LORD JESUS CHRIST, the helper of the helpless; my Dad, late Mr. Samuel Akinola Omitola with whom I conceived the idea of the PhD programme; and to the loving memory of my son, Oluwakorede Akinloye Akinmurele, whom God brought my way briefly in the course of the programme.

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LIST OF ABBREVIATIONS

A. boonei	*Alstonia boonei*
A. congensis	*Alstonia congensis*
ABL	*A. boonei* leaf
ABLA	Aqueous fraction of *A. boonei* leaf
ABLC	Crude extract of *A. boonei* leaf
ABLD	DCM fraction of *A. boonei* leaf
ABLE	Ethyl acetate fraction of *A. boonei* leaf
ABSA	Aqueous fraction of *A. boonei* stem-bark
ABSb	*A. boonei* stem-bark
ABSC	Crude extract of *A. boonei* stem-bark
ABSD	DCM fraction of *A. boonei* stem-bark
ABSE	Ethyl acetate fraction of *A. boonei* stem-bark
ABSE	Ethyl acetate fraction of *A. boonei* stem-bark
ACL	*A. congensis* leaf
ACLA	Aqueous extract of *A. congensis* leaf
ACLC	Crude extract of *A. congensis* stem-bark
ACLD	DCM fraction of *A. congensis* leaf
ACSA	Aqueous extract of *A. congensis* stem-bark
ACSb	*A. congensis* stem-bark
ACSC	Crude extract of *A. congensis* stem-bark
ACSD	Crude extract of *A. congensis* stem-bark
CC	Column chromatography
CTAB	Cetyl trimethyl ammonium bromide
DCM	Dichloromethane
DEPT	Distortionless Enhancement Polarisation Transfer
DNA	Deoxy ribonucleic acid
dNTPs	Dinucleotide tri phosphates
ETOAC	Ethyl acetate
ETOH	Ethanol
High [K^+]	High potassium ion concentration

HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Coherence
ITS	Internal Transcribed Spacer
MeOH	Methanol
NaCl	Sodium chloride
NaOCl	Sodium hypochlorite
N-HEX	Hexane
PCR	Polymerase Chain Reaction
RLS	Radial longitudinal Section
TAE	Tris base Acetic Acid
TLC	Thin layer chromatography
TLS	Tangential Longitudinal Section
TS	Transverse Section
UPGMA	Unweighted pair group mea with arithmetic mean
^{13}CNMR	Carbon 13 Nuclear Magnetic Resonance
^{1}HNMR	Proton Nuclear Magnetic Resonance

CHAPTER ONE

1.0 INTRODUCTION

1.1 Medicinal plants

Medicinal plants, since primordial times and in all the cultures of the world, have been a major source of natural therapy for various diseases. In modern times, they are a major source of new chemical substances having potential healing effects (Makrane *et al*., 2018). In addition, the success of modern medical science has originally and largely depended on materials obtained from natural sources (Dhami, 2013). In Africa, over 80% of the population use medicinal plants for treatment of different ailments. Similarly, natural products have recently gained wide attention in developed countries as sources of new, safer and effective bioactive constituents of high therapeutic properties (Nitta *et al*., 2002). Many of the drugs prescribed in official health care facilities today all over the world are, either directly or indirectly, derived from plants, hence, the significance of plant species as major sources of new drugs (Oksman-Caldentey and Inze, 2004). However, the manufacture and therapeutic utilisation of herbal medicines have become more challenging than it would necessarily have been, due to variations in biological or chemical constituents of medicinal plants. Such variations are largely caused by genetic and environmental factors. In addition to therapeutic implications, safety of therapy can also be affected by this variation. Bisset (1994) and Nikam *et al*. (2012) aver that several biologically active substances contained in a medicinal plant, affected by genetic variation (besides other factors), could pose undesirable toxic side effects. The need to combat the challenges posed by the variation in the biological or chemical constituents of medicinal plants has continued to constitute a major focus in research on medicinal plants and their preparations.

Base on preliminary review, studies on medicinal plants have focused on bioactivity,

ethnobotany, pharmacology and taxonomy. Meanwhile, there has been little or no research attention given to their standardisation, including safety. To ensure the desired safety benefits, it is essential that individuals (consumers of herbal drugs) doses within the required limit, over a specific duration from a professionally standardised herbal preparation. Furthermore, the increasing trend in the use of medicinal plant products necessitates the need to establish the quality control standards, prior to granting marketing authorisation for human consumption (Jamshid-Kia *et al*., 2018). World Health Organiation (WHO) in 2017 establishes that quality control standard is particularly necessary because the quality of the raw materials and the finished drug products have implications for the safety and efficacy of herbal medicines. An important factor, which can contribute to the consistent quality and acceptance of herbal products, is to have adequate standardisation of all production processes from herbal procurement to marketing. Hence, the development of a set of standards, required for quality assurance and as contained in the official pharmacopoeial monograph of the particular medicinal plant raw material, is very crucial. This is the subject matter of this study.

1.2 Medicinal plants as natural laboratories for natural product synthesis

Plants are considered as natural laboratories for the synthesis of variety of phytochemicals as secondary metabolites with structural diversity and different therapeutic values (Yang *et al*., 2016). For instance, Harvey *et al*. (2015) estimate that about 200,000 secondary metabolites are present in the plant kingdom, some of which include alkaloids, flavonoids, saponins and so on. About 12,000 of these secondary metabolites (less than 10% of the total phytochemical constituents synthesised by plants) have been isolated from plants (Falodun and Usifoh, 2006). While most of the secondary metabolites (such as alkaloids) serve as plant defense mechanisms against predation by microorganisms, insects and herbivores, some others are synthesised to heal up wounds caused by biotic or abiotic predators or wind (Raghueer *et al*., 2015). These secondary metabolites are used by man as natural drug products due to their disease-inhibiting activities and innocuousness. For instance, quinine and artemisinin, which are important anti-malarial drugs were derived from *Cinchona succirubra* Pavon ex Klotzsch (Rubiaceae) and *Artemisia annua* L. (Asteraceae),

respectively (Qui, 2007). These attest that plants are natural laboratories for natural product synthesis and drug discovery.

1.3 Herbal medicine

Herbal medicine is the most significant and rational part of traditional medicine. It is a prominent and inseparable component of traditional medicine in which the practitioner specialises in the use of herbs for the treatment of diseases. As defined by the World Health Organisation (2017), herbal medicine (herbal materials, herbal preparations or finished herbal products) comprise, as active constituents, parts of plants, plant materials, or combination of both. Herbal medicines may include herbs, resins, gums, leaves, bark, roots or rhizomes in its crude form, plant extracts, fresh juices, fixed oils, or essential oils, as active ingredients to meet health needs. The centrality of herbs as sources of herbal medicines has brought about some designations such as botanical medicine, phytomedicine or plant medicine, as alternative terminologies or synonyms for herbal medicine.

Gradually in human history, different groups of people and tribes added the medicinal values of herbs in their environment to their knowledge base, and this, over the years, enhanced development in the use of herbal medicines. Such developments are, usually, outcomes of observations of wild animals, signature, and intuitions; and by trial and error. By observation, people logically collected information on medicinal plants and developed their own record of medicinal plant usage. Consequently, today's herbal pharmacopoeias utilised a lot of documentations from the herbal knowledge base of the indigenous populations. It is worth mentioning that herbal medicine actually orchestrated the development of modern medicine in some notable ways. In reality, many of the orthodox drugs in use today are from herbal sources (Nyika, 2006).

Though herbal practitioners believe that the healing potency of plants is embedded in the synergistic interaction of all its ingredients, both known and unknown (Adelaja, 2006), modern pharmacology is interested in specific active compounds, and seeks to isolate each specific compound to the exclusion of all the others. In this regard, most of the researches on plants continue to focus on isolating and identifying pure active ingredients, rather than studying the medicinal properties of the whole plants.

1.4 Standardisation of herbal medicines

Standardisation of herbal medicine is the process of setting up and prescribing a set of peculiar identities, specific characteristics or standards, which are generally unique and of unshared qualities (Elujoba, 2005). It involves a step-wise pharmacognostic studies (Ozarkar, 2005). It is essential that herbal medicines should be of uniform quality in identity, chemical composition, efficacy and safety through the process of standardisation. The specific characteristics or standards are derived by experimentation and observation, which then leads to the formulation of the pharmacopoeial monograph.

The danger in the use of unstandardised herbal medicine begins from wrong identification of the plant raw materials. One of the most common sources of error is the existence of the same vernacular name for more than one plant species or several vernacular name for the same plant species (Chanda, 2014). Hence, from the plant material identification, through to the various production stages and then to the final finished products, must be standardised. Explicit identification and authentication of the plants used for the production is, therefore, an elementary but critical step towards an extensive quality assurance process. It is when the plant component of herbal medicine has been correctly identified, collected at the right time, from a right place, extracted by a specific method and hence contains the right active substance at the right concentration that a specific standard dose, constant for the herbal medicine can be dispensed to produce a uniform, reproducible pharmacological effect in patients, all the time (Sofowora, 1992). Thus, each batch of the finished products will then comply with the pharmacopoeia monograph. Hence, the role of standardisation as a crucial component of the quality control process is to ensure that a predefined amount of the preparation produces the required therapeutic effect of the active ingredient (s) in each dose, whenever it is dispensed to a patient (Zafar *et al*., 2005).

Herbal products cannot be considered acceptable and fit for registration until they have been well-standardised for ensuring reproducibility and repeatability of batch-to-batch manufacturing. Moreover, many dangerous and lethal side effects (such as allergic reactions, contaminant and herb-drug interaction effects) have recently been reported (Vaidya and Devasagayam, 2007). In addressing such issues, guidelines for standardisation of herbal medicine have been set by concerned regulatory bodies such as including Food and Drug Agencies (FDAs) and World Health Organisation (WHO). Published monographs

(stating the quality control standards, dosage regimens, uses, efficacy, pharmacology and safety of commonly-used medicinal plants) in national herbal pharmacopeias are the most practical guides for achieving standardised herbal drug formulations. Some of the easily-accessible herbal pharmacopoeias include: Nigerian Herbal Pharmacopoeia (NHP), Ghana Herbal Pharmacopoeia (GHP), West African Herbal Pharmacopoeia (WAHP), African Herbal Pharmacopoeia (AfriHP), British Herbal Pharmacopoeia (BHP), Chinese Herbal Pharmacopoeia (CHP), Indian Herbal Pharmacopoeia (IHP).

1.4.1 Conventional methods of standardisation of herbal medicines

Standardisation of crude drugs of plant origin include macroscopic and microscopic examination, evaluation of chemical composition by different chromatographic, and other techniques such as spectroscopic, biochemical, physicochemical as well as evaluation of biological activities of plant extracts. Generally, for standardisation and quality assurance purposes, the authentication, purity and assay of medicinal plants must be established and guaranteed prior to other studies.

The primary and critical step at the onset of any standardisation process (hence, subsequent quality assurance) of any medicinal plant and its products is the explicit identification and authentication. Starting any standardisation process with the wrong plant leads to a wrong monograph and, eventually, wrong medicines from it. This is implied from the World Health Organisation (WHO) guidelines (2011), specifying the macroscopic and microscopic data as the first documentation towards the authentication of any medicinal plant material. Purity of a medicinal plant refers to the detection of adulterants present in the plant material sample, which could affect the efficacy, safety and reproducibility of its medicinal products. This can be achieved through the physico-chemical analysis of the medicinal plant such as total ash determination, elemental analysis and so on. Chemical and biological assays carry out measurements of the percentage chemical constituents and bioactivities of the medicinal plant or its products. The various types of chemical fingerprinting of plant material extracts (chromatographic, densitometric, spectroscopic, metabolomic, and so on) can serve as an invaluable support in standardising medicinal plants. Specifically, they serve as matching markers in the quality control of medicinal end-products (Torey *et al*., 2010). It is necessary to provide appropriate systems and methodologies for standardisation and quality control of

herbal medicines so as to improve their acceptability as well as reducing the dangers of toxicity and adverse herbal reactions (Elujoba, 1998).

1.4.2 WHO guidelines for standardisation of herbal medicines

a) Botanical standards which include macroscopic (sensory evaluation, morphological description and macro-morphometric) and microscopic (histological, histochemical and micro-morphometric) evaluation.

b) Physicochemical (ash values, moisture contents, extractive values, volatile oil determination, and the like) and chemical identity (fingerprints, chromatographic and quantitative estimation of phytochemical constituents).

c) Estimation of biological activities (bitterness, haemolytic index, astringency, foaming index, swelling factor, etc.)

d) Toxicity (pesticide residues, microbial contamination, heavy metals, among others)

e) Radioactive contamination

1.4.3 Molecular authentication of medicinal plants

Deoxyribonucleic acid (DNA) fingerprinting has been proven to be a substantial complementation for old chemical and morphological markers for authentication in herbal drug standardisation. The DNA is the basic structural component of all living cells, often arranged in base-pair sequences, which is central to the determination of traits and physical characters of the cells in individual organism. The DNA fingerprinting has been increasingly applied in the past few decades to determine the origin of plants, animals and other microorganisms. A crucial significance of DNA fingerprinting of plant species and strains is that its genotypic characterisation may show considerable variation between strains. In relation to commercial herbal drugs, DNA fingerprinting allows for the availability of intact genomic DNA from plant samples after they are processed.

In addition, DNA fingerprinting enables drug authentication as it helps to reveal adulterants, even in processed samples (Mihalov *et al*., 2000). Further, the strength of DNA fingerprinting relates to the fact that, though the phytochemical constituents may vary with the part of plant used, physiology and environment, it keeps genome intact, irrespective of the plant part used (Choudary and Sekhon, 2011). A number of DNA-based molecular

techniques are utilised to evaluate DNA polymorphism, which include hybridisation-based method, Polymerase Chain Reaction (PCR)-based method and sequencing-based method (Srivastava and Mishra, 2009).

1.5 Justification for the study

Currently, there is difficulty in distinctly identifying and distinguishing *Alstonia boonei* from *Alstonia congensis* due to their very close morphological similarities. Hence, this study seeks to set standards and compile separate monographs for the two plants to facilitate correct identification and differentiation. Moreover, scientific evaluation of the *A. boonei* leaf and stem-bark antidiarrhoeal activities is still missing in the literature. The same is as well lacking for *A. congensis* stem-bark. Hence, the study seeks to validate the ethnomedical claims of the two plants as antidiarrhoeal agents. Also, there is no DNA barcoding of *Alstonia boonei* and *Alstonia congensis* using ITS primer for molecular authentication.

1.6 Research hypotheses

i. Pharmacognostic standards and ITS molecular marker can provide distinguishable characters for correct identification and authentication of *A. boonei* and *A. congensis*

ii. Aqueous extracts and fractions of *A. boonei* and *A. congensis* possess antispasmodic and antidiarrhoeal activities.

iii. The compounds responsible for the antispasmodic activities can be isolated, characterised and identified.

1.7 Aim and objectives

This study aims to develop pharmacognostic and molecular standards which could be used to compile individual monographs for *Alstonia boonei* and *Alstonia congensis* stem-barks for their anti-diarrheoeal activities and drug formulation purposes.

The study's specific objectives are to:

i. provide pharmacognostic standards for the leaves and stem-barks of the plant samples;

ii. authenticate the closely related *A. boonei* and *A. congensis*, using DNA barcode (ITS 1 and 4);

iii. determine the phytochemical constituents and specify the elemental compositions of the plant samples;

iv. compare the antispasmodic and antidiarrhoeal activities of the leaves and stem-barks of the two plants;

v. isolate, characterise and identify the antispasmodic compound (s)

vi. formulate antidiarrhoeal drug (syrup and powder) from *A. boonei* stem-barks

vii. compile necessary pharmacopoeial monographs for *A. boonei* and *A. congensis* stem-barks.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Standardisation of medicinal plants

2.1.1 Pharmacognostic standardisation of medicinal plants

2.1.1.1 Macroscopic standards

Macroscopic study of medicinal plants as raw materials for herbal medicinal products involves the description of the morphological plant parts, which are visible to the eye or visible through an enlarging hand lens (Chanda, 2014). Prior to processing any plant into a finished herbal product, botanical identification of the plant species is mandatory to ensure correct collection of the raw material, to avoid arriving at wrong medicine.

Plant identification involves specifying plant name as well as its botanical source, using the binomial system of nomenclature to include the family name, the authority and the variety. The geographical source must also be specified. This is necessary to avoid confusion arising from the existence of several tribal (native) names for the same plant species or the same tribal names for different plant species (Elujoba, 1998). Macroscopic studies of herbal raw materials include macro-morphometry (size determination), visual appearance (to the naked eye) and the careful judgment on its sensory characteristics such as colour, odour and taste sensation. Macroscopic evaluation of herbal raw materials is dependent on the morphological part from which the herbal product is obtained.

The useful parameters for assessing the identity of herbal materials (such as bark, leaf, flower, fruit, seed, whole plant and root) morphologically include the shape (curved, recurved, channelled, quill, double quill); fracture (splintery, fibrous, laminated, short or granular) in the case of bark drugs; leaf morphological description include leaf shape (lanceolate, emarginate, linear, cordate, cuneate, rotund, ovate, obovate), leaf base (cuneate, cordate, decurrent, assymetrical), leaf apex (obtuse, acute, cuspidate, retuse, emarginate,

acuminate), leaf margin (entire, serrate, biserrate, dentate, ciliate, crenate), leaf surface (glabrous, hairy), venation pattern on the leaf surface (parallel, reticulate), leaf arrangements (whorl, opposite), flowers, presence or absence and types of calyx, corolla, androecium/gynoecium and inflorescence; while the fruit will specify type of ovary, placentation and then seed description (Dutta, 2001; WHO, 2011). Since drug substitutes or adulterants may have similar morphological characters, macroscopic standards would require a twin-support from microscopic characters and other sets of standards (Elujoba, 1998; WHO, 2011)

2.1.1.2 Microscopic standards

Microscopic analyses deal with exposition and visualisation of tissue under the microscope, in order to provide closer cellular discrimination in their intact natural arrangements, as well as for quantitative measurements of their internal structures. Such standardisation requirements are crucial to ensuring the identity of plant material, which is primary in screening for structural and elemental impurities. Microscopic evaluation may be done under ordinary light microscope (at different objective lenses) or scanning electron microscope. A variety of procedures are usually involved in carrying out light microscopy. For dried materials for instance, it is necessary to soften the samples before preparation for microscopic studies. Softening may be carried out by moistening or soaking the samples in water if tender parts like leaf or flower are involved. In the case of hard parts of plants such as the root and stem-bark, boiling is often required.

Observation of structures under a light microscope is commonly preceded by preparation of slides, which involves clearing (with the use of clearing agents), staining and mounting of samples. Thereafter, anatomical analysis would require preparation of transverse, radial and longitudinal sections of the plant materials, by conventional micro-techniques (Silva *et al*., 2008). Further, microscopic evaluation of medicinal plant tissues could be presented in two ways, namely: qualitative and quantitative microscopy).

2.1.1.2.1 Qualitative microscopy

The use of qualitative diagnostic characters such as type, presence or absence of cells (vascular bundle, vessels, medullary rays, stomata, trichomes, starch grains, sclereids,

among others) are requisite for the identification and quality control of herbal drugs in powdered and whole forms. Examination of transverse sections through the midrib, the lamina and surface preparations of both epidermises as well as cleared leaf portions of the whole leaf are important qualitative diagnostic investigations. In some cases, application of histochemical tests for detection and localisation of some chemical constituents may be necessary. Structures frequently present in powdered leaves are whole or fragments of epidermis and stomata, with trichomes, palisade parenchyma cells, cellulose parenchyma, starch grains, vascular elements, spongy parenchyma cells, calcium oxalate crystals and so on (Serrano *et al*., 2010). Microscopic characterisation of the stem-bark in relation to the cork, type of sclereids, phellogen and phelloderm, presence or absence of secretory cells, phloem fibres, contents of the medullary rays and presence or absence of starch grains are the most important features for botanical diagnosis in bark samples. When calcium oxalate is present, their distribution is also studied. Transverse and longitudinal sections as well as macerates and powdered samples are always used for microscopic evaluation (Serrano *et al*., 2010).

2.1.1.2.2 Quantitative microscopic

Quantitative microscopy involves accurate cellular micrometry or measurements of tissues such as palisade ratio, stomatal number, size of stomata, vein-islets, vein termination number, stone cells, vessels, length of trichomes, and so on (Africa Pharmacopoeia, 1986). Palisade ratio is the average number of palisade cells inside four contagious epidermal cells. Stomatal number is the average number of stomata per square millimeter area of epidermis. Vein-islet number refers to the average number of vein islet per square millimeter of the leaf surface midway between the midrib and margin. Vein termination number is the average number of vein terminations per square millimeter of the leaf surface between midrib and the margin.

2.1.1.3 Physico-chemical analysis

The parameters, which are studied under physico-chemical evaluation represents pharmacopeial standards for authenticity, quality, identity and purity for assessing herbal drug products. It involves assessment of parameters such as moisture content, ash values

(total, acid-insoluble, water-soluble) and extractive values (alcohol and water-soluble), contents of fibre, protein and carbohydrate. In such analysis, ash values, for which a 'not-less-than' standard is recommended (Elujoba, 1998; Kunle *et al*., 2012), are used to determine the quality and purity of crude drugs; it reveals the presence of various impurities like carbonate, oxalate and silicate. The total ash values are meant to set standards for the level of silicate and mineral matter, exposing low quality herbal drugs. The acid insoluble ash consists of mainly silica, and indicates contamination with earthy material. A standard expressed as not-more-than value is recommended for both total and acid-insoluble ash values (African Pharmacopoeia, 1986). The water-soluble ash value is used to estimate the amount of inorganic compounds present in crude drugs. It could be used to detect water exhausted drugs, which may have been used as admixture to substitute or adulterate the genuine drugs.

Moisture content analysis is the determination of water molecules or moisture contained in the crude drugs. Minimal level of moisture content in drugs is required to lessen inaccuracies in the estimation of actual weights of plant materials extracted, to discourage the growth of micro-organisms and thereby preventing spoilage (WHO, 2011). Extractive values (water- soluble or alcohol-soluble) determine the amount of the extractable chemical constituents of crude drugs under different solvent environments. Extractive values could be used to detect water or alcohol exhausted crude drugs, which might have been used as adulterants or substitute for the genuine crude drug.

2.1.1.4 Fluorescence analysis

Fluorescence analysis relates to the reaction of powdered samples with different freshly-prepared chemical reagents. Colour reaction is observed in the daylight and under the Ultra Violet lights at both short (254 nm) and long (365 nm) wavelengths. The reactions at different radiations are recorded. The ultra violet light produces fluorescence to many natural products which do not normally fluoresce in daylight. Hence, crude drugs could be qualitatively investigated by this means as an important physicochemical parameter for crude drug evaluation (Zhao *et al*., 2011).

2.1.1.5 Elemental analysis

Contamination of herbal raw materials by heavy metals such as copper, cadmium, arsenic and mercury, which poses clinically significant health dangers to herbal materials users, may be attributed to a number of factors, among which is environmental pollution. (Shrikumar *et al*., 2006). Engaging in elemental analysis by means of varied available methods such as Atomic Absorption Spectrophotometry (AAS), Inductively-Coupled Plasma (ICP) and Neutron Activation Analysis (NAA), is therefore crucial for the determination of heavy metals present in herbal raw materials.

2.1.1.6 Biological Assay

Each plant is known for its therapeutic properties due to its active bioactive constituents. Biological assay is the evaluation of the therapeutic effect of plant materials and their products on living organisms, isolated organs or enzymes for their therapeutic efficacy against particular ailments, thus establishing the dose-action relationship. Consistency in the biological activity of herbal medicine is dependent on the consistency of the phytochemical constituents of the medicinal plant (Torey *et al*., 2010, Sumitra and Arunachalam, 2014). Bioassays help in evaluation of the strength of the drug or its preparation (Shulammithi, *et al.*, 2016). The different techniques employed in carrying out biological assay are *in vivo, in vitro* and *ex vivo* techniques.

In vivo technique, meaning "within the living", involves the use of a living organism. This is why *in vivo* technique often involves animal studies and clinical trials. Example of animal study is antidiabetic assay using wistar albino rats, antidiarrhoeal assay usually carried out on Balb/c mice and so on. *In vitro* technique, meaning "within the glass", usually involves test tubes experiment; it involves carrying out an experiment in a controlled environment outside a living organism or cells (Shulammithi, *et al.*, 2016). Example is an antioxidant assay. *In vivo* testing is often preferred to *in vitro* technique because the former allows observation of the overall effects of an experiment on a living subject. It has the potential to offer definite understanding about the nature of medicine and disease (Kinnings *et al*., 2009). *Ex vivo* takes place outside an organism. In this technique, an assay is carried out on tissue from an organism in an external environment with minimal alteration of its usual natural condition. *Ex vivo* allows experimentation on an organism's cell or tissue under more

controlled conditions than it could be done in a whole organism, at the expense of altering the natural environment (Dusinska *et al*., 2012). Examples of *ex vivo* study are Chick Chorioallantroic Membrane assay (CAM), Antispasmodic assay.

2.1.1.7 Chromatographic and spectroscopic analyses

Chromatographic and spectroscopic analyses, which often successfully reveal the similarities and differences between various herbal samples, can be used to identify the phytochemical constituents in herbal drugs (Wani *et al*., 2007). Thus, chromatographic fingerprint could be used to evaluate the quality of herbal medicines, considering the multiple constituents present in them (Nikam *et al*., 2012). Different techniques of chromatography such as Thin Layer Chromatography (TLC), Column chromatography (CC), High Performance Thin Layer Chromatography (HPTLC), High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC) and other hyphenated techniques are used for the analysis of phytochemical constituents (Kirti *et al*., 2014). Chromatograph and spectroscopy can be employed for identification and authentication of medicinal plants, as well as determination of various adulterants and contaminants, all towards standardisation purposes. One unique advantage of spectroscopy over other methods discussed earlier (macroscopic, microscopic and other molecular biological methods) is its application to pharmaceutical formulations, which is beyond the herbal raw medicinal materials.

2.1.2 Molecular standardisation of medicinal plants

The set of standards used in herbal industry for standardisation of herbal raw materials is the herbal pharmacopoeia which carries a set of data collectively known as the monograph. The morphological, microscopical, biological and chemical methods are some of the mostly identified standards used since inception of the subject of Pharmacognosy as currently described in the herbal pharmacopoeia globally. However, well known sets of pharmacognostic standards are not without some setbacks (Adeniran *et al*., 2018). The consistency and relevance of these techniques are based on technical know-how with wide knowledge of diagnostic characters for bioactive compounds to be separated for closely related species with similar morphological or phytochemical characters (Liu *et al*., 2011).

Physiological and environmental conditions such as age, geographical location, seasonal variation and time of collection, diagnostic features of the tissue and chemical composition of plants do affect morphology of plant parts. Hence, there is need for additional means of identification of medicinal plants, which can complement the traditional methods of identification of medicinal plants for consistency and accuracy. This motivated the development of genome-based methods for the identification of medicinal plants in the early 1990s (Sucher and Carles, 2008).

It is worthy of note that standardisation of herbal medicine at molecular level is DNA-oriented, involving the use of molecular markers which are often DNA-based. Molecular markers, which are genes or nucleotide sequence located on a chromosome, evince the presence of metabolism or a chemical or physical process, which can be used in cells, individuals or species differentiation (Ganie *et al*., 2015). Usually, DNA markers are not affected by environmental and physiological factors. Thus, they can be detected at any stage of plant development, hence its usual preference in studies targeted at standardisation.

As a DNA sequence whose inheritance can be easily monitored, molecular markers are used based on the naturally occurring DNA polymorphism, which forms the basis for designing strategies to exploit for applied purposes. Thus, a marker must be polymorphic; that is, it must exist in different forms so that chromosome carrying the mutant genes can be differentiated from the chromosomes with the normal gene by a marker it also carries. Genetic polymorphism is defined as the simultaneous occurrence of a trait in the same population of two discontinuous variants or genotypes. This definition explicates the preference of DNA markers as the best candidates for efficient evaluation and selection of plant material. In addition to this, DNA can be easily extracted from plant materials and its analysis can be cost and labour effective (Kumar *et al*., 2009). In addition to genetic polymorphism, molecular markers are required to exhibit easy and fast assay, high reproducibility, frequent occurrence in genome, selective neutral behaviours, easy access (availability) and codominant inheritance. Authenticating medicinal plants by DNA means can be a veritable tool, for of herbal products' quality and safety, which will lead to improvement in medicinal potential and commercial profitability of traditional medicine (Ganie *et al*., 2015).

2.1.2.1 Types of molecular markers

A wide variety of molecular markers have been documented in the literature. Some of these include Amplified Fragment Length Polymorphism (AFLP), Allele Specific Polymerase Chain Reaction (AS-PCR), DNA amplification fingerprinting (DAF), Degenerate Oligonucleotide Primed PCR (DOP-PCR), Diversity Arrays Technology (DArT), Inter-Simple Sequence Repeat (ISSR), Inverse PCR (IPCR), Restriction Fragment Length Polymorphism (RFLP), Selective amplification of microsatellite polymorphic loci (SAMPL), Sequence characterised amplified regions (SCAR), Sequence specific amplification polymorphisms (S-SAP), Single stranded conformational polymorphism (SSCP), Sequence tagged microsatelite site (STMS), Sequence tagged site (STS), Simple sequence repeats (SSR), Strand displacement amplification (SDA), Site-selected insertion PCR (SSI), Variable number tandem repeat (VNTR). The newest developments are the Next Generation Sequencing (NGS) based markers.

These various molecular markers can be classified into different groups based on (i) mode of transmission (biparental nuclear inheritance, maternal nuclear inheritance, maternal organelle inheritance, or paternal organelle inheritance); (ii) mode of gene action (dominant or codominant markers) or (iii) method of analysis (hybridisation-based or PCR based markers). Also, each of the techniques targeted towards a particular component of the genome has unique methodological, technical and material challenges. Therefore, individual researcher's aim determines the choice of specific markers (Ganie *et al*., 2015).

2.1.2.2 Techniques used for DNA polymorphism Evaluation

Evaluation of DNA polymorphism may be carried out through diverse types of DNA-based molecular techniques. They include hybridisation-based methods, polymerase chain reaction (PCR)-based methods and sequencing-based methods.

2.1.2.2.1 Hybridisation-based method

Hybridisation-based method cloned DNA elements (such as random genomic clones, cDNA clones, probes for microsatellite and minisatellite sequences) or synthetic oligonucleotides as probes to hybridise the DNA of interest. Radioisotopes or conjugated enzymes are needed to catalyse the reaction (Houghton and Mukherjee, 2009). Presence or absence of bands

upon hybridization are indicators of polymorphisms. This method involves Restriction Fragment Length Polymorphism (RFLP) and Variable Number Tandem Repeats (VNTR) (Joshi *et al*., 2004).

Restriction fragment length polymorphism (RFLP) is a system associated with origination of molecular genetics field. This system is constructed on the principle of variation caused by mutation occurrence in restriction enzyme binding and cleavage sites. Also, the system involves reshuffling in the genomic region flanked by restriction sites, by which the genomes distribution is disrupted, thereby bringing about polymorphism (Ganie *et al*., 2015). The RFLP is not only time consuming, but also involves radioactivity; it is strenuous and problematic to automate.

2.1.2.2.2 Polymerase Chain Reaction (PCR) - based method

Polymerase chain reaction (PCR) is the in vitro enzymatic multiplication of specific DNA sequence with the help of arbitrary oligonucleotide primers (Kumar *et al*., 2009). PCR could be deployed to amplify RFLP regions in a situation of identified flanking regions of nucleotide sequence. The steps involved in PCR are denaturation, annealing and extension. Various PCR based techniques include Inter Simple Sequence Repeats (ISSR), Randomly Amplified Polymorphic DNA (RAPD), Arbitrarily Primed Polymerase Chain reaction (AP-PCR), Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeats (SSR).

Inter Simple Sequence Repeats (ISSRs) is one of the dominant PCR based techniques. It involves the use of microsatellites (di-penta nucleotide) as primers targeted at amplifying DNA segment present at a specifically increasing distance in-between two identical microsatellites repeat regions when arranged in opposite direction. Inter simple sequence replicates of the genomic DNA of diverse sizes are amplified by the microsatellites which are aimed at multiple genomic loci. ISSRs, on another hand, engenders the annealing of the primers, and when this is done at higher temperatures, it often leads to higher stringency. Detection of DNA fragments, which are mostly 0.5–2.0 kb long, may be achieved by means of polyacrylamide and agarose gel electrophoresis (Techen *et al*., 2014).

2.1.2.2.3 Sequence-based method

Sequence-based method involves the use of DNA sequences for identification of plants at several taxonomic levels. The DNA sequencing can also be used as a definitive means for identifying species. Using such method, information on a defined locus can be found as variations due to transversion, insertion or deletion can be obtained directly. Genetic variation occurs widely at the single nucleotide level and direct sequencing can effectively identify such single nucleotide polymorphisms depending greatly on how closely related the organisms are. Sequence-based method involves DNA barcoding, Next Generation Sequence (NGS), Sequencing-based markers (SCAR).

The DNA barcoding is a unique technique designed to correctly identify and differentiate closely related species of biological organisms using sequences from a short, nuclear or organelle DNA genome (Techen *et al.*, 2014). According to Kress *et al.* (2005) and Hollingsworth *et al*. (2013), DNA sequence could serve as barcode when it has the following basic characters: (i) meaningful genetic variability for discrimination of species; (ii) a short sequence length to enhance DNA amplification and iii) conserved flanking regions for universal primers development across the different taxa. The DNA barcoding is a method used for species identification applying a short standardised DNA sequence instead of a whole genome (Sukrhamit, 2015).

Herbert *et al*. (2003) posit DNA barcoding involves the use of state-of-the-art biotechnology in a quick and accurate manner to identify plant species. They added that it entails collection of data from one or a couple of regions in the genome to identify all the species in a particular class. An instance of such DNA genomic region is analysis of the variable internal transcribed spacer (ITS) sequences of ribosomal DNA (rDNA). The level of ITS sequence variation suitable for phylogenetic analysis is found at various taxonomic levels within families, depending on the linkage. Joshi *et al*. (2004) have also sequenced other regions of DNA such as trnK of chloroplast and spacer region of 5s rDNA as diagnostic tools for authentication purpose. The ITS has the potential to be used as a standard DNA barcode to identify medicinal plants and their close relatives since it has demonstrated excellent species resolution even for closely related species (Liu *et al*., 2011; Selvaraj *et al*., 2012). Recently, rDNA-ITS, matK and rbcL regions have frequently been applied to diverse herbal medicines to authenticate medicinal plant species and determine the molecular phylogeny

of medicinal herbs (Kim *et al*., 2016). The DNA barcoding is notably applied in identification of medicinal plants as well as authentication of herbal drugs.

The DNA barcoding has been found to be an effective ethno-genomics tool to identify the cryptic species, phytomedicines and biological authentication of raw materials. Thus, it enhances the value of both traditional ethno-botanical and scientific knowledge (Newmaster and Ragupathy, 2010). The ITS barcode region has been used for the molecular identification of medicinal plants including medicinal plants of Selaginellaceae (Gu *et al*., 2013). The DNA barcoding system is widely used in plant identification, in forensic science, verification of herbal medicines, and foodstuffs (Costion *et al.,* 2011), and resolving ambiguity of species in plant systematics (Balachandran *et al*., 2015).

Also, DNA barcoding has been used to check the authenticity of natural products, which provide authentic assessment of commercial herbal products within the marketplace. This includes reports on detecting adulteration through DNA barcoding in commercial tea samples (Stoeckle *et al*., 2011) and natural health products (Wallace *et al*., 2012).

2.2 Gastrointestinal disorders

Gastrointestinal (GI) disorder is one of the most commonly-encountered diseases in man (Choung *et al*., 2006). The gastrointestinal tract is under the control of the sympathetic and parasympathetic arms of the autonomic nervous system (Ibeh *et al*., 2018). The interchange between these two arms brings about balance and helps maintain normal peristaltic roles in charge of the headlong movement of intestinal contents (Osim, 2002). Excessive involuntary contraction of muscle (spasm) associated with superfluous release of acetylcholine, a neurotransmitter which mediates parasympathetic functions causes increased peristalsis, which leads to gastro intestinal disorder such as incontinence, cramps, gastritis, peptic ulcers, diarrhoea and ulcers due to increased gastric secretions (Jackquelyn, 2013).

2.2.1 Antispasmodic activity

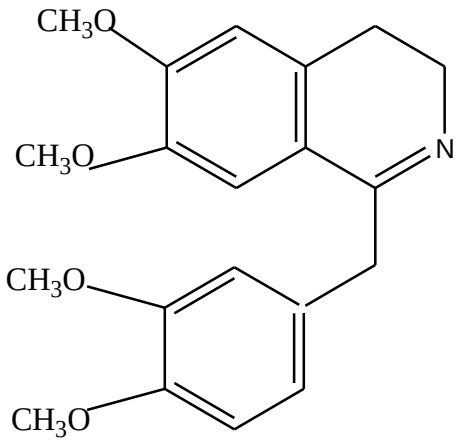
Spasm is a sudden uncontrollable muscular contraction of smooth muscles or transitory constriction of a passage such as the intestine, bladder, lungs and stomach, which is usually accompanied by pain and may be induced by endogenous acetyl choline (Alam *et al*., 2016).

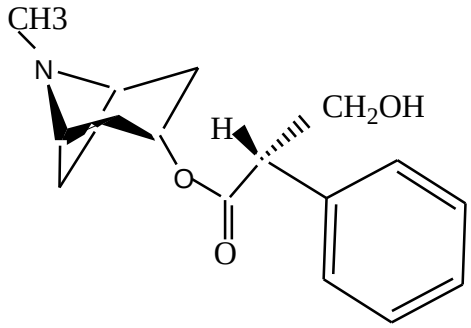
Majority of gastrointestinal (GI) disorders like colic, abdominal pain, flatulence, diarrhoea, cramping and irritable bowel syndrome are caused by spasm. Spasm of the smooth muscle is caused by antagonistic muscarinic actions, which are mediated by M3 receptors (Khana and Gilani, 2009). Such antagonistic muscarinic actions can be combatted by antispasmodic agents— substances which relax and subdue the muscle spasms or contractions. They prevent spasm of the stomach and intestine by blocking the action of acetylcholine neurotransmitter in the parasympathetic system, thereby inhibiting cholinergic nerve impulses (Ibeh *et al*., 2018). Antispasmodics are classified into anticholinergic (dicyclomine and hyoscyamine) and musculotropic (mebeverine). Each of them, however, shows various side effects such as dry mouth, narrow angle glaucoma, tachycardia, and the obstructive disease of gastrointestinal tract (Hadley and Gaader, 2005).

Plant-derived antispasmodic agents are considered relatively safer and cost-effective treatments. They include some tropane alkaloids (atropine, hyoscine, scopolamine, hyoscyamine, opium alkaloids (paperverine, codeine and morphine), flavonoids (luteolin, quercetin, rutin, apigenin, kaemferol, and so on) and essential oils, obtained from caraway, peppermint and garlic, among others (Bayad, 2016). Medicinal plants are rich in phytochemical constituents with antispasmodic properties, which relieve GI pains and contractions. Some antispasmodic medications of herbal origin are already being used in the orthodox medical practice. They are very important in the treatment of gastrointestinal motility disorder (Sadraei *et al*., 2003). They are useful for relieving or calming down colics, resulting from the spasms of the gastro intestinal muscles as well as diarrhoea of gastro intestinal hypermotility origin. Paperverine from *Papaver somniverum* L. (Papaveraceae) is notably used in treating colic, while atropine (*Atropa belladonna* L. (Solanaceae)) is an antimuscarinic agent for relieving spasm of gastrointestinal tract, induced by acetylcholine (Broadley and Kelly, 2001).

Traditionally, some plant products such as pepermint oil and bamboo shoot have been used as antispasmodic and gastro intestinal symptoms (Pittlier and Erst, 1998). Drugs of plant origin such as atropine and hyoscyamine from Solanaceous plants (for example *Datura* species), or papaverine from *Papaver somniferum* (opium poppy) have been used over a long period of time for the treatment of spasms of smooth muscles. The chemical structures

of some isolated plant constituents used for treatment of smooth muscle spasm is shown in Figure 2.2.1





Papaverine (alkaloid) (Evans, 2008, p360) Atropine (alkaloid) (Evans, 2008:126)

Figure 2.2.1: Chemical structures of plant constituents used for inhibition of smooth muscle spasm

2.2.2 Diarrhoea

The gastrointestinal tract (GIT) is saddled with the responsibility of absorbing and processing nutrients and fluids, which is necessary for the upkeep of good health (Martinez-Augustin *et* a*l*., 2009). Maintenance of balance between intestinal motility and the retention of intestinal fluid volume is a necessary condition for optimal GIT function. Any uproar in this homoeostatic process will result in GIT disorders like diarrhoea or constipation. Diarrhoea is defined as urgent and frequent passage of formless faeces three or more times within a period of 24 hours or more frequently than normal for an individual (Schiller *et al*., 2017). It is an outcome of hypermotility which often results from imbalance of the absorptive and secretory mechanisms in the intestinal tract, bringing about dehydration (Sharma *et al*., 2015; Nemeth and Pfleghaar, 2017). It is one of the leading clinical signs of gastrointestinal diseases and can be an indicator of a serious health condition such as ulcerative colitis, inflammatory bowel disease, and Crohn's disease (Mishra *et al*., 2016).

Brijesh *et al*. (2011) aver that, a wide range of agents could be responsible for diarrhoea episode. Some of these agents are entero-pathogenic microorganisms (*Escherichia coli*, *Shigella flexneri, Staphylococcus aureus*, *Candida albicans* and *Salmonella typhi*), bile salts, alcohol, irritable bowel syndrome, secretory tumors, hormones, and intoxication. Diarrhoea could be infectious and, in some cases, it could be non-infectious (Palomo, 2006). For immune-competent individuals, diarrhoea may be abrupt and self-limiting at the onset, and then aggravate to persistent chronic diarrhoeic condition, even with therapy. Chronic diarrhoea is however more common among people with underlying debilitating clinical conditions such as HIV/AIDS and diabetes mellitus, or individuals with an aging immunity (Joseph *et al*., 2017). Lawrence and Schiller (2007), however, assert that diarrhoea can be a good means of rapidly wiping out toxins from the body.

2.2.2.1 Pathophysiology of diarrhoea

Bacteria and viruses are the most common causal agents of diarrhoea. Some diarrhoea could be caused by several types of bacteria infected through contaminated food or water. Examples include *Campylobacter, Salmonella, Shigella,* species and *Escherichia coli*. Also, viruses like rotavirus, norovirus, herpes simplex virus, and hepatitis virus can cause

diarrhoea. For instance, infection with the rotavirus is the most common cause of acute diarrhea in children. It usually resolves in 3 to 7 days but can pose problem in digesting lactose for up to a month or longer. Parasites are also causative agents of some conditions of diarrhoea. Parasites can be ingested through food or water, and settle in the digestive system. Diarrhoea causative parasites include *Giardia lamblia, Entamoeba histolytica,* and *Cryptosporidium spp.* In addition to the foregoing, diseases such as inflammatory bowel disease, ulcerative colitis, Crohn's disease, and celiac disease can lead to diarrhoea. There are also evidences that **s**ome people have difficulty in digesting certain food composition such as lactose, complex carbohydrates. Some may experience diarrhoea if they consume certain types of sugar substitutes like sorbitol or mannitol. Furthermore, antibiotics, cancer drugs and antacids can trigger diarrhoea (Vitali *et al.,* 2006). One or more of the following four mechanisms could be involved in the pathogenesis of any diarrhoea case.

2.2.2.1.1 Osmotic diarrhoea

Osmotic diarrhoea is caused by lack of brush border enzymes required for the digestion or ingestion of poorly absorbed complex solutes (Baldi *et al*., 2009). Absorption of water in the intestine depends on the right amount of solute absorbed. When solutes are poorly absorbed and excess of it is retained in the intestinal lumen, the gradient of water absorption toward fluid retention in the intestinal lumen is altered, leading to diarrhoea (Fernandez-Banares *et al*., 2009). Examples of diarrhoea induced by osmotic basis include malabsorption states such as celiac disease, bacterial overgrowth, osmotic laxatives, antacids ($MgOH_2$) and maldigestion of complex carbohydrate such as lactose, fructose or sugar alcohol (mannitol, sorbitol) due to deficiency in disaccharidase (Schiller *et al*., 2017). Steatorrhea and azotorrhea (passage of fat and nitrogenous substances into the stool) may result from osmotic diarrhoea without rectal bleeding (Camilleri, 2015). A distinguishing feature of osmotic diarrhoea is that it stops after the patient is fasted or stops consuming the poorly absorbed solute.

2.2.2.1.2 Secretory diarrhoea

Secretory diarrhoea is always caused by secretagogue, which is usually induced by decreased sodium absorption, uncontrolled water secretion, increased chlorine secretion and

increased mucosal permeability (Ravikumara, 2008). Other agents such as laxatives, bile acid, antidepressant drug, congenital disease and caffeine can induce secretory diarrhoea. In most cases, secretory diarrhoea will not resolve with fasting prescription.

2.2.2.1.3 Inflammatory and infectious diarrhoea

Disruption of intestinal epithelium mucosa (due to infection or inflammation) is a very common cause of diarrhoea. It always results in exudation of serum and blood into the lumen and also inefficient absorption of water from the intestine (Groschwitz and Hogan, 2009). This diarrhoeic condition is prompted by the damage or killing of intestinal epithelial cells, caused by reactive oxygen species from leukocytes. The damaged or killed cells are thus replaced with immature cells that, typically, are deficient in the brush border enzymes and transporters necessary for absorption of nutrients and water. In this way, components of osmotic (malabsorption) diarrhoea are added to the problem. Presence of mucus, pus and blood in the stool is suggestive of inflammatory diarrhoea associated with abdominal pain.

2.2.2.2 Epidemiology of diarrhoea

Diarrhoea is a global problem, which affects people of all ages. Episodes of it may range from a mild, temporary condition, to prolonged and potentially life-threatening episodes (Joseph *et al*., 2017). Riddle *et al*. (2016) assert that diarrhoea is a leading cause of outpatient visits, hospitalisation, and loss of quality of life among the adult populations. As documented by UNICEF/WHO (2009) and WHO (2017), severe cases of diarrhoea, which is marked by dehydration and loss of electrolytes, may lead to death. According to the World Health Organisation, diarrhoea affects 3-5 billion people globally per year. WHO (2017) further adds that children less than 5 years of age are more susceptible to the disease with over 5 million deaths of children per annum, as a result of their relative low level of immunity as compared to adults.

2.2.2.3 Diagnosis of diarrhoea

Diagnostic tests that could be used to detect the cause of diarrhoea include medical history and physical examination, stool culture, blood test, fasting test, and sigmoidoscopy or colonoscopy. A medical history of persons affected by diarrhoea can be accessed when the

physician probes the patients' way of living, family history, current medication, and performs a physical examination to look for signs of illness (Caliendo *et al*., 2013). In another approach, a sample of the stool may be analysed in a laboratory to check for bacteria, viruses, parasites or other microorganisms. Fasting test helps to detect if diarrhoea is caused by food intolerance or allergy. Sigmoidoscopy (also known as colonoscopy) may be used to detect the signs of intestinal diseases that have caused chronic diarrhoea (Steffer *et al*., 2012).

Based on duration of symptoms during diagnosis reported in extant literature, diarrhoea is broadly classified into three types, namely: acute, persistent and chronic diarrhoeas. Acute diarrhoea is usually referred to as gastroenteritis and often caused by enteric pathogenic infections, intoxicants or food allergy. It involves frequent passage of loose stool without visible blood and could be accompanied by fever and vomiting. This type of diarrhoea is self-limiting without any therapeutic intervention and can resolve within two weeks (usually 7 days) from onset (Riddle *et al*., 2016).

Persistent diarrhoea, on the other hand, often results from secondary infections accompanied with complications such as malabsorption and dehydration (Peter and Umal, 2018). It will often last for more than 14 days. It is usually marked by weight loss, loss of fluid and electrolytes leading to dehydration.

Chronic diarrhoea is associated with a number of non-infectious agents such as hereditary metabolic disorder lasting longer than 4 weeks (Joseph *et al*., 2017). It mostly results from hereditary defects of digestion and absorption such as deficiency of lactase, which results to intolerance of lactose or irritable bowel syndrome. Pharmacologic agents for treatment of chronic diarrhoea include antispasmodic such as atropine, loperamide, 5-hydroxytryptamine type 3 (5-HT_3) receptor or anti-inflammatory agents (Lee, 2015). Peter and Umal, (2018) establishes that there are two potentially-serious complications of diarrhoea, namely: dehydration, which occurs with acute or chronic diarrhoea; and malabsorption accompanied with chronic diarrhoea.

2.2.2.4 Treatment of diarrhoea

Many casual diarrhoeal cases as well as those resulting from excess consumption of fruits and/or green vegetables, described as mild, often last for very short periods of 1 or 2 days and will normally cease without requiring any treatment. On the other hand, any persistent and chronic diarrhoea should be properly diagnosed and the underlying cause should be treated in addition to the accompanying diarrhoeal symptoms. Possible treatments include rehydration, routine zinc therapy, antidiarrhoeal medications (including antibiotics) and diet. Rehydration is implicated in children or adult diarrhoeas especially whenever dehydrated. Thus, rehydration is needed in combatting diarrhoeal manifestations. The WHO/UNICEF (2009) submits that ORS (oral rehydration therapy) can safely and effectively treat dehydration. Routine zinc therapy is also recommended by WHO/UNICEF (2009) for children diarrhoea, irrespective of the types.

Furthermore, antidiarrhoeal medications using therapeutic agents can also be found effective and implicated in the treatment of diarrhoea. For example, loperamide, an antimotility drug, is used to treat adults and children (> 2 years) with acute diarrhea and irritable bowel syndrome (Hanauer *et al*., 2007). Loperamide, like many other antidiarrhoeal medications, can reduce the duration of loose stools and can relieve cramps and urgency Hanauer *et al*., 2007). Antibiotics (such ciproflaxin and metronidazole) are used in the treatment of infectious diarrhoea, diagnosed to be infected by microorganism. Lawrence and Schiller (2007) also reported that antisecretory agents such as bismuth subsalicylate and crofelemer in HIV-associated) diarrhoea, can reduce diarrhoeal stool output in adults and children.

Diet has also been found to be a very crucial factor in the treatment of diarrhoea. The World Gastroenterology Organisation (WGO, 2012) recommends that patients should eat 2-4 hours after rehydration. This suggests that food starvation during diarrhoea episode is anomalous. However, there may be exceptions such as in cases of severe dehydration where intravenous rehydration has to take place. The WGO (2012) further recommends that after each loose stool, the lost fluids should be replaced with at least one cup of liquid. Also, it is advised that patients should take high-potassium foods and liquids, such as diluted fruit juices, potatoes without the skin, and bananas. Fabio *et al*. (2018) submit that a potential antidiarrhoeal agent may, as a sign of its effectiveness, inhibit the intestinal smooth muscle

spasm (antispasmodic), gut motility, gastrointestinal transit, electrolyte outflux and increased water absorption. Necessarily therefore, there has to be reduction in the consumption of foods that may worsen diarrhoea, such as creamy, fried, sugar free gum and drinks, caffeinated drinks, sugary foods, fructose in high amounts, fruit juices, grapes, honey, dates, nuts, figs, soft drinks, and prunes, dairy products and magnesium.

2.2.2.5 Medicinal plants as antidiarrhoeal agents

The ethno pharmacological knowledge and familiarity of people living in the underdeveloped and developing regions with nature has helped them to often avert and cure various diseases, including diarrhoeal episodes with medicinal plants in their vicinity. In traditional medicine, a number of plants are known to be effective against diarrhoea (Kumar *et al*., 2009). WHO has continued to advocate and encourage developing countries to use of medicinal plants for treating diseases in health care of their people including cases of GIT disorders (Bellini *et al*., 2014,Schiller, 2017). Thus, the regulatory body recognizes the potency of medicinal plants in the treatment of diarrhoea. Most of the herbal medicines with antidiarrhoeal activities act as antispasmodic agents through delayed processes of GIT motility, absorption of fluid and as antimicrobials in infectious diarrhoeas (Maroyi, 2016). Table 2.2.2.5 below details some locally sourced diarrhoea-mitigating plants

Table 2.2.2.5: Medicinal plants with antidiarrhoeal activities

Plants	Family	Parts used
Catharanthus roseus (L). G. Don	Apocynaceae	Leaves
Mangifera indica L.	Anacardiaceae	Bark, leaves, seed
Alepidea amatymbica Eckl. & Zeyh.	Apiaceae	Rhizomes, root
Psidium guajava L.	Myrtaceae	Stem bark
Tithonia diversifolia	Compositae	Leaves
Euphorbia hirta L	Euphorbiaceae	Leaves, root
Aegle marmelos (L.) *Corr. Serr.*	Rutaceae	Ripened fruit
Allium cepa L.	Amaryllidaceae	Bulb
Curcuma longa Linn.	Zingiberaceae	Rhizome
Cocos nucifera L.	Arecaceae	Flesh
Hibiscus sabdariffa Linn	Malvaceae	Flower
Phyllanthus amarus Schum. & Thonn.	Phyllanthaceae	Whole plant
Garcinia cola Heckel	Clusiaceae	Seeds and leaves
Annona muricata L.	Annonaceae	Leaf, fruit, stem bark
Dioscorea bulbifera L.	Dioscoreaceae	Root
Brypohyllum pinnatum (Lam) Oken	Crussulaceae	Leaf
Picralima nitida Stapf. Th. and H. Dur.	Apocynaceae	Seeds
Kigellia Africana and Schinz	Bignoniaceae	Fruit and leaves
Ocimum gratisimum L.	Lamiaceae	Leaves

Adapted from Maroyi (2016)

2.3 The family Apocynaceae

The Apocynaceae (also known as Dogbane or Oleander family) is a large Angiosperm family. It is made up of 424 genera in five subfamilies: Rauvolfiodeae, Apocynoideae, Periplocoideae, Secamonoidea and Asclepiadoideae. The habit includes trees, shrubs and herbs, and members of the family exhibit diagnostic feature of latex production (Rijhwani, 2013). They are distributed in tropical rainforest. However, some are found in tropical dry, xeric environments, while some flourish in temperate area. The leaves are simple, opposite and decussate or whorled, without stipules; flowers are usually showy, radially symmetrical, cymose or racemose inflorescences, ovary is usually superior while fruits are drupe, capsule, follicle or berry. The plants from this family have high economic and medicinal values, e.g. *Catharanthus roseus*, which is an ornamental plant and from which anticancer agents; vinblastine and vincristine have been isolated (Rijhwani, 2013).

2.3.1 Genus *Alstonia*

Alstonia is a widespread genus of evergreen trees and shrubs of the dogbane family (Apocynaceae). It is pan tropically distributed, consisting of about 40 species. Of the 40 species, only two of them (*Alstonia. boonei* De Wild. and *Alstonia congensis* Engl.) are indigenous to Africa. These two are are morphologically similar, and thus, are always assumed to be same plant (Palla, 2005).

2.3.1.1 *Alstonia boonei* De Wild.

Alstonia boonei is commonly known as pattern wood or stool wood. As recorded by Okoye (2014), it is locally called ahun/awun (Yoruba), egbu/egbu-ora (Igbo**),** ukhu (Bini), okugbe (Itsekiri), ukpukuhu (Urhobo). It is widely distributed in the tropics such as Senegal, Gambia, Ivory Coast, Ghana, Nigeria and Cameroon, in West Africa. It is commonly found in rocky sites or dry land but sometimes also in marshy areas (Okoye *et al*., 2014).

2.3.1.1.1 Botanical description of *Alstonia boonei* De Wild.

It is one of the two indigenous species of *Alstonia* in Africa (Palla, 2005). It is a large evergreen tree of about 25-40 m high and 1.2-1.4 m in diameter, known for its plenteous milky sap production. Stem-bark is scaly, grey to greyish green in colour, inner bark is off

white to creamy to yellowish colour. Leaves are simple and entire, arranged in whorls of 4–8, subsessile to petiolate, glabrous, no stipules, petiole 0.5-1cm long and stout; blade is oblanceolate to obovate, apex is acute to emarginate, base cuneate. The leaf margin is entire with coriaceous texture and dark green above and light green underneath, midrib is more conspicuous below, veins are very close to margin, parallel; flowers are regular and hermaphroditic, white to creamy or off white in colour. The fruits are made of two linear follicles, greenish in colour with hairy flat seed. Shown in figure 2.3.1.1 below is the habit photograph of *Alstonia boonei* in its natural habitat

Taxonomy of *Alstonia boonei*

Kingdom: Plantae
Division: Sympetalae
Order: Gentianales
Class: Asterids
Family: Apocynaceae
Subfamily: Plumerioideae
Tribe: *Alstoninae*
Genus: *Alstonia*
Species: *Alstonia boonei* De Wild.

Figure 2.3.1.1: *Alstonia boonei* De Wild. (Apocynaceae) showing the tree, florescence, leaves, flower and fruit in its natural habitat (behind Physiology Department, University of Ibadan)

2.3.1.1.2 Ethnomedical uses of *Alstonia boonei*

Alstonia boonei is a common medicinal plant used all over Africa for the treatment of various diseases. Abbiw (1990) maintains that its stem bark is one of the effective analgesics available in nature. For instance, he reports that the hardened latex (from the plant) is used for the treatment of yaws. Again, a decoction of stem bark of *A. boonei*, *Khaya grandifolia with Cleistopholia patens* and some quantity of red small pepper (*Capsicum frutenscens* fruit) in palm wine is used to treat malaria. Similarly, Igberaese and Ogbole (2018) assert that half a glass cup of *A. boonei* leaf squeezed into water is used to treat malaria, diarrhoea and dysentery. Also, the stem bark decoction is administered for the management of tooth ache, and with other preparations in the treatment of fractures or dislocation and jaundice. Furthermore, its fresh infusion is used as an effective antidote against snake, rat or scorpion poisons. Asuzu and Anaga (1991) establish that *A. boonei* stem bark is also used in treating painful micturition and rheumatic conditions. In addition, *A. boonei* leaf and latex are used topically to reduce swellings for the treatment of rheumatic pains, muscular pains and hypertension (Iwu, 1993). While an infusion of the stem bark is taken to expel round worms and other intestinal parasites in children, a brew the root, stem bark or the leaf is found to be effective remedy for asthma. Akinmoldun *et al*. (2007) report that *Trema orientalis* and decoction of the stem bark of *A. boonei,* mixed with the root and stem bark of cola and fruits of *Xylopia parviflora* with hard potash is orally administered to asthmatic patients. Further, Orwa *et al*. (2009) report *A. boonei* as one of the few herbs with potential anti-HIV indication.

The medicinal relevance of *A. boonei* in procreation/fertility as well as urethra related issues have also been registered in the literature. The stem-bark of *A. boonei* and a bunch of *Piper guineensis* fruit are infused in local gin and teaspoon of the infusion is administered daily for treatment of impotence. According to Orwa *et al*. (2009), *A. boonei* latex, when taken as a purgative, does not only effectively relieve painful urethritis common with gonococcus or other microbial infections in men but also aids in expelling retained products of conception and afterbirth. As further demonstrated by Adotey *et al*. (2012), the application of *A. boonei* can be used to combat gynaecological problems, and particularly dysmenorrhoea, especially when associated with uterine fibroid or ovarian cysts in women.

2.3.1.1.3 Pharmacological activities of *Alstonia boonei*

Extant studies have documented a number of scientific proofs for the biological activities of *A. boonei*, in relation to some compounds isolated from the plant. Okoye *et al*., (2014) for instance, reports the anti-inflammatory activity of *A. boonei*, and attributes it to the presence of α-amyrin acetate and β-amyrin. This is also supported by Akinnawo *et al*. (2017) who claim that aqueous fraction of *A. boonei* leaf exhibited substantial active anti-inflammatory and antioxidant activities. Also, Akinloye *et al*. (2013) has reported the hypoglycaemic properties of aqueous extract of *A. boonei* stem bark. In addition, Okoye and Okoye (2016) report that 5-caffeoylquinic acid (Chlorogenic acid) and 4,5-dicaffeoylquinic acid isolated from the ethyl acetate fraction of the methanol leaf extract of *A. boonei* showed good antioxidant activities on DPPH free radical scavenging model.

Ali *et al*. (2017), show that ethanol and hot water extracts of *A. boonei* with *V. doniana* had high antityphoid activity. Ogueke *et al*. (2014) also report that ethanol extract of *A. boonei* stem-bark possessed high antibacterial activity. In a study in which aqueous, ethanol and methanol extracts of *A. boonei* leaf were screened for larvicidal activity against different instars of mosquito larvae, Omoya *et al*. (2012) demonstrated the high anti-larvicidal activity of the plant against mosquito larvae. This report is supported by Oigiangbe *et al*. (2013) who maintain that the leaf alkaloid of *A. boonei* has larvicidal activity. Moreover, Iniaghe *et al.* (2012) report the analgesic properties of *A. boonei* methanol leaf extract in chemical and thermal pain models and suggested that the analgesic effect of the *A. boonei* leaf extract may be mediated *via* both central and peripheral mechanisms. Ethanol and aqueous extracts of *A. boonei* stem-bark have shown chemo-suppressive activity on early malaria in *Plasmodium berghei berghei* infected mice (Anyalogbu *et al*., 2013). The ethanol extract of *A. boonei* stem bark has also been reported to produce dose dependent anti-malarial activity. Onyishi *et al*. (2020) have also reported the anti-malarial effect of the aqueous extract of *A. boonei* leaf and root. *Alstonia boonei* stem-bark has been listed in Ghana Herbal Pharmacopoeia (GHP, 2007) as an antimalarial drug.

Also, the possible nephrotoxic effect of aqueous ethanol extract of *A. boonei* at higher doses and long-term administration has been reported by Oze *et al*. (2006). Various levels of toxicity of *A. boonei* to the liver, kidney or spleen were attributed to the triterpenes which have anti-arthritic (Kwefio- Okai and Caroll, 1992) and antiproteolytic activities

(Chaturvedi *et al*., 1974). Hence, long-term administration of *A. boonei* extracts may limit its benefits as an antiarthritic drug (Kweifio-Okai, 1991a). Other therapeutic effects of *A. boonei* that have been subjected to pharmacological investigation include its anti-cancer activity (Kamarajan *et al*., 1991; Jagetia *et al*., 2005); neuroleptic and anxiolytic properties (Elisabetsky and CostaCampos, 2006); and antihelminthic property **(**Klu *et al*., 2016).

2.3.1.2 *Alstonia congensis* Engl.

Alstonia congensis is found in tropical rainforest, occurring from the south-western Nigeria, Central African Republic, Democratic Republic of Congo and northern Angola, Cameroon and Liberia. Specimens for scientific identification were first collected from Congo. Hence, the specific epithet was coined from the Country (Orwa *et al*., 2009). The species usually occurs in swampy area with *Elaeis guineensis, Dioscorea preusii, Cnestis hirsutus*, *Funtumia africana*, Raphia, although it is sometimes found on drier soil.

2.3.1.2.1 Botanical Description of *Alstonia congensis* Engl.

Alstonia congensis is an evergreen tree of about 30 m high, with copious latex. Its stem bark is greyish and rough, with large lenticels. The fresh inner bark is creamy, but brownish when dried. Its leaves are arranged in whorls of 6-8, simple, entire, stipules absent, petiole 0.5 to 1 cm, flattened and grooved with narrowly triangular colleters at base. The blade is obovate with cuneate base; the leaf may assume rounded, acuminate or retuse shapes at the apex.

Taxonomy of *Alstonia congensis*

Kingdom: Plantae
Division: Sympetalae
Order: Gentianales
Class: Asterids
Family: Apocynaceae
Subfamily: Plumerioideae
Tribe: *Alstoninae*
Genus: *Alstonia*
Species: *Alstonia congensis* Engl.

The habit photograph of *Alstonia congensis* is shown in figure 2.3.1.2. below;

Figure 2.3.1.2: *Alstonia congensis* Engl. (Apocynaceae) showing the tree, leaves and florescence in its natural habitat (Itokin Village, off Epe/ Ikorodu Express way)

2.3.1.2.2 Ethnomedical uses of *Alstonia congensis*

Decoction of *A. congensis* leaf is used to treat diarrhea both in adult and children in DR Congo (Nsaka *et al*., 2012). Neuwinger (2000) posits that *A. congensis* stem bark decoction is used to treat malaria, gonorrhea, rheumatic pain and dysmenorrhea. He adds that the bark, in addition to its galactagogue and anthelmintic effects, is also applied as an antidote against arrow poison. The latex is used to treat leucorrhoea, ulcers, scabies, yaws and headache, and its lightly roasted leaves may be smoked in a pipe, as a remedy for cough (Burkhill, 1985).

2.3.1.2.3 Pharmacological activities of *Alstonia congensis*

Extant studies have documented few pharmacological activities of *A. congensis*. They include antidiarrhoeal activity (Nsaka *et al*., 2012); antihyperglycaemic and antidiabetic activities (Nsaka *et al*., 2016); and antiplasmodial activity (Cimanga *et al*., 2019).

2.3.1.3 Phytochemistry of the genus *Alstonia*

Alstonia species are rich in wide range of compounds, from simple but rare monosaccharide to complex secondary metabolites, including alkaloids, terpenes, flavonoids saponins and so on.

Alkaloids are a large group of pharmacologically active nitrogen containing organic compounds of plants, microbial or animal origin. They can be classified in terms of their chemical structures (the nucleus must contain nitrogen) or their biosynthetic pathway. Pyridines, pyrroles, indoles, pyrrolidines, isoquinolines, quinolone and piperidines are some of the common alkaloid ring structures. Indole alkaloids and quinolone alkaloids are predominantly contained in the genus *Alstonia*. These alkaloids are assumed to originate from the condensation of tryptophan with secologanin (Arulmohzhi *et al*., 2007). Several plants of the *Alstonia* species have been reported to contain the monoterpene indole group of alkaloids including echitamine, echitamidine and their derivatives, akuammidine, augustilobines and so on. Echitamine had been reported to have a wide array of pharmacological activity from diuretic, antispasmodic, hypotensive to sedative activities (Ojewole, 1984).

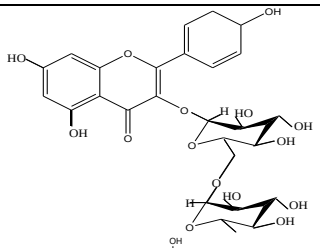
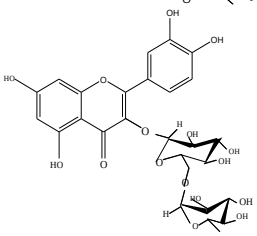
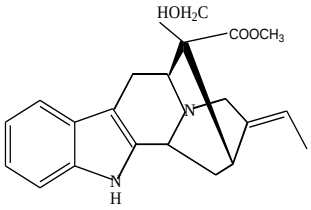
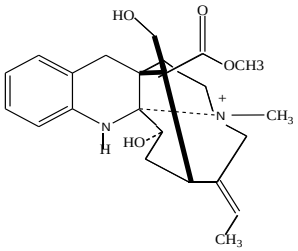
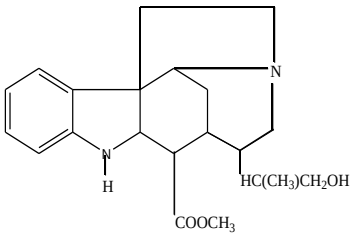
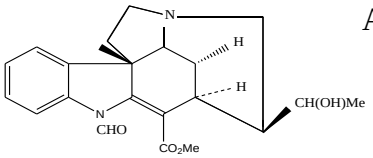
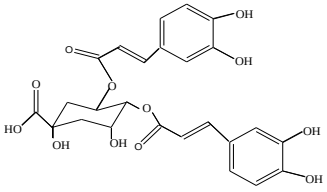
Fifteen alkaloids have been previously isolated from the root bark, stem bark and leaf of *A. boonei and A. congensis.* These include echitamine, echitamidine, akuammicine, 12-methoxyakuammicine, 12-methoxy-N (4) methylakuammicine, tubotaiwaine, 12-methoxytubotaiwaine, angustilobines A and B, 6-7 seco angustilobines A and B, angustilobines B- N (4) oxide, akuammidine, nor-echitamine, and 17 acetoxy- nor-echitamine (Caron *et al.,* 1989).

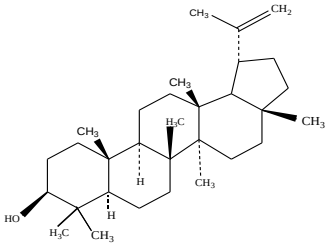
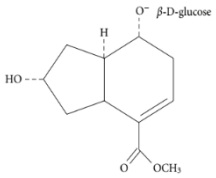
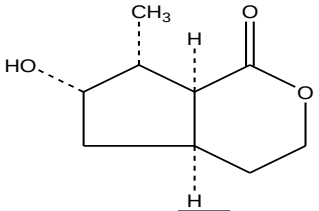
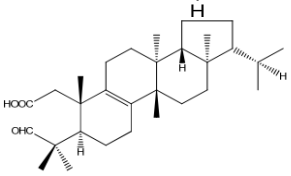
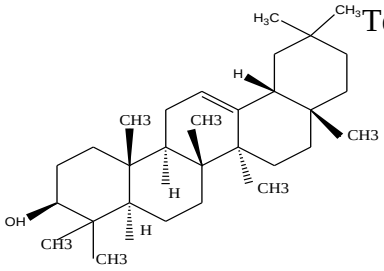
Terpene, a generic name of a group of natural products structurally based on the isoprene (Isopentenyl) units, is one of the largest and most diverse groups of plant secondary metabolites. Terpenes are normally grouped based on the number of isoprene units from which they are biogenetically synthesised and based on their skeleton type. The various groups include hemiterpenes (C5), Monoterpenes (C10), Sesquiterpenes (C15), Diterpenes (C20), Triterpenes (C30), Tetrapenes (C40). Compounds such as isoprene, iridoids, as well as α and β amyrin have been reported on these terpenes. Also, the compounds are reported to have shown a broad range of biological activities such as an antioxidative, antibacterial, antifungal, antitumoral, hepatoprotective, cardioprotective, anti-inflammatory, antiprotozoal and anti-insect, antiarthritic, weak cytotoxicity activity, anti-arthritis, ursolic and betulinic acid and anti- lung cancer properties (Amaral *et al*., 2013; Wang *et al*., 2017). Specifically, Adotey *et al*. (2012) has isolated α-β amyrin, ursolic acid and lupeol from different species of *Alstonia.*

Flavonoids are class of plant secondary metabolites which are soluble in water. 'Flavonoid' is generally used to describe wide group of natural products that possess a C6-C3-C6 skeleton, or more specifically a phenylbenzopyran function (Marais *et al*., 2007). Flavone ring is the nucleus of diverse flavonoid molecules or the backbone of flavonoid structure. They generally occur in plants as glycosylated derivatives and contribute to the brilliant shades of colour from blue, purple, scarlet and orange in leaves, flowers and fruits. They have high range of pharmacological activities which include anti-oxidant, anti-tumour, anti-inflammatory, antidiarrhoeal and anti-osteoporosis (Lydeking-Olsen *et al*., 2004; Kamlesh *et al*., 2016).

Table 2.3.1.3 shows some of the phytochemical constituents isolated from the genus *Alstonia*

Table 2.3.1.3: Some isolated compounds from the Genus *Alstonia*

Compound	Source	Structure	Class of compound
Kaemferol 3-O-rutinoside	*A. boonei*		Flavonoid
Quercetin-3-O-rutinoside	*A.boonei* leaves		Flavonoid
Akuammidine	*A. congensis*, *A. scholaris*, *A. boonei*		Alkaloid
Echitamine	*A. boonei* *A. congensis* *A. scholaris*		Alkaloid
Echitamidine	*A. boonei* *A. congensis* *A. pneumatophora*		Alkaloid
Nα-formylechitamidine	*A. boonei*, *A. congensis*		Alkaloid
4,5dicaffeoylquinic acid	*A. boonei* leaf		Caffeic acid derivative

Lupeol	*A. boonei*		Terpene
Loganin	*A. boonei*		iridoid
Boonein	*A.boonei*		iridoid
Alstonic acid A	*A.scholaris*		Terpene
β-amyrin	*A. boonei*		Terpene

Adapted from Adotey *et al.,* (2012) and Okoye *et al*., (2014)

2.4 Formulation of herbal medicine

Herbal formulations, otherwise known as herbal dosage-forms, in many cases contain two or more medicinal plant materials, and they may be in liquid, solid, powder or capsule dosage forms. Although single-plant formulations also exist in herbal practice, there is universal belief that the concept of two or more different plant materials in the same herbal formulation (multicomponent or polyherbalism preparations) can ensure higher therapeutic outcomes than single-plant therapy. Studies have therefore proved that, combination of 2 or more medicinal plants in the same formulation, in the right optimum proportion, can give an enhanced therapeutic efficacy, and may reduce the toxicity of individual component plants in the mixture (Falodun *et al.,* 2015). In Ghana, for instance, a common Ghanian antiarthritis polyherbal formulation consists of *A. boonei* root bark (90%), *Rauwofia vomitoria* root bark (5%) and *Elaeias guineensis* nut without pericarp (5%). This combination is believed to be in commensurate proportion of each of the plants incorporated and also effective in neutralising the toxicity of one of the constituents (Kweifio- Okai, 1992).

Thus, in polyherbal prescriptions in which two or more independent phytomedicines are used for the same condition, the components usually display potential interaction effects, demonstrating mutual enhancement or additive action, mutual assistance or synergistic effect, mutual restraint and mutual antagonism by the virtue of individual associated diverse constituents present (Ramaiah *et al.*, 2013). Where the interaction is beneficial, the pharmacological principle works in a dynamic or pharmacokinetic way to produce maximum therapeutic action with minimum side effects (Benzie and Watchel-Galor, 2011). In orthodox therapy also, using two or more independent conventional drugs for the same condition usually produces a promising or otherwise effect over a single-drug therapy for the same disease (Aslam *et al*., 2016). In the recent past, a patient suffering from malaria would not be treated with chloroquine alone but combination of chloroquine, paracetamol and piriton; the paracetamol will reduce the body temperature of the patient while the piriton would subdue the itching side effect of chloroquine, the combination thus gave the desirable effects (Risberg *et al*., 2011).

Herbal formulations in their whatever forms (liquid, powder, capsules and so on) often involves a number of processes such as extraction, distillation, expression, fractionation,

purification, concentration or fermentation of herbal substances. Whole, fragmented or plants cut into pieces, plant parts, algae, fungi, lichen in an unprocessed, usually dried form but sometimes fresh, could as well be used in the preparations of herbal drug (Attila, 2013). Common methods of herbal drug formulation and administration include the following.

***Hot Infusion*:** Infusion involves preparation of herbal drug by soaking herbs in boiled potable water, covered and steeped for 10-20 min. This method of herbal formulation is usually used for the delicate herbs, leaves and fresh tender plants. Preparing an infusion is like making a cup of tea. A ceramic pot is the suitable container for preparing an infusion; a metal pot should be avoided (Ogbonna *et al.*, 2012).

***Decoction*:** In decoction method of herbal formulation, the plant material is boiled for 1 h. This method is often chosen when tough and fibrous plant, stem bark and root are to be used (Taylor, 2004). The proportion of herbs to water is usually a ratio of 1 teaspoon powdered herbs or 2 teaspoons of cut herbs to 237 mL of water. The extract could be strained through a tea strainer into a teacup (Ogbonna *et al*., 2012).

***Tincture*:** Herbal formulation extracted with mixture of alcohol and water is called a tincture. Tincture is the choice when water cannot readily extract the plants' active constituents and/or when a larger quantity is prepared for convenience, and wanted for longer term storage. If tinctures are properly prepared, they can last several years or more without losing potency. The shelf life is determined by the percentage of alcohol usually; the higher content of alcohol used, the longer the shelf life since alcohol has an inherent preservative property. The herb is sometimes the determinant of the ratio of alcohol and water to be used for extraction (Mazza, 1998).

***Poultices and Compresses*:** Poultices involves mashing or chewing up fresh leaf or root and spitting them on the skin. In this method, the poultice is bound to the area being treated as a light cotton bandage. In tradition/remote settings (such as in the jungle) where bandage may not be available, a nice large flexible leaf is commonly employed and tied with a bit of twine). Compresses, on the other hand, involve soaking a cloth in a prepared infusion, tincture or decoction and laying the cloth onto the affected part of the body/skin (Taylor, 2004).

***Steaming/hot baths*:** This method has been in used for thousands of years in which the medicinal plant is boiled and poured in a container. The patient is asked to inhale the steam or vapour coming out of the extract. Two-three thick wrappers are used to cover the patient together with the hot extract. This is usually used for mature children and not for toddlers. Another means is for the patient to bath with the extract or the patient is asked to stay in the extract for a period of time. This method is similar to the currently evolving dermal delivery systems for drug absorption being employed in conventional medicine (Taylor, 2004).

***Inhalation therapy*:** The use of therapeutic material whether powders, liquids, vapours or gases through the inspired air has revolutionised into a medical sub-speciality known as inhalation therapy. In some contexts, the method is denoted with the term "Respiratory Therapy." While the term appears to be related to inhalation, it is however limited in that it covers only the treatment of the respiratory troubles, ignoring the other body parts. Today, nearly all medical specialties have one or more procedures in which the medications are taken through inhalation by different means for many body ailments. Forms of drug inhalation in traditional medicine are smokes of burnt reeds, plants or minerals or powder sample for snuffing. For instance, *A. boonei* leaf with some other plant materials can be burnt and the ashes are snuffed for treatment of respiratory disorder (Wollman and Smith, 1980).

***Powdery form*:** Powdered herbs could be sprinkled on food or taken with water or pap according to the instruction given by the herbalist. It can be applied topically as dusting powder to the skin or mixed with oil to rub the body (Ogbonna *et al.*, 2012).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Chemicals and instrument/equipment items

3.1.1 Chemicals and solvents

Methanol, Distilled water, Dichloromethane, Ethyl acetate, Butanol, *n*-hexane (Analar grade), CTAB (Cetytrimethylammonium bromide (Sigma- Aldrich), NaCl, EDTA (Ethylenediaminetetraacetic acid disodium salt dehydrates (Sigma- Aldrich). Internal Transcribed Spacer 1 and 4 (ITS1 and 4) (Bioscience), Dinucleotide triphosphates (10 mM dNTPs) Tris base, Ethanol, DMSO, Agarose gel (Sigma- Aldrich), Ethidium bromide, DNA loading dye, Taq buffer, Taq polymerase. Gene ruler (100bp) (bioscience), Silica gel (70-230 mesh, Scharlau, Spain), TLC plate (silica gel 60 F_{254} , (Merck, USA), β-mercaptoethanol, Isoporopanol, Iso amyl alcohol, H_2SO_4, 1 mL syringe, Castor oil B.P (Druggists Ltd, England), Potassium chloride (KCl (Sigma)), Magnesium chloride ($MgCl_2$), Sodium hydrogen phosphate (NaH_2PO_4), glucose ($C_6H_{12}O_6$), sodium bicarbonate ($NaHCO_3$), Acetylcholine (Sigma (St. Louis, MO, USA), Calcium chloride ($CaCl_2$) (Merck (Darmstadt, Germany), HNO_3 (nitric acid), per chloric acid, $FeCl_3$, HCl, Safranin O, Alcian blue, Potassium nitrate (KNO_3), Glycerol, Acetycholine, TE buffer X1 (Tris base and EDTA), Isopropanol, 0.1 % Monothioglycerol, Sodium hypochlorate (NaOCl).

3.1.2 Instruments and equipment items

Organ bath (AD Instrument), Desktop computer, Rotary evaporator (Buchi, Switzerland), Freeze drier (Gallenhamp, UK), Thermal cycler (Mycycler- USA), Centrifuge (Thermoscientific, USA), NMR (BRUKER), Furnace. Weighing meter, refrigerator -20 and

-80^{o}C (Bruhm,), PCR (PE Applied Biosystems Inc.), Microscope (Nikon eclipse), Sledge microtome (Richet), Cotton wool, Hand gloves, Animal cages, Animal feed, Oral cannular, Micropipette (Thermoscientific, USA), Falcon tubes, Eppendorf tubes, Spatulas, Standard volumetric flasks, Beakers, Macerating jars, Funnels and Measuring cylinders, Water bath, Column glass (Sigma-Aldrich), Nanometer (Thermo Scientific, USA), Camel brush, Glass petri dish, Porcelain boats, Micro slides and Cover slip, Crucibles, Furnace, NMR, Mass spectrometer, UV lamp, Surgical scalpel, Distilled water, Experimental animals (Wistar albino rats and albino mice), Aqueous crude extracts, Fractions (DCM and ethyl acetate) of *A. boonei* and *A. congensis* stem-bark, a cardboard box of appropriate size was constructed containing the following vital labelling data: (a) Composition: *Alstonia boonei* stem-bark (100)% powder; (b) Indication: It is to be used for diarrhoea; (c) Dosage: following ethnomedical (5 mL teaspoonful=2.82 g) and animal studies (0.5 g/ kg body weight), for Adult: 1-2 tablespoonfuls once or twice daily; Children above 5 years: 1-2 teaspoonfuls once or twice daily. If, however, there is no improvement after 24 h of administration, medication should be stopped and physician should be consulted.

3.2 Research design

Alstonia boonei De Wild. and *Alstonia congensis* Engl. in the family Apocynaceae are two closely related species with similar morphological characters which often leads to misidentification of the two plants. The research aims at developing standards for identification of the plants, ascertaining their antispasmodic activities in ordere to validate their ethnomedical uses as antidiarrhoeal remedies and formulating an antidiarrhoeal medicine from the research.

3.3 Plant materials

Samples of *A boonei* De Wild. and *A congensis* Engl. (Apocynaceae) were collected in September, 2015 behind the Department of Physiology, University of Ibadan, Ibadan, Nigeria and Itokin, along Ikorodu road, Ijebu-Ode, Ogun State, Nigeria, respectively and were authenticated by Mr. Ifeoluwa Ogunlowo, the plant curator at the Obafemi Awolowo University Department of Pharmacognosy Herbarium, Nigeria with FPI numbers 2169 and 2170, respectively. The fresh leaves of the two plants were preserved in 50% ethanol for the

anatomical analysis, while some of the leaves of the plants were air-dried at room temperature, pulverized and stored in air-tight containers for other analyses.

Leaf, stem-bark, root-bark, stem wood and root wood were preserved in fixative (50% ethanol) for anatomical analysis while the leaf and stem-bark of the plant species were air dried at room temperature, pulverised and stored in air tight container for biological assay.

3.4 Pharmacognostic procedures

The studies consisted of evaluation of macro-morphology, microscopy, organoleptic study, fluorescence analysis, physicochemical analysis, elemental analysis, phytochemical screening, *ex vivo* antispasmodic assessment of crude extracts, fractions and isolated compounds on Isolated animal tissue, *in vivo* antidiarrhoeal activity of crude extract and DCM fractions of *A. boonei* and *A. congensis* stem-bark and the formulated drug from *A. boonei* stem-bark(Diastill syrup).

3.4.1 Macroscopy

The vegetative characters of the plants visible to the eyes such as leaf apex, leaf base, leaf colour, leaf shape, margin type, venation pattern and leaf arrangement were evaluated.

3.4.2 Microscopy

3.4.2.1 Surface tissue preparation

3.4.2.1.1 Epidermal layers preparation

Epidermal studies were carried out using modified Shultze's method of maceration according to Ogundipe *et al*., (1991) and Sonibare *et al*., (2014). Median portions of the fresh leaves were boiled for 5-10 min with concentrated nitric acid in a petri dish placed on a water bath. The formation of bubbles on the leaf surfaces indicated the separation of the upper and the lower epidermises from the mesophyll. The adaxial and abaxial epidermal peels obtained were carefully removed with forceps and camel hair brush and rinsed severally in water and bleached in 20% sodium hypochlorite to remove the chlorophyll. The peels were stained in Safranin O; the stained epidermal peels were rinsed in water, dehydrated in graded series of ethanol (50%, 70%, 90% and 100%) and mounted in dilute

glycerol. Observations including presence or absence of stomatal, type of stomatal, stomatal size and measurements of epidermal structures were made with a light microscope (Olympus microscope) using calibrated micrometer eyepiece. Twenty-five measurements of each structure were randomly made from each specimen and the mean and standard error calculated.

3.4.2.1.2 Stomatal frequency determination

Stomata number per square mm of the cleared epidermal peel at x100 magnification using square graticule was counted and the average number of the stomata was recorded as stomata frequency. Twenty-five fields of view from five slides were observed.

3.4.2.2 Transverse section

The transverse sections of the leaves were obtained by free hand sectioning using a scalpel. The leaf midrib (1 cm x1 cm) was cut and embedded in pawpaw for easy sectioning. Sections were cleared in 20% w/v sodium hypochlorite for 2-5 min, rinsed severally in water, stained in Safranin O and counter-stained in Alcian blue. Each section was passed through graded ethanol (50%, 70%, 90% and 100%) for dehydration and differentiated and mounted in dilute glycerol. The distribution of tissue through the mid rib and lamina was observed under the light microscope.

3.4.2.3 Palisade ratio determination

Alstonia boonei and *A. congensis* leaf palisade ratios were determined by adopting modified method of Ahlam and Bouran (2011). Leaf median portion (3-4 cm) of each species was cut and soaked in chloral hydrate and boiled on water bath for 15-20 min. This was rinsed in water and cleared in 20% sodium hypochlorite for 20 min. It was mounted in dilute glycerol and observed under the light microscope. The number of palisade cells contained in four joined epidermal cells was counted and divided by four. This was done for twenty-five field of views.

3.4.2.4 Venation pattern

For the purpose of studying the venation patterns of the leaves, modified method of Hickey (1973) was adopted. Sizeable portions of the matured leaf of each of the species were obtained from the median portion of the leaf, chlorophyll was removed by boiling in 90% ethanol at 20°C for 10-15 min and rinsed severally in water. The portions were then transferred into 5% NaOH, boiled for 15 min, rinsed in distilled water to remove 5% NaOH and further cleared in 20% sodium hypochlorite for 20-30 min under sunlight. It was rinsed severally in water and stained in Safranin O and mounted with 25% glycerol for observation under the light microscope.

3.4.2.5 Sectioning of stem and root (bark and wood)

Using Richet sledge microtome, wood (stem and root) of *A. boonei* and *A. congensis* were sectioned at 10 µm thickness to obtain the transverse section (TS); tangential longitudinal section (TLS); and radial longitudinal section (RLS)}, while the stem and root barks were sectioned transversely (TS) and longitudinally (LS). Sections were stained in Safranin O and counter stained in Alcian blue for 3 min, rinsed in water, treated with graded solutions of ethanol (50%, 70% and 100%) for dehydration and differentiation and mounted on a glass slide with dilute glycerol for observation under the light microscope.

3.4.2.6 Maceration and staining of stem and root (bark and wood)

Bark and wood of *Alstonia* species were lacerated into pieces, drenched in Schultz's fluid (a equi-variant mixture of 10% chromic acid [1g potassium nitrate (KNO_3) in 50 mL concentrated nitric acid (HNO_3)] and 10% nitric acid), boiled on hot plate in a fume hood at 30^{o}C for 20 min and the macerates were rinsed in water (five changes) and stained with Safranin O (Akinloye *et al*., 2015).

3.5 Molecular study

3.5.1 DNA extraction method

Doyle's and Doyle's (1990) method was modified and used for DNA extraction in this study. 100 mg (w/v) of tender leaf tissue was ground thoroughly in 1 mL of Cetyltrimethylammonium bromide (CTAB) buffer and the homogenous mixture was

transferred into 1 mL Eppendorf tube, mixed and incubated at 60^{o}C for 10 min. Thereafter, 600µL of phenol: chloroform: isoamyl alcohol (25:24:1) were added to the mixture, vortexed and centrifuged at 12,000 xg for 10 min. Then, 450 µL of the supernatant was transferred into a clean Eppendorf tube, mixed (by inverting 4-5 times) with 300 µL of cold isopropanol and incubated at -20^{o}C for at least 1 h.

The sample was centrifuged at 12,000 xg for 10 min and isopropanol was carefully discarded. The pelletised DNA was washed by adding 70% ethanol and centrifuged for 2 min, this was repeated twice. After carefully discarding the ethanol, the DNA was air dried to remove the last trace of ethanol. The pellet was dissolved in 50 µL of 1xTE buffer and stored at -20^{o}C.

3.5.2 Determination of DNA quality and quantity

The quality and quantity of genomic DNA was assessed using Nanodrop UV/Vis spectrophotometry (Thermo Scientific, USA). Sample purity was assessed using the ratio determination of absorbance at 260 nm/280 nm (A260/A280). Blank absorbance was measured using 1 µL of 1XTE buffer and DNA concentration was determined by the equation (Weising *et al*., 2005):

1 Optical Density (OD260 unit) = 50 ng/ µL

Optical surface was cleaned with a sterile optical tissue and 1 µL of DNA sample was pipetted on the optical surface. Readings were taken three times.

3.5.3 Agarose gel electrophoresis

Agarose 1.5% (w/v) was mixed with 100 mL of 1X TAE (distilled water —1 L, acetic acid —1.15 mL, Tris base —40 mM, pH 8.0, and EDTA— 1 mM,), warmed, gently shaken; to this was added 0.5 µg/mL of ethidium bromide. It was thereafter poured on 60 well agarose gel tank. The DNA sample was shaken briefly and then mixed with 6x loading dye. Thereafter, it was loaded on the gel and run at 70 V for 1 h.

3.5.4 Polymerisation chain reaction (PCR)

The universal primers ITS 1 (5'- TCC GTA GGT GAA CCT GCG G-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') (Kim *et al*., 2016) were used in the amplification of the ITS region under the conditions of initial denaturation at 95ºC (5 min); 35 cycles of 95ºC denaturation (30 s); 55ºC annealing (1 min); and 72ºC extension (1 min); a final extension at 72ºC for 7 min following the conditions below; PCR amplification was done in a GeneAmp® PCR System 9700 (PE Applied Biosystems Inc.) using 50 µL reaction mixture of 2 µL 10 x PCR buffer (including 2.5 mM $MgCl_2$), 2 µL dNTP Mix (2.5 mM each), 1 µL 10 µM each primer, 2-5 µg template DNA, 0.5 µL dimethyl sulfoxide (DMSO) and 1.5 U Taq DNA polymerase, made up to 50 µL by addition of DNAse free autoclaved water. Also, 1.5% (w/v) agarose TAE gel was used in separating all PCR products prior to photography under ultra violet light (Kim *et al*., 2016).

3.5.5 Purification of amplified DNA

The amplified DNA (50 µL) was loaded with 10 µL of loading dye (6x) into 1% agarose gel of capacity 70 µL, run at 70 V for 1 h and cleaned using Thermo Scientific GeneJet Gel extraction kit. Using sterilised scapel under a protected UV lamp, the DNA fragment was removed from the agarose gel, and it was then transferred into a clean 1.5 mL micro centrifuge tube. Binding buffer (100 µL) was introduced per 100 mg of the gel, crushed (to increase the surface area for the melting of the gel) and incubated at 50ºC with random overturn of the test tube until it was totally melted. Thereafter, 800 µL of the melted gel was transferred into Genejet purification column, centrifuged at 27ºC at 10,000 rpm for 60 s and washed with 700 µL of wash buffer by centrifuging at 27ºC at 10,000 rpm for 60 s. The GeneJet purification column content was transferred into a clean micro-centrifuge tube (1.5 mL), washed twice. Using the elution buffer as control, the purified DNA contents were run on agarose gel by electrophoresis to determine the concentration using NanoDrop spectrophotometer (Adeniran *et al*., 2018)

3.5.6 Sequence alignment and data analysis

Confirmation of the DNA barcodes of the Alstonia accessions was done by downloading the *ITS* sequences of the species from GenBank for comparison. Sequencence alignment

was done using clustal W (Thomson *et al*., 1997) and unweighted pair group method with arithmetric mean (UPGMA) trees based on *ITS* constructed with MEGA 7.0 (Tamura *et al*., 2011). Bootstrap support analyses each clade of 1000 replications were done and identified gaps were assumed missing

3.6 Proximate and physicochemical analyses of *Alstonia boonei* and *Alstonia congensis* leaves and stem-barks

3.6.1 Determination of moisture content

Samples (2 g of each) were weighed into a crucible and oven-dried to a constant weight at 105^{o}C (WHO, 2011)

$$\% \text{ Moisture Content} = \frac{W_L}{W_S} \times 100$$

W_L = weight of moisture loss;

W_S = weight of powdered sample

3.6.2 Determination of ash values

3.6.2.1 Determination of total ash

Samples (2 g of each) were weighed into a dry silica crucible with constant weight, incinerated into ash in a muffle furnace at 450^{o}C (until it was ascertained to be free from carbon), cooled in a desiccator and weighed (WHO, 2011). The percentage of total ash, with reference to the dried plant sample was evaluated as follows:

$$\% \text{ Ash Content} = \frac{W_A}{W_S} \times 100;$$

W_A = weight of ash;

W_S = weight of powdered sample

The result was expressed as ≤ value.

3.6.2.2 Determination of Acid- Insoluble ash

The total ash was boiled with 25 mL of 2 N HCl for 5 min and the insoluble matter was collected in an ashless filter paper, washed with boiled water, ignited, cooled and weighed. The percentage acid- insoluble ash was calculated with reference to the total ash, using the formula below:

$$\% \text{ Acid-insoluble ash} = \frac{W_{AS}}{W_S} \times 100$$

W_{AS} = weight of residual ash;

W_S = weight of powdered sample

The acid- insoluble ash was expressed as ≤ value.

3.6.3 Determination of crude protein

Using the micro Kjeldahl method, the crude protein was determined as a function of Nitrogen (AOAC, 2004). Samples of 0.2 g each were weighed into Kjeldahl flask containing a Kjeldahl tablet ($Na_2S_2O_8/CUSO_4$) and 25 mL of conc. H_2SO_4. The flask was swirled, clamped to a retort stand and heated in a fume hood until a clear solution was obtained. The solution was cooled, transferred into a 100 cm^3 volumetric flask, made up to mark with distilled water and 10 mL of the mixture was measured into the distillation set. Also, 2 drops of methyl orange and 40% NaOH were added to the flask containing the digested sample. The addition of NaOH liberated the NH_3 from the sample and made it alkaline, changing the red colour to yellow. Thereafter, 5 mL of 2% boric acid was pipetted into a 100 mL conical flask, placed at the receiving end of the distillatory (the conical flask was placed such that the delivery tube entirely dipped into the boric acid solution). The trapped NH_3 in boric acid solution was titrated against 0.1 M HCl until the first permanent colour change was observed. A blank titration was also carried out.

$$\% \text{ N} = \frac{M_{HCl} \times 0.014 \times T \times D_f}{W_S} \times 100$$

N= Nitrogen

M_{HCl} = molarity of hydrochloric acid;

T = titre value;

D_f = Dilution factor;

W_S = weight of powdered sample

% Crude protein = % N × 6.25

3.6.4 Determination of crude fat

Sample (5 g of each) was extracted with *n*-hexane in the extraction thimble of the Soxhlet extractor. The extract was evaporated to dryness with rotar vapor. The fatty residue was then weighed.

$$\% \text{ Fat (w/w)} = \frac{M_f}{M_S} \times 100$$

M_f = mass of crude fat;

M_S =mass of powdered sample

3.6.5 Determination of crude fibre

To determine the crude fibre of the powdered samples of the selected plants, 200 mL of freshly prepared 1.25% H_2SO_4 was added to 2 g of the residue after the extraction of fat, boiled for 30 min, filtered and washed thoroughly to ensure it was free from acid. The residue was poured into a digestion flask in which 1.25% NaOH was added, and the mixture was boiled for 30 min. The mixture was filtered and residue was washed three times with methylated spirit and petroleum ether (to remove alkali), drained, transferred into a silica dish and dried to a constant weight at 105^{o}C. The organic matter of the residue was burnt in a furnace at 600^{o}C, cooled and weighed and the loss in the burning off was calculated as crude fibre (Pearson *et al*., 1981).

$$\% \text{ Crude fibre} = \frac{L_S}{W_S} \times 100$$

L_S = loss in weight of sample;

W_S = weight of powdered sample

3.6.6 Determination of carbohydrate content

Carbohydrate content was determined as spelt out by AOCS (2000), indicated below:

% Carbohydrate = 100 – (Ash + Moisture content + crude fat + crude fibre + Protein)

3.6.7 Determination of soluble extractives

3.6.7.1 Determination of water-soluble extractive values

Air-dried (coarsely powdered) plant material (4 g) was weighed in closed bottles, soaked with 100 mL of water for 18 h, and shaken frequently for 6 h. It was, rapidly filtered and 25 mL of the filterate was carefully transferred into previously dried and weighed evaporating dish. Filterate was evaporated to dryness at 100^{o}C and dried in the oven at 105^{0}C for 6 h, cooled (using a dessicator) and weighed. Percentage of water soluble extractive was calculated with reference to the powdered plant material. This was done in replicate of five, recording the value as ≥. The calculation followed formula below:

$$\% W_V = \frac{W_E}{W_S} \times 100$$

W_V = water extractive value;

W_E = weight of extract in 25 mL;

W_S = weight of powdered sample

3.6.7.2 Determination of alcohol-soluble extractive values

Air-dried (coarsely powdered) plant material (4 g) was soaked with 100 mL of distilled ethanol in closed bottles for 24 h, shaken frequently for 6 h, rapidly filtered, evaporated to dryness (using 25mL of the filterate carefully tansferred into previously dried and weighed evaporating dish) on water bath at 100^{o}C. It was further dried in the oven at 105^{o}C for 6 h, cooled in a dessicator and weighed. Percentage of alcohol soluble extractive was calculated with reference to the powdered plant material, using the following formula. This was replicated for five times (WHO, 2011):

$$\% E_V = \frac{W_E}{W_S} \times 100$$

E_V = ethanol extractive value;

W_E = weight of extract in 25 mL;

W_S = weight of powdered sample

The results were recorded in ≥ value.

3.7 Elemental analysis

Powdered plant samples (0.25 g each) were digested in 6.5 mL acid solution (HNO_3, H_2SO_4, $HClO_4$ in ratio 5:1:0.5). The suspension was heated on hot plate inside the fume hood until the appearance of white fumes. This was made up to 50 mL with distilled water and filtered with Whatman filter paper no.1. Furthermore, standard working solutions of elements of interest were prepared for standard calibration curve. Using the atomic absorption spectrophotometer (Perkin Elmer precisely, AAnalyst 700), the metallic constituents in each sample was determined, and fifteen metals (Na, Mg, K, Ca, Cd, Mn, Si, Co, Zn, Ni, Fe, Cu, Pb Cr and Hg) were determined.

3.8 Phytochemical analysis

Powdered samples of leaf and stem-bark of *Alstonia boonei* and *Alstonia congensis* were screened for several phytochemical constituents including; alkaloids, cardiac glycosides, tannins, saponins, flavonoids, steroids, anthraquinones (Evans, 2008).

3.8.1 Alkaloids

Powdered sample (1 g) was boiled with 10% HCl (1 mL) on a water bath, and the extract was cooled and filtered. The filtrate was divided into three portions I, II and III; and Dragendorff's, Mayer's and Wagner's reagents, respectively were added. The formation of orange, cream and reddish brown precipitates, respectively confirmed the presence of alkaloids.

3.8.2 Flavonoids

Powdered sample weighed at 0.5 g was boiled in 5 mL of distilled water for 5 min, filtered, cooled, and 2 mL of 10% aqueous NaOH solution was then added. The solution was observed for the presence of yellow colouration. A change in colour from yellow to colourless on addition of dilute HCl indicated the presence of flavonoids (Evans, 2008).

3.8.3 Tannins

Powdered sample (1 g) was boiled in 20 mL of distilled water for 5 min, cooled, filtered and made up to 20 mL by adding water. Thereafter, 1mL of the resultant solution was diluted

in a test tube to 5 mL with water and 2-4 drops of 0.1 % $FeCl_3$ was added. A blue black or green colouration confirms the presence of tannins.

3.8.4. Test for cardiac glycosides

Powdered sample (1 g) was extracted with 10 mL of 80% methanol for 5 min on a steam bath, extract was filtered and diluted with equal volume of distilled water and cooled. This was divided into two portions.

3.8.4.1 Keller-Killani test

To a portion of the cooled extract from 3.8.4 above, 3 mL of Ferric chloride reagent (0.3 mL of 10% ferric chloride in 50 mL glacial acetic acid) was added, and therafter, 2 mL of H_2SO_4 was cautiously poured down the side of the test tube and the resultant liquid was checked for presence of a reddish-brown ring at the interface and a green colour in the acetic layer, which confirms the presence of 2- deoxy sugars.

3.8.4.2 Kedde's test

To the other portion of the cooled extract from 3.8.4 above, 1 mL of 2% 3, 5- dintrobenzoic acid in methanol was added; the solution was made alkaline with 5% NaOH and the presence of brown purple colouration shows the presence of unsaturated lactones in the cardenolides.

3.8.5 Saponins

Powdered sample (50 mg) was boiled with 10 mL of distilled water for 10 min, filtered, filtrate was allowed to cool. Then, (5 mL) of the filtrate was measured into a test tube and diluted with 10 mL of distilled water, shaken vigorously for 2 min and observed for stable frothing formation.

3.8.6 Test for anthraquinones

Powdered sample weighing about 1 g was mixed with 5 mL of chloroform, 2 mL of ferric chloride solution and 1 mL of hydrochloric acid. The mixture was boiled for 10 min, and cooled, after which equivalent volume of benzene was added. The benzene portion was carefully separated into another test tube with the addition of 2 mL of 10% ammonia

solution. Pink colouration in the layer containing ammonia indicated to discern the presence of combined anthraquinones.

3.9 Procedure for plant extraction

Lichens and dirt were removed from the leaves and stem barks of *Alstonia boonei* and *Alstonia congensis,* respectively by washing in tap water. The leaves were detached from the stem and stem-barks were cut into pieces to hasten air-drying under shade for 7-14 days. The dried samples were ground into coarse powder and stored in air-tight container. Powdered sample (4 kg each) of *A. boonei* and *A. congensis* leaves and stem-barks were macerated in distilled water for 72 h, filtered and concentrated *in vacuo* at 50^{o}C using Rotavapor (Buchi-Germany). The extract was thereafter freeze-dried using freeze-drier (Gunman, Germany) to get a crunchy dried brownish extract, weighed and refrigerated until they were needed. The calculation of extracts percentage yield was based on the formula below:

$$\% \text{ Yield of crude extract} = \frac{W.E}{W_S} \times 100$$

W.E = weight of extract;

W_S = weight of powdered sample

3.10 Solvent-solvent partitioning of aqueous extracts

Aqueous extract (100 g each) of *Alstonia boonei* and *Alstonia congensis* leaf and stem-bark was successively partitioned into dichloromethane, ethyl acetate and the residues which were aqueous fractions.

3.11 Biological activities of extracts and fractions

3.11.1 *Ex vivo* antispasmodic assay

3.11.1.1 Experimental animals for antispasmodic assay

Wistar albino rats (150-200 g) of either sex were used for this study. They were housed, fed with standard diet and water *ad libitum*, and kept at 23-25^{o}C at the Animal House of the

COMSATS University of Technology (CIIT), Pakistan. The animals were fasted 18 h prior to experiment with water *ad libitum.*

3.11.1.2 Procedure for antispasmodic assay

Experimental animals were sacrificed by cervical dislocation, and antispasmodic activity of the extracts was studied on isolated ileum of matured albino Wistar rats as described by Gilani *et al.* (2007). The segmented ileum of 2 cm length was suspended in a 10 mL tissue bath filled with Tyrode's solution. The solution (composed of $NaHCO_3$ 11.90, $MgCl_2$ 1.05, KCl 2.68, NaCl 136.9, $CaCl_2$ 1.8, glucose 5.55 and NaH_2PO_4 0.42, all in mM) was bubbled with carbogen gas at 37^oC. A constant resting tension of 1 g was applied to the tissues (ileum) throughout the experiment. Isometric contractions were recorded using force displacement transducers connected to a Power Lab Data Acquisition System (AD Instruments, Sydney, Australia) attached to a computer installed with labchart software (version 6). Tissues were equilibrated for a minimum of 30 min, stabilised with sub-maximal concentration of acetylcholine (0.3 μM) which was washed off immediately and replaced with Tyrode solution before the start of the experiment. The spontaneous rhythmic contractions exhibited by the rat ileum under the above experimental condition gave room for the testing of the antispasmodic activity directly without the use of any agonist. Dosing of test drugs was done cumulatively by serial dilution of 300-3 $mgmL^{-1}$ at 0.01- 10 $mgmL^{-1}$, with each succeeding test delayed for 3-5 min interval. High potassium ion concentration (high $[K^+]$) (80 mM) was applied to depolarise the preparations (as described by Farre *et al*., 1991); this was to assess whether the antispasmodic effect of the extracts was mediated through calcium channel blockade (CCB). The addition of high $[K^+]$ to the tissue bath resulted in a sustained contraction. Relaxation of ileum pre-contracted with $[K^+]$ by the extract was expressed as percent of the control response mediated by high $[K^+]$.

3.11.2 *In vivo* antidiarrhoeal assay

3.11.2.1 Experimental animals for antidiarrhoeal assay

Healthy Swiss albino mice (both male and female) weighing between 25-30 g were procured from Central Animal House, University of Ibadan, Ibadan, and acclimatised for two weeks in the Department of Pharmacognosy Animal House, University of Ibadan with access to

food (Ladokun feed) and water *ad libitum*. The animals' management and treatments conformed to the University of Ibadan Animal Care and Use Research Ethics guidelines (UI-ACUREC/19/0135).

3.11.2.2 Antidiarrhoeal activity of aqueous crude extracts and fractions of *Alstonia boonei* and *Alstonia congensis* stem-barks on albino mice

Using the method reported by Nsaka *et al*. (2012), sixty animals were randomised into twelve Groups (A-L) of five animals each; Group A (diarrhoeal control) received distilled water in a drop of Tween 80 (0.5 $mLkg^{-1}$ *b.w*), Group B received Loperamide (5 $mgkg^{-1}$ *b.w*). *A. boonei* stem-bark aqueous crude extract was administered to animals in Groups C and D at 100 $mgkg^{-1}$ and 200 $mgkg^{-1}$, respectively, while Groups E and F respectively received 100 $mgkg^{-1}$ and 200 $mgkg^{-1}$ stem-bark aqueous crude extract of *A. congensis*.

Groups G, H and I received 50, 100 and 200 $mgkg^{-1}$ *b.w* of dichloromethane fraction of *A. boonei* stem-bark, respectively while Groups J, K and L respectively received 50 $mgkg^{-1}$, 100 $mgkg^{-1}$ and 200 $mgkg^{-1}$ of DCM fraction of *A. congensis* stem-bark. All administrations were done orally. Thirty minutes after the respective treatment, oral administration of 0.5 mL castor oil was administered to each animal for inducing diarrhoea. Each animal was placed in an individual cage (the floor of which was laid with plain sheet of paper) and were observed for 4 h to record the following: time elapsed between administration of the cathartic agent and the excretion of the first diarrhoeic faeces (wet faeces that sample a halo on the filter paper), total number of faecal output; and total number of diarrhoeic faeces excreted. The percentage diarrhoea drop inhibition was calculated using the formula below:

$$\% ID = \frac{D_C - D_S}{D_C} \times 100$$

Where,

ID = inhibition of diarrhoea

D_C = mean number of drops caused by castor oil;

D_S = mean number of drops caused by the test sample.

3.11.2.3 Preparation of mixtures *Alstonia boonei* stem-bark and *Picralima nitida* seed *decoction* as antidiarrhoeal herbal standard

Stem-bark of *A. boonei* (AB) and seed of *Picralima nitida* (PN) were bought from Bode Market, Molete, Ibadan and identified at the University of Ibadan Herbarium (UIH), Botany Department by Mr. Donaltus. Extraction was done by aqueous decoction of three different ratios of the plant mixtures and filtered. Filtrate was concentrated *in vacuo*, freeze dried and stored in air tight containers. The various ratios included:

AP1- **AB + PN (1+1)**; ratio **1:1**

AP2- **AB + PN (1:2)**,

AP3- **AB + PN (2:1).**

Where,

AB = *Alstonia boonei* stem-bark

PN = *Picralima nitida* seeds

A mixture of *A. boonei* stem-barks and *P. nitida* seeds was formulated into an antidiarrhoeal remedy (DIASTOP) by the Village Chemist,Obafemi Awolowo University (OAU), Ile-Ife, prior to the study.

3.11.2.4 Formulation of Diastill Syrup (antidiarrhoeal drug)

A decoction in distilled water was prepared by boiling 15 g of powdered *Alstonia boonei* stem-bark for 1 h, filtered plus 30 mL Simple Syrup B.P was added to the filterate plus sufficient amount of preservative and the mixture was made to 300 mL with distilled water (5% w/v). The finished product was dispensed into 5 bottles of 60 mL each. And labelled as DIASTIL Syrup following after the production of process DIASTOP by the Village Chemist, Obafemi Awolowo University (OAU), Ile-Ife

3.11.2.5 Antidiarrhoeal activity of mixtures of *Alstonia boonei* stem-bark and *Picralima nitida* seed decoction as herbal reference standard on albino mice

The method explained in section 3.11.2.2 was adopted for this experiment. Seventy healthy albino were randomised into fourteen Groups (A-N). Animals in Group A (diarrhoeal control) received distilled water in a drop of Tween 80 (0.5 $mLkg^{-1}$ *b.w*), Group B received reference drug Loperamide (5 $mgkg^{-1}$ *b.w*). Groups C, D, E and F received 100, 200, 400

and 500 mgkg^{-1} *b.w* of AP1 decoction, respectively. Groups G, H, I and J received 100, 200, 400 and 500 mgkg^{-1} *b.w* of AP2 decoction, respectively. Groups K, L, M and N respectively received 100, 200, 400 and 500 mgkg^{-1} *b.w* of AP3 decoction. All administrations were done orally and 0.5 mL castor oil was administered (30 min post-treatment) to the animals to induce diarrhoea. The animals were singly accommodated in cages under-laid with plain sheet of papers then housed. All animals were observed for 4 h to determine the same parameter assessed in 3.11.2.2 above. The percentage diarrhoea drops inhibition was calculated using the formulae below:

$$\% \text{ ID} = \frac{D_C - D_S}{D_C} \times 100$$

Where,

ID = inhibition of diarrhoea

D_C = mean number of drops caused by castor oil;

D_S = mean number of drops caused by the test sample.

3.11.3 Statistical analysis

All results were expressed as mean ± SEM. Data from antispasmodic experiments were analysed by non-linear regression. Data from antidiarrhoeal experiments were evaluated by one-way analysis of variance (ANOVA) and means were compared using Tukey's Multiple Comparison posthoc as a test at a statistically significant of $\alpha_{0.05}$. Graph pad prism program 5 (GraphPad, San Diego, CA) was used for the analysis.

3.12 Isolation of compounds from dichloromethane fraction of *Alstonia boonei* stem-bark

3.12.1 Column chromatography

One hundred and fifty grams (150 g) of silica gel (70-230 mesh sizes) was made into slurry with 100% *n*-hexane and packed into a 40 mm × 600 mm glass column. It was eluted with 100% *n*-hexane till it was compactly packed. Five grams (5.0 g) of the dichloromethane (DCM) extract pre-adsorbed on silica gel (5 g) was loaded into the column. The loaded sample was eluted with gradient elution method starting with *n*-hexane (100%), *n*-

hexane:DCM (90:10), DCM:ethyl acetate (90:10), and ethyl acetate:methanol (90:10). Absolute methanol polarity was achieved, fractions were collected in 100 mL volume beakers, concentrated with rotary evaporator and transferred into small vials, which resulted into 150 fractions and pooled with the aid of TLC to 10 fractions (I-X). Fractions II and V were further purified with column chromatography using DCM: ethyl acetate (80:20) in increasing polarity until absolute ethyl acetate polarity was achieved for fraction II and *n*-hexane: ethyl acetate (80:20) for fraction V. Fractions II and V afforded us compounds A and B, respectively.

3.12.2 Thin Layer Chromatography

Using thin layer chromatography (TLC) with UV absorbance at 254 nm and 365 nm, fractions derived from Column chromatography were monitored, and similar fractions were pooled together on the basis of TLC similarity. The TLC was used to optimise solvent system for running purification of the column for isolation of pure compounds.

3.12.3 Determination of antispasmodic effect of isolated compounds from DCM fraction of *Alstonia boonei*

The procedure for the antispasmodic assay of crude extracts and fractions of the selected plants as presented in section 3.11.1.2 above was followed for the determination of the antispasmodic effects of *A. boonei* DCM fraction-isolated compounds on spontaneous and high [K^+] induced contraction of isolated rat ileum. However, the procedure differs slightly from the former in terms of dosage. For the determination of the antispasmodic effects of isolated compounds, dosing of test drugs was done in cumulative manner by serial dilution of 300-3 µg/mL at 0.01- 10 µg/ mL at 3-5 min interval.

3.12.4 Statistical analysis of antispasmodic effect of isolated compounds

All results were expressed as mean ± SEM and in isolated tissue experiments, data were analysed by non-linear regression. Differences were considered statistically significant when the probability value $P < 0.05$. Graph pad prism program 5 (GraphPad, San Diego, CA) was used for the analysis.

3.12.5 Identification and structure elucidation of isolated compounds by spectroscopic techniques

The ^{1}H NMR, ^{13}C NMR using Bruker Ascend 400 MHZ spectrometer (Bruker Instruments Incorporation, Billerica, MA USA), Mass spectroscopy (EI/ mz) and other 2D-NMR spectroscopic techniques including COSY, NOESY, DEPT-90, DEPT-135, HSQC, HMBC, were applied to identify and elucidate the structures of the pure isolated compounds from the dichloromethane fraction of *A. boonei* stem-bark. Compounds were dissolved in deuterated pyridine (C_5D_5N). Chemical shifts (∂) were recorded in parts per million (ppm); coupling constants (*J*) were recorded in Hz. and trimethylsilane (TMS) was used as internal standard. Carbons were distinguished and classified as methene, methylene or methyl (CH), (CH2), (CH3) using distortionless enhancement by polarization transfer (DEPT). Also, heteronuclear single quantum coherence (HSQC) was used to directly correlate- proton and carbon nuclei through one bond, hetero multinuclear bond correlation (HMBC) was used to obtain long range correlations of proton and carbon nuclei through two, three or four bonds and mass spectroscopy (EI-MS) was utilised to substantiate the molecular weight of the compounds.

CHAPTER FOUR

4.0 RESULTS

4.1 Macroscopic and organoleptic evaluation of *Alstonia boonei* and *Alstonia congensis*

The morphological characters of the tissues and powder of *A. boonei* and *A. congensis* are summarised in this section. Figures 4.1.1 and 4.1.2 reveal diagnostic macroscopic features of the selected plants for this study. The leaves of *A. boonei* and *A. congensis* are both green in colour but the shade of *A. boonei* greenish colour is darker than that of *A. congensis*. The upper surface leaves of both species green colour are darker and glossy than the lower surface. The leaf apex of *A. boonei* assumes acute to emarginate shape, while the apex of *A. congensis* leaf assumes short acuminate to acute shape; the leaves of the two samples are in whorl form. The leaves of both species are coriaceous in texture and glabrous. Midrib is more prominent on the lower surfaces of both species. Main lateral nerves are 40-60 in *A. boonei*, and 40-50 in *A. congensis*, closed, parallel and nearly horizontal, joining an intramarginal one, nervation is inconspicuous.

Figure 4.1.3 shows the macroscopic features of the stem-barks of *A. boonei* and *A. congensis*. Stem-barks of both species are slightly curve; fracture is short, greyish brown, rough on the outer surface and creamy to yellowish colour on the inner surface when wet and brownish in colour in dry state. Figure 4.1.4 shows the diagnostic characters of the organoleptic evaluation of stem-bark powdered samples of *A. boonei* and *A. congensis* while Table 4.1.1 gives the morphological description of *A. boonei* and *A. congensis*. Tables 4.1.2

and 4.1.3 give the morphological description of *A. boonei* and *A. congensis* leaf and stem-bark powdered samples. The stem-bark of *A. boonei* has carton yellow colouration, while *A. congensis* is light yellow, having bushy odour, coarse texture and bitter taste.

Figure 4.1.1: Macroscopic features of *Alstonia boonei* leaves

A. boonei morphological characters which include **a:** Upper surface, **b:** Entire margin, **c:** Whorled arrangement of leaves, **d:** Emarginate apex, **e:** Fruit, **f:** Prominent mid-rib, **g:** Lower surface

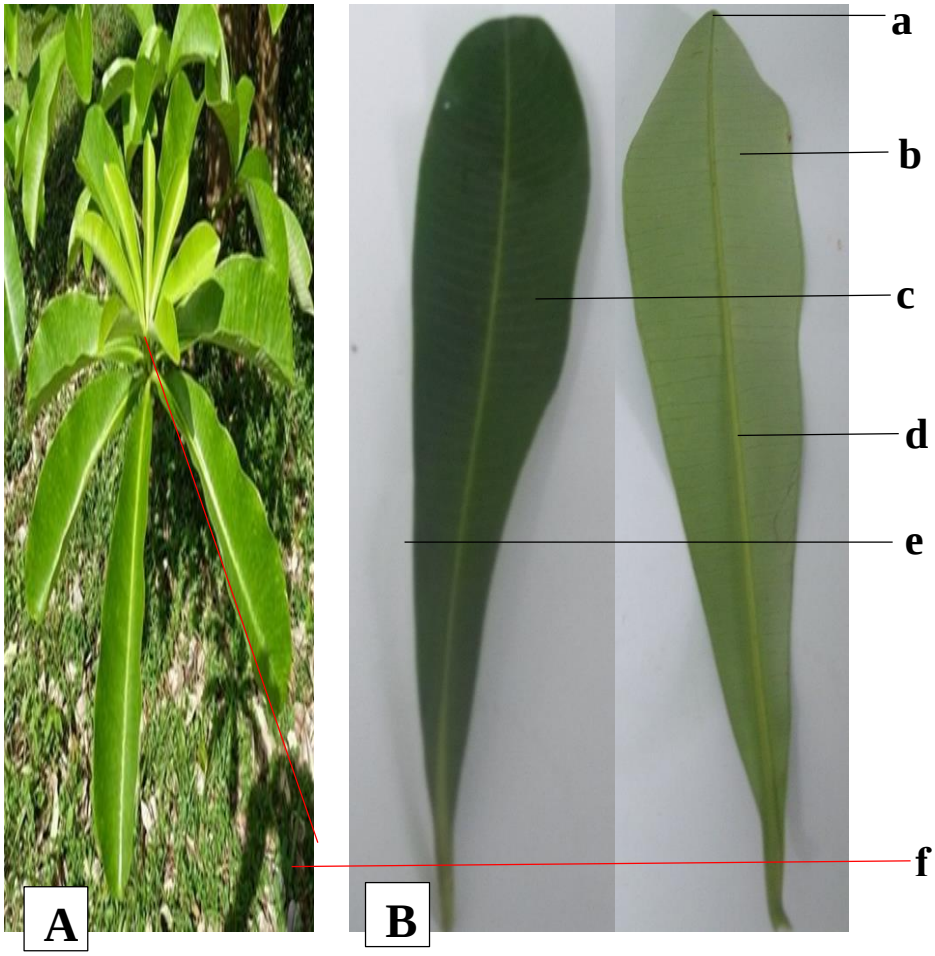


Figure 4.1.2: Macroscopic features of *Alstonia congensis* leaf

A and B show the morphological features of *A. congensis* leaf which include **a:** acuminate apex, **b:** Lower surface, **c:** Upper surface, **d:** Prominent mid rib, **e:** Entire margin, **f:** Whorled arrangement of leaves

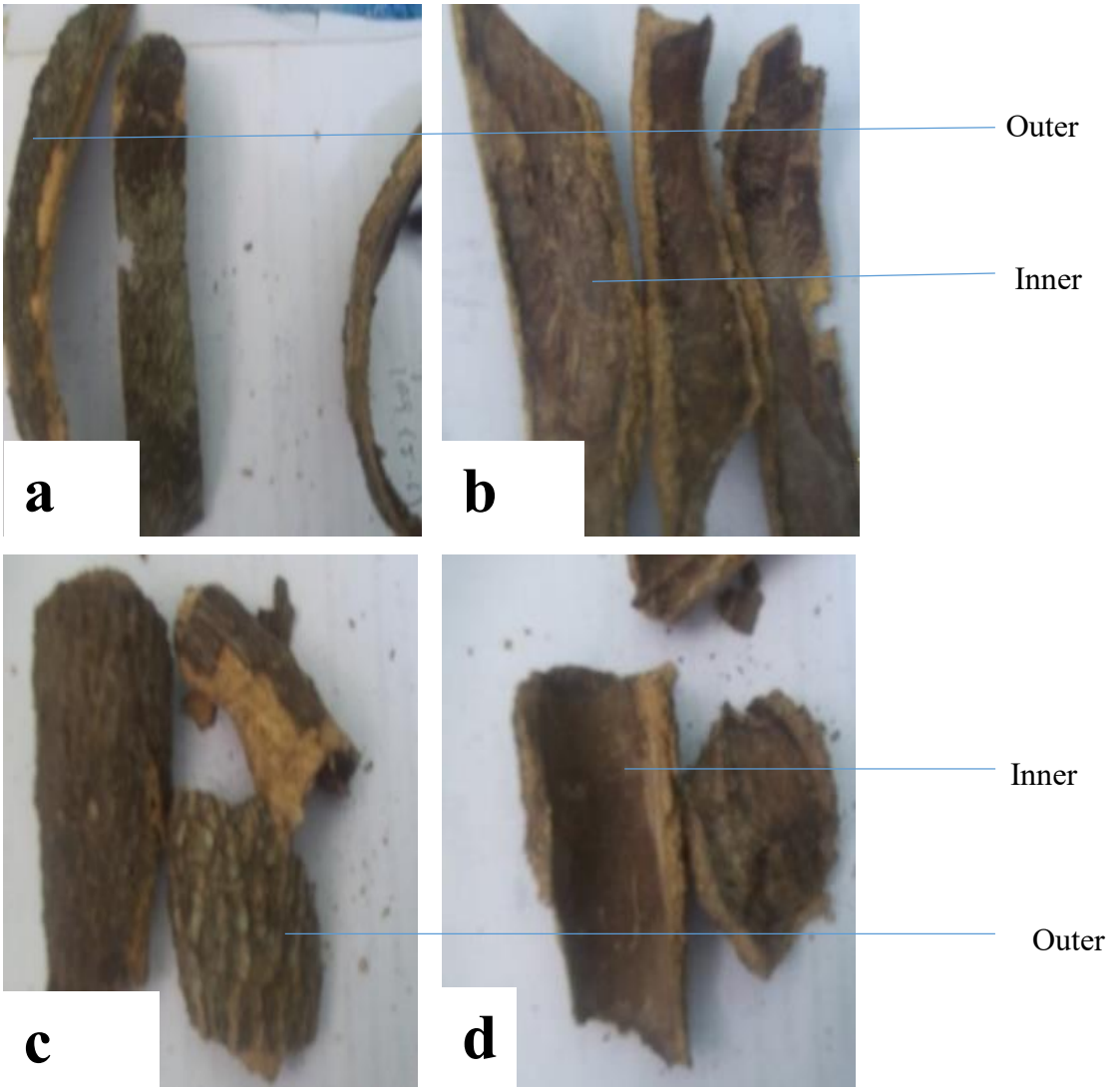


Figure 4.1.3: Macroscopic features of *Alstonia boonei* and *Alstonia congensis* stem-bark. a and b show the outer and inner surface and curvature type of *A. boonei* dried stem-bark while c and d show the outer and inner surface and curvature type of dried stem-bark of *A. congensis*, respectively; both have greyish brown colouration

Figure 4.1.3: Macroscopic features of *Alstonia boonei* and *Alstonia congensis* stem-bark powders.

a (*A. boonei*) has carton yellow colouration while b (*A. congensis*) has light yellow colouration

Table 4.1.1: Morphological features of *Alstonia boonei* and *Alstonia congensis*

Morphological part	***A. boonei***	***A. congensis***
Habit	Tree	Tree
Leaf arrangement	Whorl 5-8	Whorl 4-6/8
Blade	Oblanceolate –obovate	Obovate
Apex	Acute-emarginate	Shortly acuminate
Base	Cuneate	Cuneate
Margin	Entire	Entire
Colour	Dark green (upper surface) light green (lower surface)	Dark green (upper surface) light green (lower surface)
Texture	Coriaceous/pubescent	Coriaceous/pubescent
Venation pattern	Pinnate	Pinnate
Leaf surface	Glabrous	Glabrous
Midrib	Pronounced at the back	Pronounced at the back
Flower colour	Cream yellow	Creamy
Fruit colour	Greenish	Greenish
Stem bark colour	Greyish brown	Greyish brown
Fracture type	Short	Short
Curve type	Slightly curved	Slightly curved

Table 4.1.2: Morphological features of *Alstonia boonei* and *Alstonia congensis* leaf powder

Features	*A. boonei*	*A. congensis*
Colour	Brownish green	Brownish green
Taste	Bitter	Bitter
Texture	Fine	Fine
Odour	Bushy	Bushy

Table 4.1.3: Morphological features of *A Alstonia boonei* and *Alstonia congensis* stem-bark powder

Features	*A. boonei*	*A. congensis*
Colour	Yellow	Light yellow
Taste	Bitter	Bitter
Texture	Coarse	Coarse
Odour	Bushy	Bushy

4.2 Microscopic evaluation of *Alstonia boonei* and *Alstonia congensis*

4.2.1 Qualitative and quantitative description of epidermises

Figure 4.2.1.1 shows the diagnostic characters of adaxial surface of *A. boonei* and *A. congensis*. The adaxial epidermises of *A. boonei* and *A. congensis* have coastal cells, which are square-like to polygonal shape; thick and straight anticlinal wall with epidermal cells polygonal in shape, trichomes and stomata are absent (figure 4.2.1.1). Starch grains spherical in shape were contained in the adaxial epidermal cells of *A. boonei* but not observed in the adaxial epidermal cells of *A. congensis* (figure 4.2.1.1). Figure 4.2.1.2 shows the diagnostic characters of abaxial surface of *A. boonei* and *A. congensis.* The abaxial epidermises of both *A. boonei* and *A. congensis* are characterised with coastal cells, thin, polygonal epidermal cells with straight anticlinal wall, which are smaller than their adaxial counter-parts; trichomes are absent on the abaxial surfaces of the two species and stomata are present on the lower surfaces of the species (figure 4.2.1.2). The stomata type found in the two species varied from cyclocytic, paracytic to anomocytic (figure 4.2.1.2 c and f). The quantitative microscopy of the leaves showed the epidermal cell lengths for *A. boonei* and *A. congensis* as 38.5 ± 0.56 and 35.5 ±1.8 (Table 4.2.1.1). Stomata numbers as 5.0 ± 1.2 and 1.8 ± 0.3, while palisade ratios were 5.2 ± 0.4 and 3.7 ± 0.2, respectively (Table 4.2.1.3).

The summary of micromorphological features of their Adaxial epidermal leaf surface and their dimensions is presented in Table 4.2.1.1. Table 4.2.1.2 reveals the microscopic features of their abaxial epidermal leaf surfaces, while Table 4.2.1.3 shows the micromorphological features of stomata, palisade cells, vein-islets and vein-termination number of *Alstonia boonei* and *Alstonia congensis*.

4.2.2 Anatomical description of transverse sections of *Alstonia boonei* and *Alstonia congensis* leaves

Mid-ribs

The leaf midribs of the two species revealed bicollateral vascular bundles, angular collenchyma cells, sclerenchyma sheath, thick cuticles, parenchyma ground tissues, absence of trichomes, prismatic calcium oxalate. Layers of angular collenchyma were observed on the upper and lower layers of both species; both contain a rectangular single layered epidermis. Prismatic calcium oxalates were observed in the ground tissue of the two species

as shown in Figures 4.2.2.1 and 4.2.2.2. The mid-rib of *A. boonei* is plano-convex in shape with almost flat adaxial layer and hemispherical abaxial layer (Figure 4.2.2.1a), while *A. congensis* mid-rib has short adaxial hump and slightly indented abaxial layer (Figure 4.2.2.2 a). Secretory canal in the ground tissue is contained in *A. boonei* but not observed in *A. congensis* (Figures 4.2.2.1b and 4.2.2.2 b). The vascular bundle in *A. boone*i was arc-shaped with xylem vessels standing in isolation, while the phloem occurs in clusters on the abaxial and adaxial portions of the xylem (Figure 4.2.2.1c) while in *A. congensi*s, a U-shaped vascular bundle was observed with xylem vessel interlocking to form a crown like connection and surrounded with phloem on both upper and lower portion of the xylem band (Figure 4.2.2.2 b and c). The sclerenchyma sheath forms a broken edge around the vascular bundle of *A. boonei*, while in *A. congensis,* the sclerenchymatous bundle sheath form an unbroken edge around the vascular bundle (Figures 4.2.2.1b and 4.2..2 b). The summary of the microscopic characters of the mid-rib is presented in Table 4.2.2A and the diagnostic features of the lamina are presented in Table 4.2.2.2.

Lamina

Leaves of *A. boonei* and *A. congensis* are dorsiventral. Epidermis was single layered in both species, while hypodermis is double layered in *A. congensis* and single in *A. boonei* (Figure 4.2.2.3). The lamina of both *A. boonei* and *A. congensis* showed 1-2 layered cylindrical-shape, and compactly packed palisade parenchyma cells. The spongy parenchyma of *A. boonei* is formed of arranged parenchyma cells, which are loosely packed, long and bone-like and/finger-like in shape with little or no air space (Figure 4.2.2.3 a, c. e). The spongy parenchyma of *A. congensis* is formed of loosely arranged parenchyma cells (with large intercellular spaces), which are short bone-like and or finger-like in shape (Figure 4.2.2.3 b, d, f). Both species have lateral veins (formed of phloem and xylem vessels) lying in the region between the palisade and spongy parenchyma, and lined with prismatic calcium oxalate (Figure 4.2.2.3 a, c, e and b, d, f)

4.2.3 Anatomical description of palisade cells and venation pattern of *Alstonia boonei* and *Alstonia congensis*

Figure 4.2.3A shows the palisade cells of *A. boonei* and *A. congensis* leaves, the palisade ratio in *A. boonei* (5.2±.2.0) is higher than that of *A. congensis* (3.7±0.4) as reported in Table

4.2.1.3. The venation pattern of *A. boonei* and *A. congensis* leaves conformed to pinnate camptodromous type with festooned brochidodromous secondaries. The secondary veins are produced on both sides of the primary vein alternately which further divide to tertiary veins. The vein terminals are dendritic in nature (Figure 4.2.3B). The striking difference between the venation patterns of these morphologically-similar species is found in their vein islet number, the vein islet number of *A. boonei* (9.0±0.7) is slightly more than that of *A. congensis* (8.8±1.8) (Table 4.2.1.3).

4.2.4 Anatomical description of wood of *Alstonia boonei* and *Alstonia congensis* stem and root samples

Figures 4.2.4.1 - 4.2.4.6 show the diagnostic characters of *A. boonei* and *A. congensis* stem wood, respectively. While the in transverse, longitudinal and radial longitudinal sections of *A. boonei* are respectively shown in figures 4.2.4.1-4.2.4.3,while those of *A. congensis* are shown in figures 4.2.4.4 - 4.2.4.6 respectively, all showing single and multiple vessels with simple perforated plate and non septate pitted fibre. The root wood anatomical features of *A. boonei* and *A. congensis* are represented in figures 4.2.4.7 -4.2.4.12, the diagnostic characters of *A. boonei* and *A. congensis* root wood in transverse, longitudinal and radial longitudinal sections, revealing the type of vessels, rays, type of parenchyma.. Table 4.2.4 presents the anatomy features of the stem and root wood of *A. boonei* and *A. congensis*.

4.2.5 Anatomical description of bark samples of *Alstonia boonei* and *Alstonia congensis* stem and root

The bark samples of *A. boonei* and *A. congensis* stem and root contain stone cells, cork, phloem and prismatic calcium oxalate (Figures 4.2.5.1, 4.2.5.2, 4.2.5.3, 4.2.5.4). While *A. boonei* stem and root barks contain both brachysclereid and osteosclereid stone cells, *A. congensis* have brachysclereid type.

4.2.6 Anatomical description of wood macerates of *Alstonia boonei* and *Alstonia congensis* stem and root samples

The wood macerates of *A. boonei* and *A. congensi*s show the diagnostic features of their stem and root (Figures 4.2.6.1 and 4.2.6.2). The wood macerates of the two species reveal

the presence of fibres, simple vessels, tracheid. The macerates of *A. boonei* stem-wood, has fusiform fibre, but it was not observed in *A. congensis*. The vessels of both *A. boonei* and *A. congensis* roots are tailed and simple (figure 4.2.6.2). Table 4.2.6 presents the micrometric description of the stem and root wood macerates of *A. boonei* and *A. congensis*

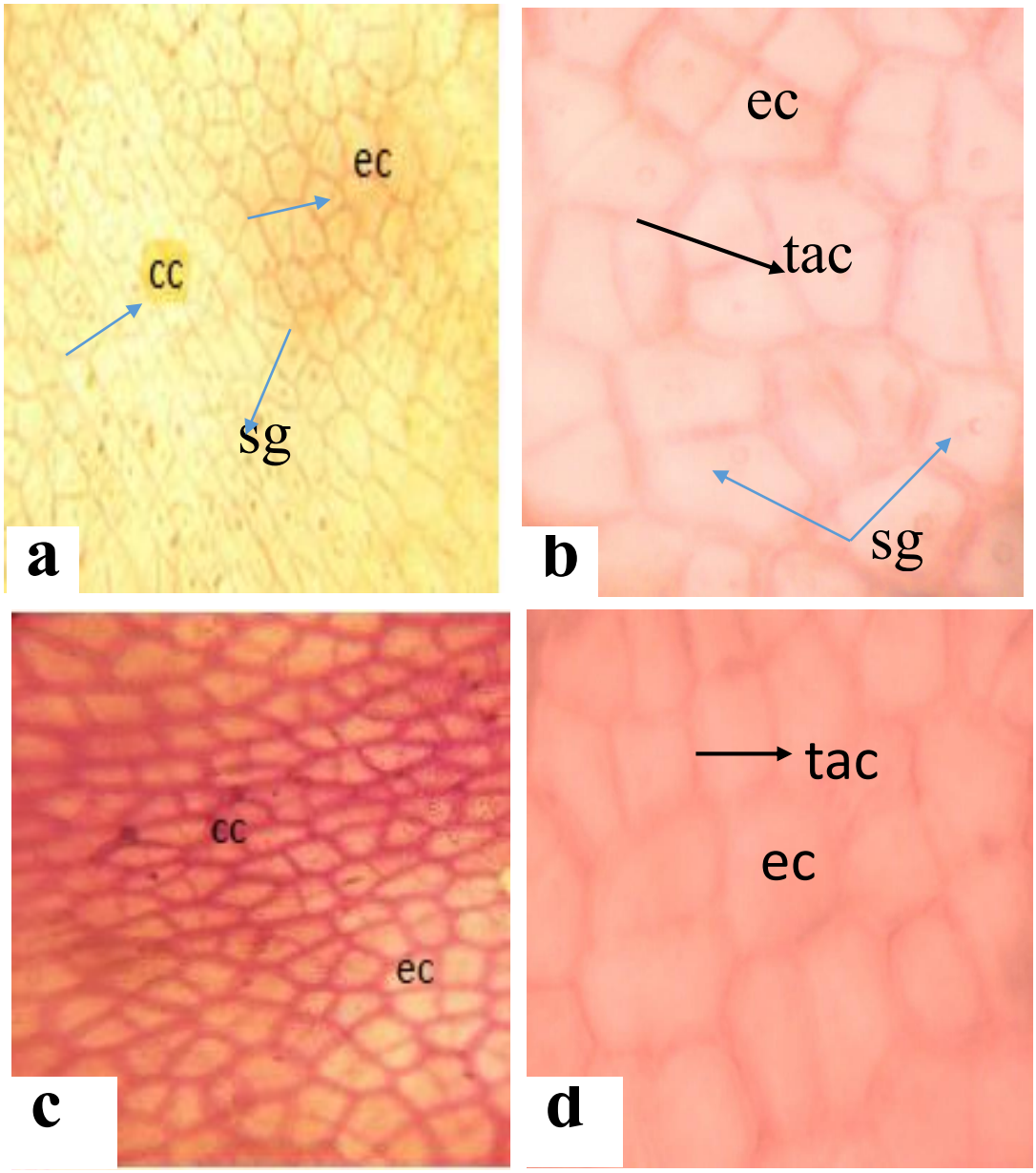


Figure 4.2.1.1: Epidermal peel of *Alstonia boonei* and *Alstonia congensis* adaxial surface: (a and b) adaxial leaf surface of *A. boonei* x100 and x400; (c and d) adaxial leaf surface of *A. congensis* x100 and x400
cc: coastal cells, ec:epidermal cells, sg: starch grains, tac: thick anticlinal wall.

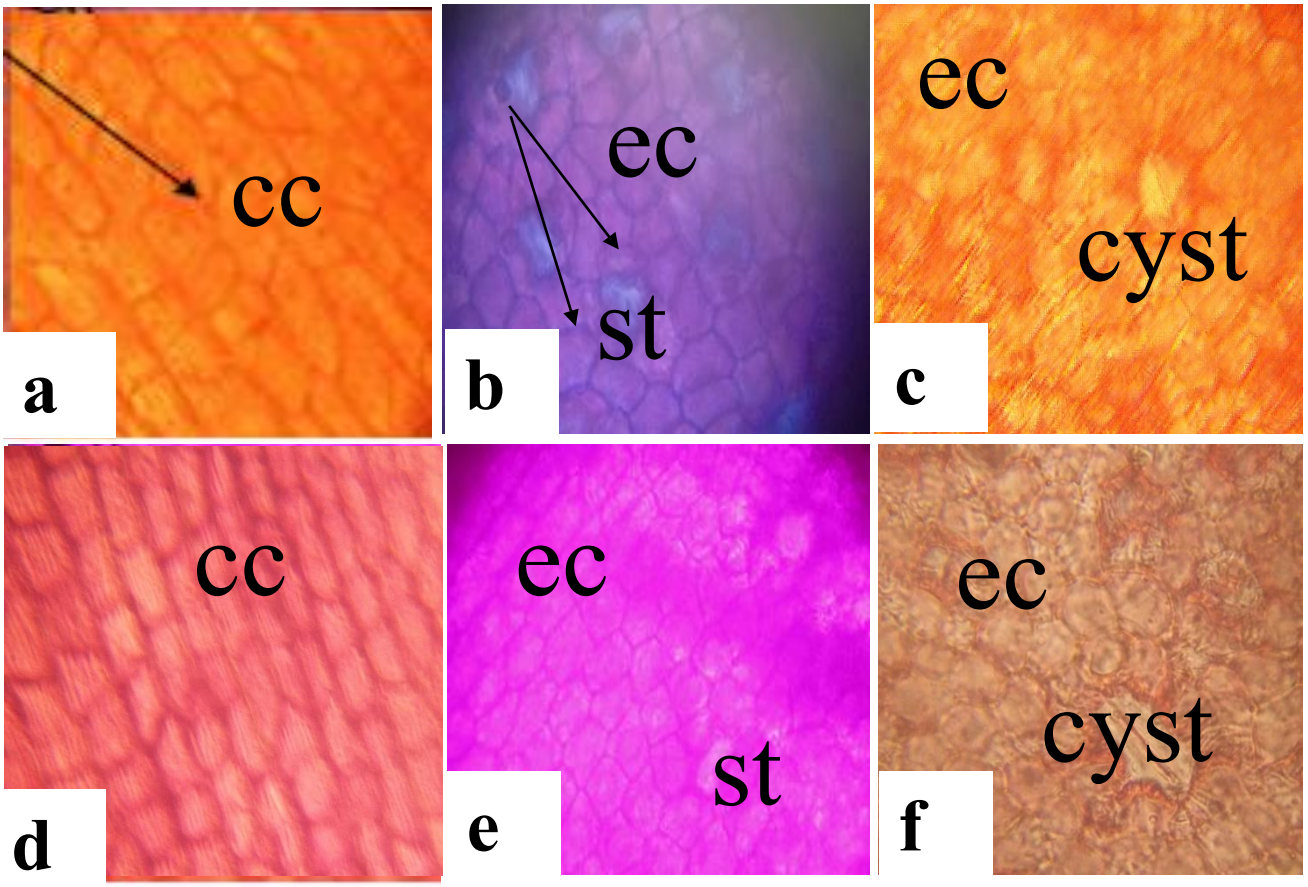


Figure 4.2.1.2: : Epidermal peel of *Alstonia boonei* and *Alstonia congensis* abaxial surface: (a,b and c) abaxial epidermal peel of *A. boonei* x100 x400; (d, e and f) abaxial epidermal peel of *A. congensis* x100 and x400

cc: coastal cells, ec:epidermal cells, cyst : cyclocytic stomata, st: stomata

Table 4.2.1.1: Micromorphological features of *Alstonia boonei* and *Alstonia congensis* adaxial epidermal cells

Taxa	Epidermal cell shape	Coastal cells	Starch grains	Stomata	Epidermal cell length (µm)	Epidermal cell width (µm)
A. boonei	Polygonal with straight and thick anticlinal wall	Present	Present	Absent	38.5±1.8	25.3±1.6
A.congensis	Polygonal with straight and thick anticlinal wall	Present	Not observed	Absent	35.2±0.9	32.0±3.8

Table 4.2.1.2: Microscopic features of *Alstonia boonei* and *Alstonia congensis* abaxial epidermal cells

Taxa	Epidermal cell description	Stomata type
A. boonei	Small and straight anticlinal wall, coastal cells present, waxy and papillose, coastal cells present, stomata present	Anomocytic, cyclotic
A. congensis	Waxy, papillose, small with straight anticlinal wall, coastal cells present, stomata present	Anomocytic, paracytic

Table 4.2.1.3: Micromorphological dimensions of stomata, palisade ratio, vein-islet and vein termination number of *Alstonia boonei* and *Alstonia congensis* leaves

Parameters	*Alstonia boonei*	*Alstonia congensis*
Stomata frequency	5.0±1.2	1.8±0.3
Stomata length (µm)	24.5±0.5	35.0±.8
Stomata width (µm)	22.0±1.2	28.0±3.0
Palisade ratio	5.2±.0.4	3.7±.2.0
Vein-islet $(mm)^2$	9.0±0.7	8.8±1.8
Vein-termination number	6.2±0.7	6.2±1.3

Data is presented in Mean ±S.E.M (n=25)

Table 4.2.2.1: Microscopic characters of *Alstonia boonei* and *Alstonia congensis* mid rib

Taxa	vascular bundle shape	Sclerenchyma sheath	Calcium oxalate	Shape of mid-rib	Vascular bundle	Secretory canal	Cuticle form
A. boonei	Arc shaped vascular bundle with xylem standing singly	Broken edge	Present in lamina	Plano-convex, slightly hump adaxial layer, hemispherical abaxial layer	Bicollateral	Present	Thick
A. congensis	U shaped with xylem vessels interlocking forming a crown-like shape	Unbroken edge	Present in lamina & lower epidermal collenchyma cells	Plano-convex, a protuberance adaxial layer and indented abaxial layer	Bicollateral	Not observed	Thick

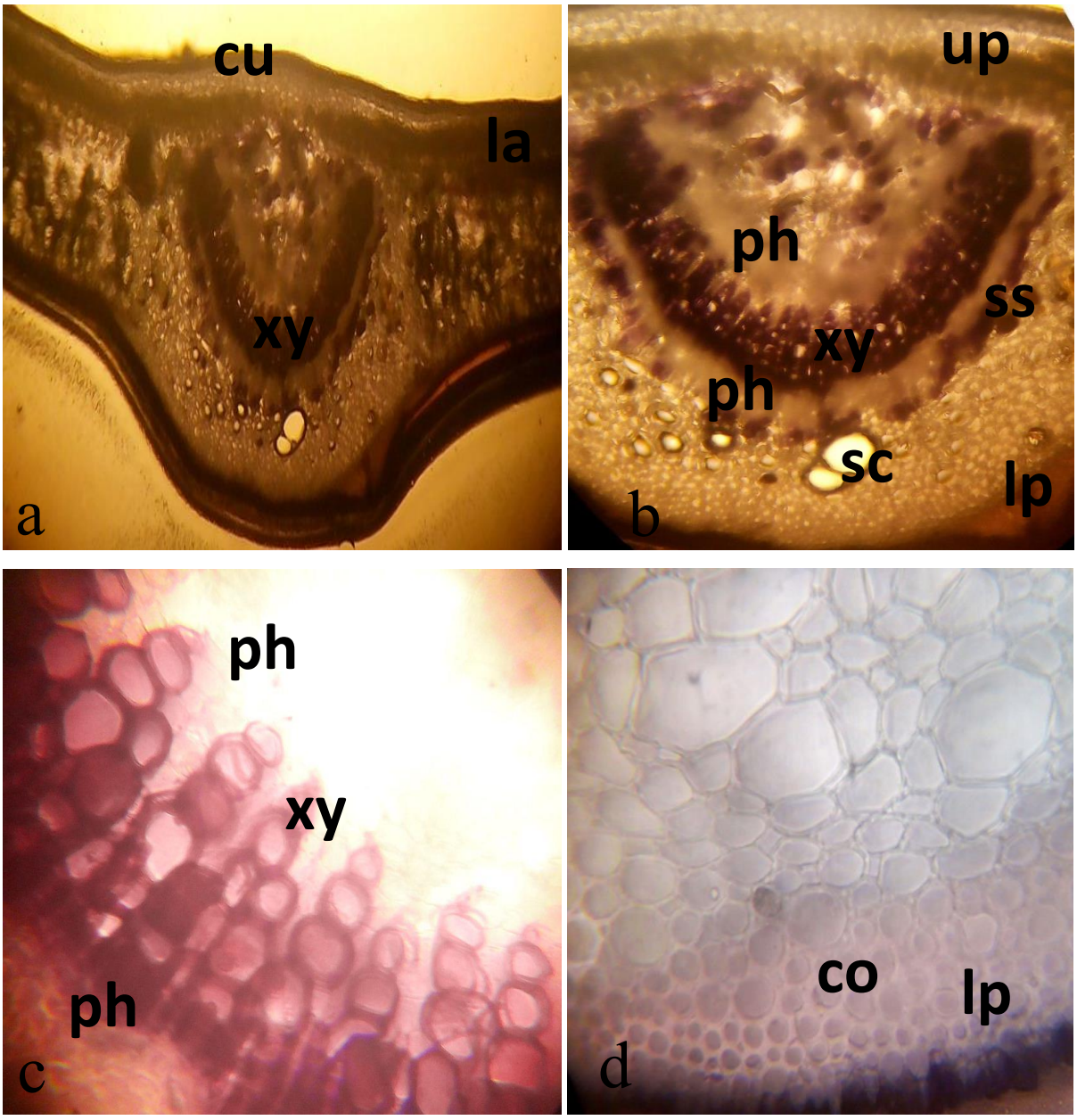


Figure 4.2.2.1: Transverse Section (TS) of *Alstonia boonei* leaf mid rib

(a and b) x40 and x100, showing the mid-rib, (c & d) x400 showing the enlarged cell inclusions of mid rib; cu: cuticle, co: collenchyma cell, la: lamina, lp: lower epidermis,ph: phloem, sc: secretory cell, ss: sclerenchymatous sheath, up:upper palisade, xy: xylem tissue

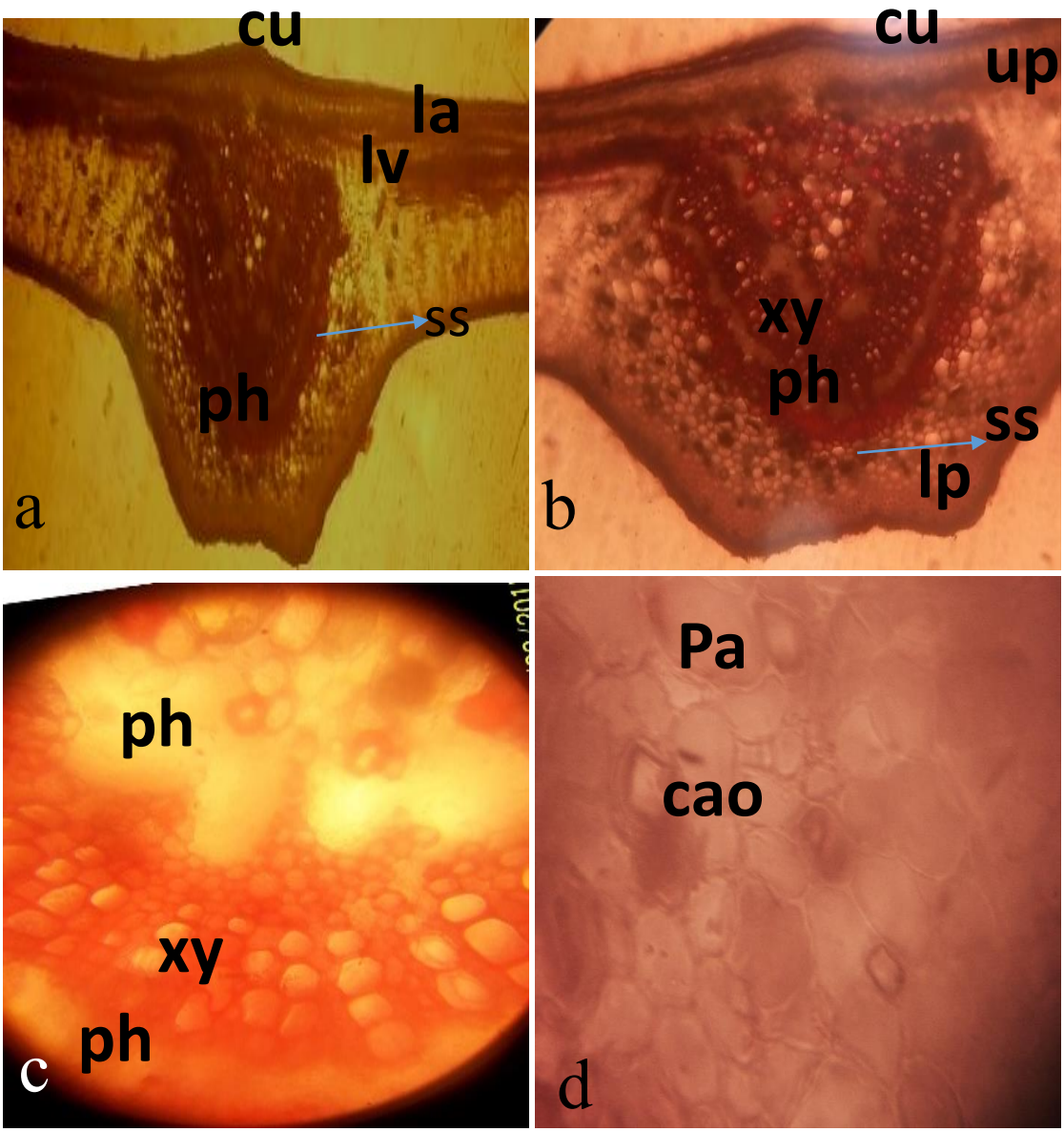


Figure 4.2.2.2: Transverse Section (TS) of *A*lstonia *congensis* leaf mid-rib

(a & b) x40 and x100, showing the mid-rib, (c & d) x400 showing the enlarged cell inclusions of mid rib; cu: cuticle, co: collenchyma cell, la: lamina, lp: lower epidermis,ph: phloem, sc: secretory cell, ss: sclerenchymatous sheath, up:upper palisade, xy: xylem tissue

Table 4.2.2.2: Microscopic characters of the lamina of *Alstonia boonei* and *Alstonia congensis*

Taxa	Epidermis type	Number and shape of hypodermis	Palisade mesophyll	Spongyl mesophyll	Fibres/calcium oxalate
A. boonei	Dorsiventral	One hypodermal layer Square like shape	Cylindrical shaped and compactly packed	Compactly packed. Long bone and finger like in shape	Fibres and calcium oxalate crystals are present
A. congensis	Dorsiventral	Two hypodermal layers Square like in shape	Cylindrical shaped and compactly packed	Loosely packed with air space Short bone and finger like in shape	Fibres and prismatic calcium oxalate are present

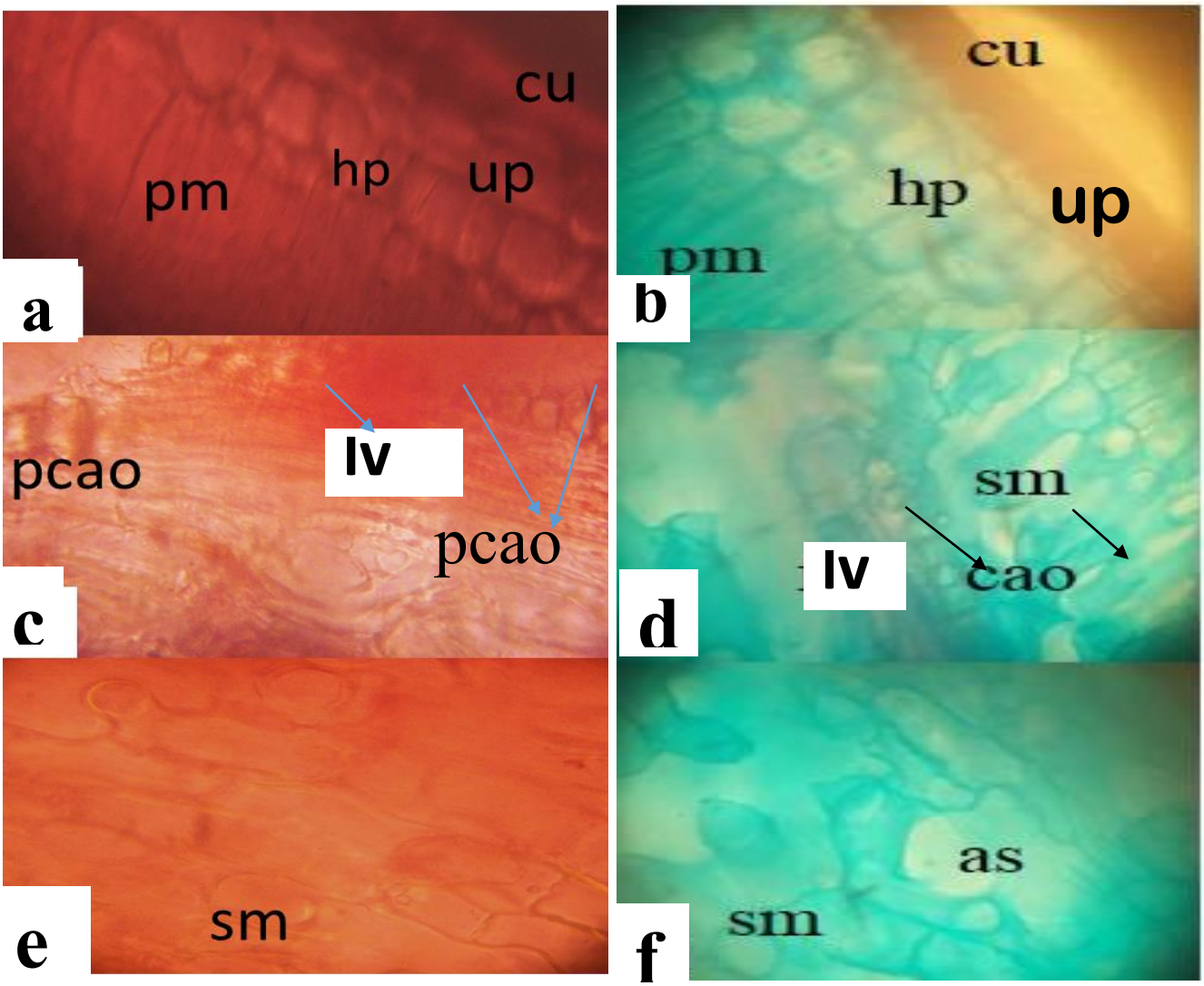


Figure 4.2.2.3: Lamina of *Alstonia boonei* and *Alstonia congensis* leaf

(a) x100, (c & e) x400 of *A.boonei* transverse section, b) x100, (d & f) x400 of *A. congensis* transverse section. pcao: prismatic calcium oxalate, cu: cuticle, lv: lateral vein, hp: hypodermis, pm: palisade parenchyma cells, sm: spongy mesophyll showing parenchyma cells

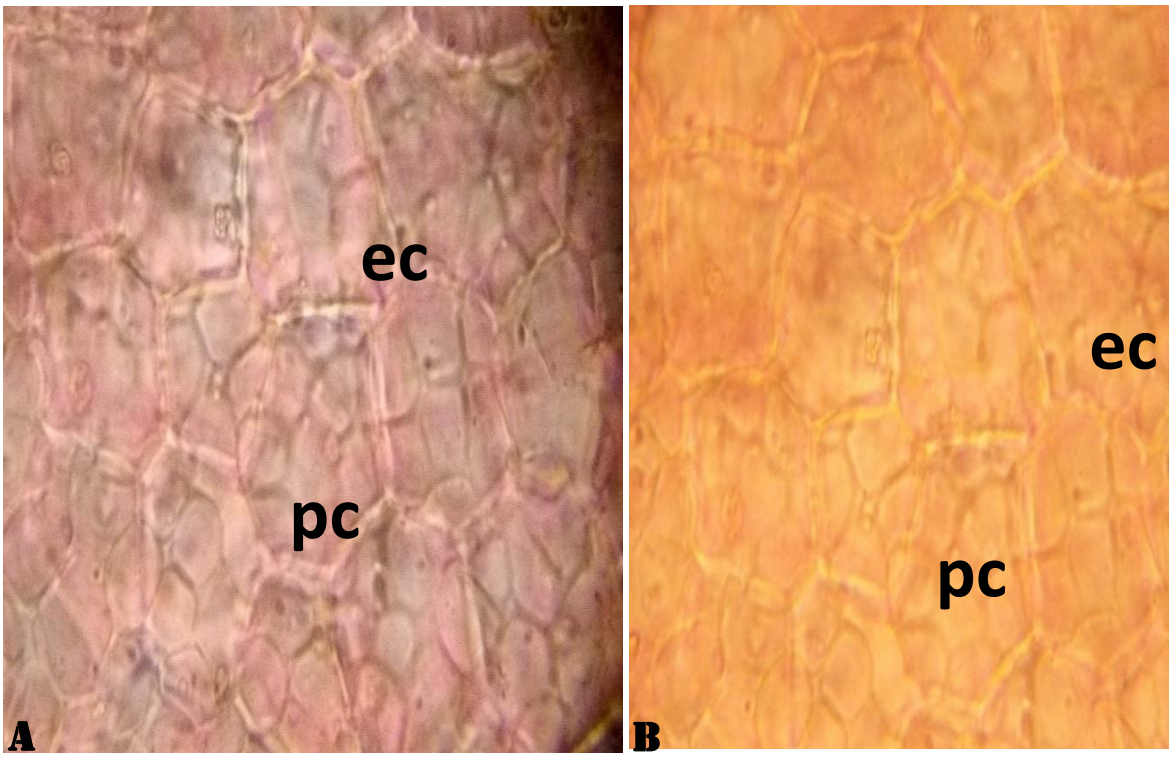


Figure 4.2.3.1: Cleared leaves of *Alstonia* species: x400 showing palisade cells of *Alstonia boonei* (A) and *Alstonia congensis* (B) leaves, respectively. ec: epidermal cell, pc: palisade cells

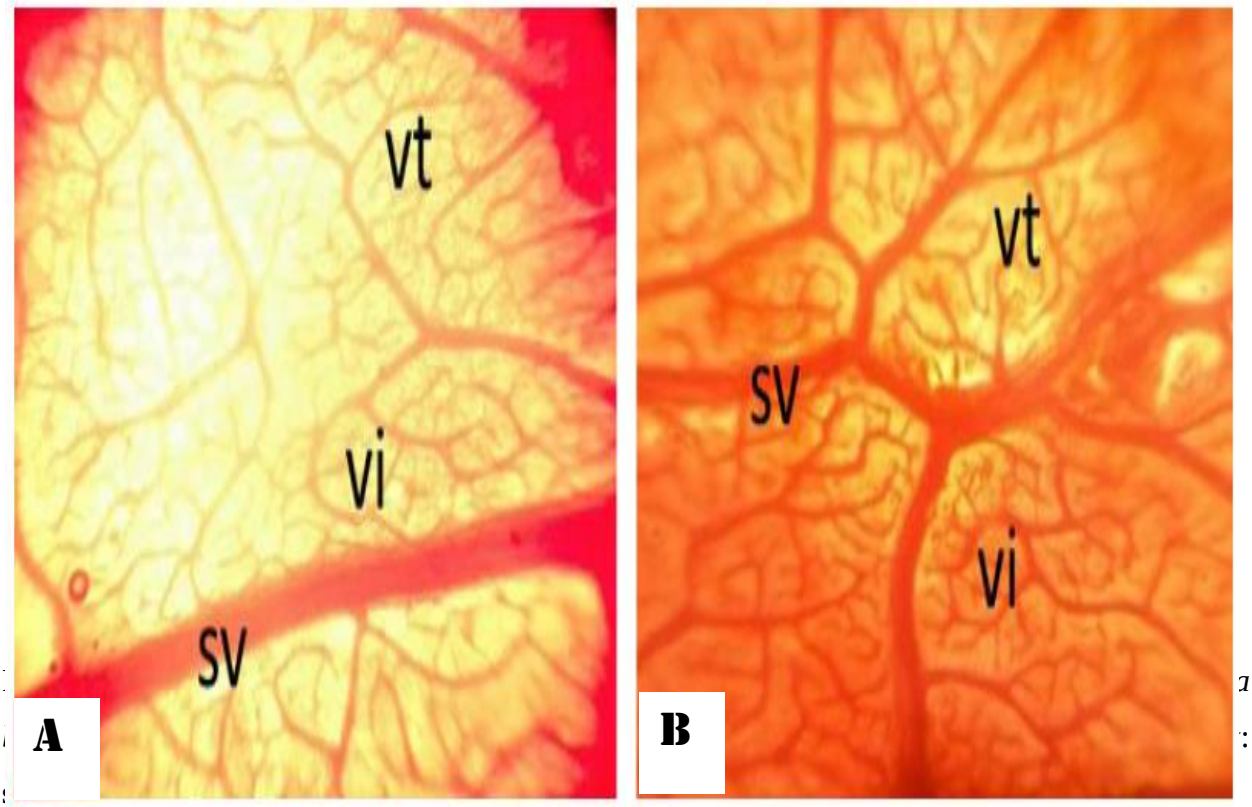


Figure 4.2.3.2: Cleared leaves of *Alstonia* species: x 100 venation pattern of *Alstonia boonei* (A) and *Alstonia congensis* (B) leaves, respectively. sv: secondary vein, vi-vein-islet, vt: vein termination

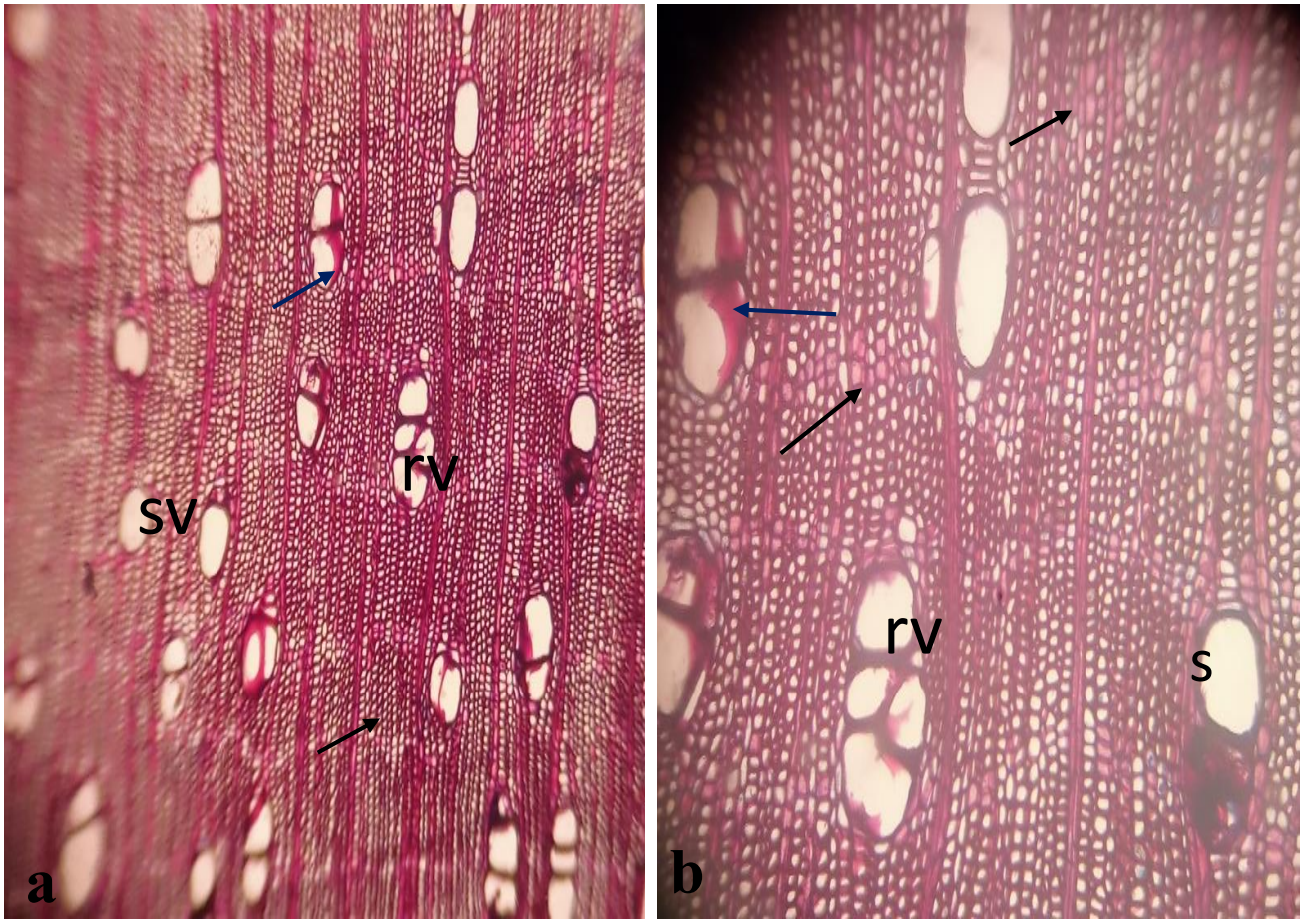


Figure 4.2.4.1: Transverse Section (TS) of *Alstonia boonei* stem wood.

(a&b) x40 & x100. Black arrows indicate diffuse in aggregates parenchyma (type of parenchyma), blue arrows show laticifer cell, sv: solitary vessel, rv: radial multiple vessel

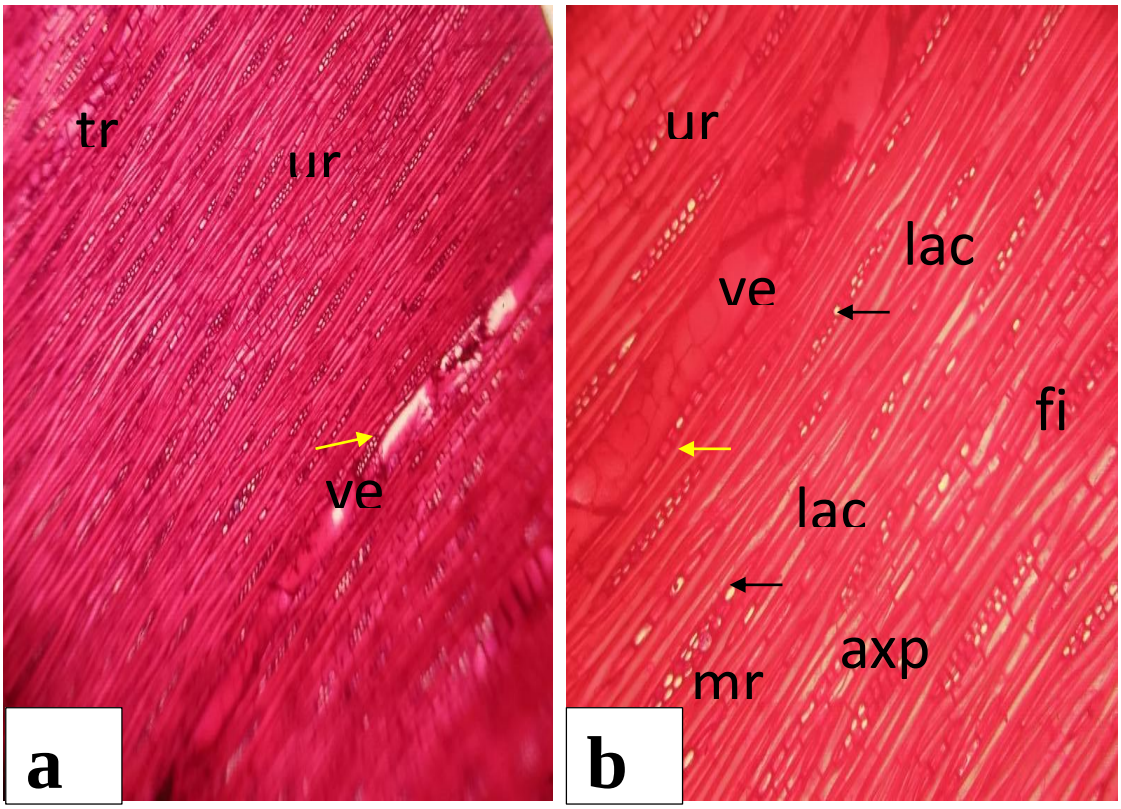


Figure 4.2.4.2: Tangentially longitudinal section (TLS) of *Alstonia boonei* stem-wood (a&b) x40 & x100.; Axp: apotracheal parenchyma, fi: non septate fibre, mr: multiseriate ray, ur: uniseriate ray, tr: tracheids, lac-laticifer, yellow arrow indicates paratracheal parenchyma, ve: vessel element

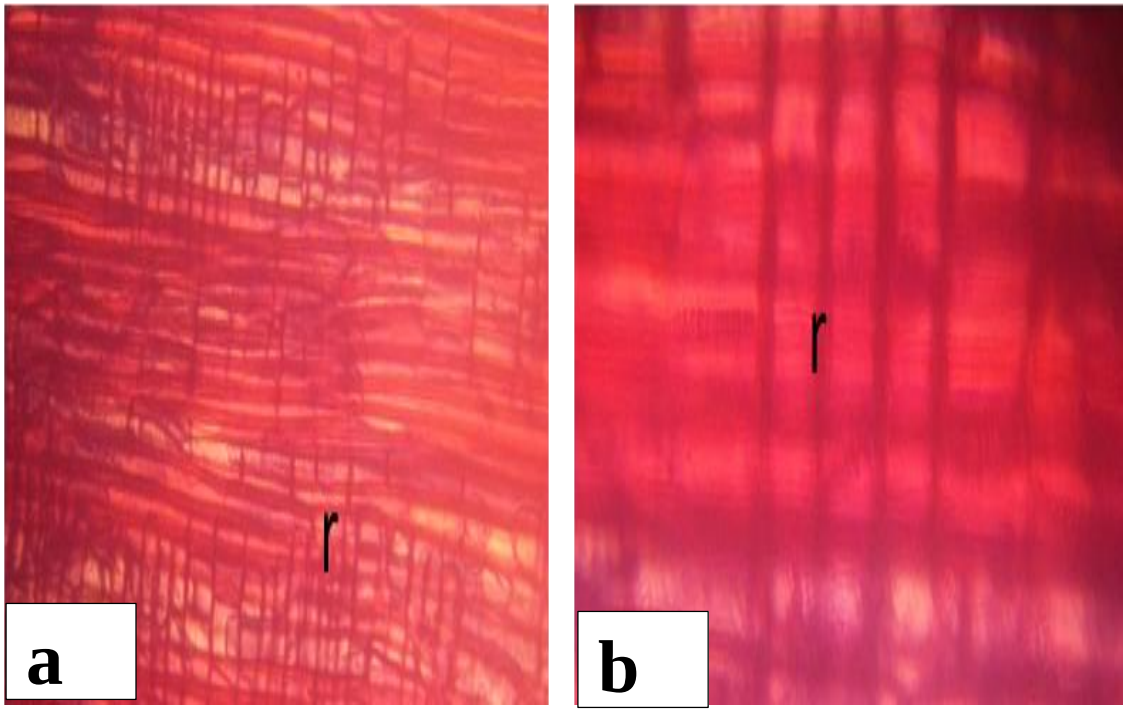


Figure 4.2.4.3: Radial longitudinal Section (RLS) of *Alstonia boonei* stem wood {a (x40) &b (x100)} r: rays

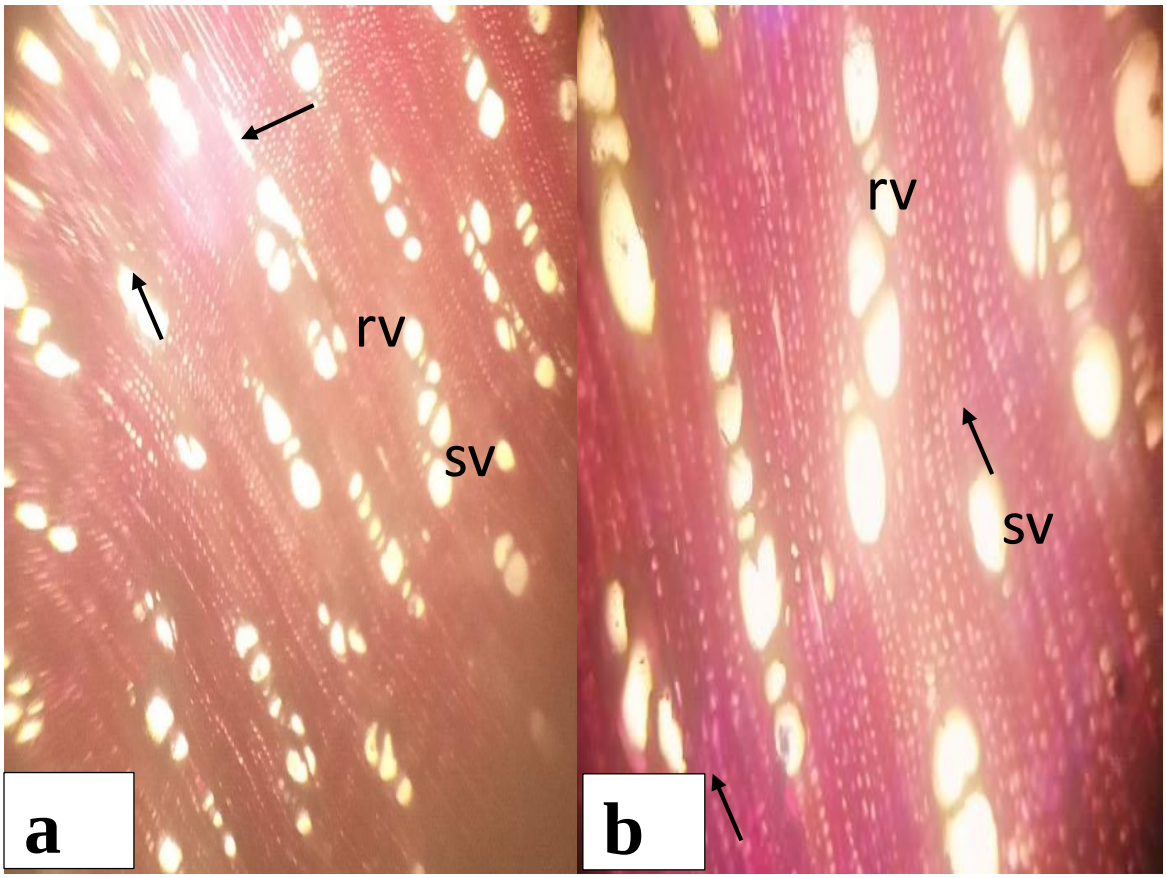


Figure 4.2.4.4: Transverse Section (TS) of *Alstonia congensis* stem wood.

a (x40) & b (x100). Black arrow indicates diffuse in aggregates parenchyma (type of parenchyma in wood), sv: solitary vessel,rv: radial multiple vessel

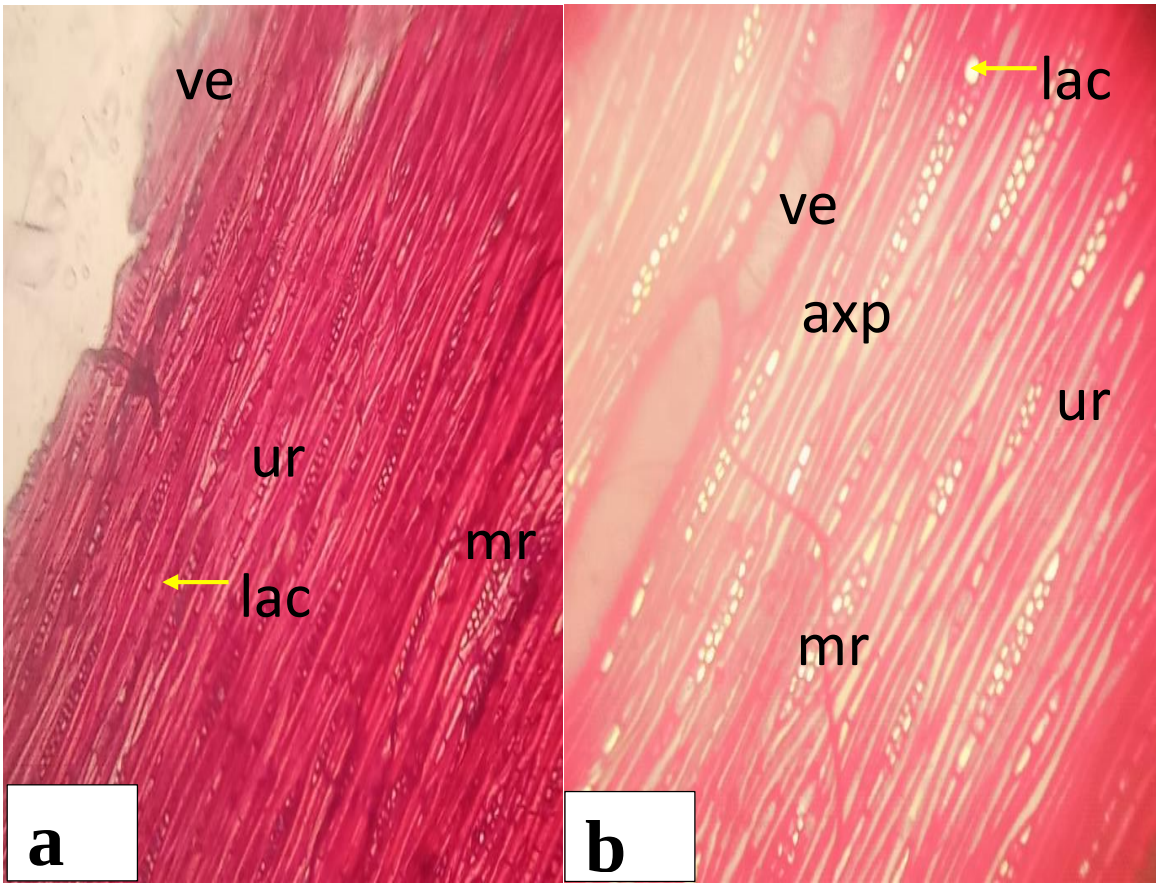


Figure 4.2.4.5: Tangentially longitudinal section (TLS) of *Alstonia congensis* stem wood. {a (x40) &b (x100)}. Axp: axial parenchyma, mr: multiseriate ray, sv: solitary vessel, ur: uniseriate ray, ve: vessel element, lac: laticifer, coloured arrow indicate paratracheid parenchyma

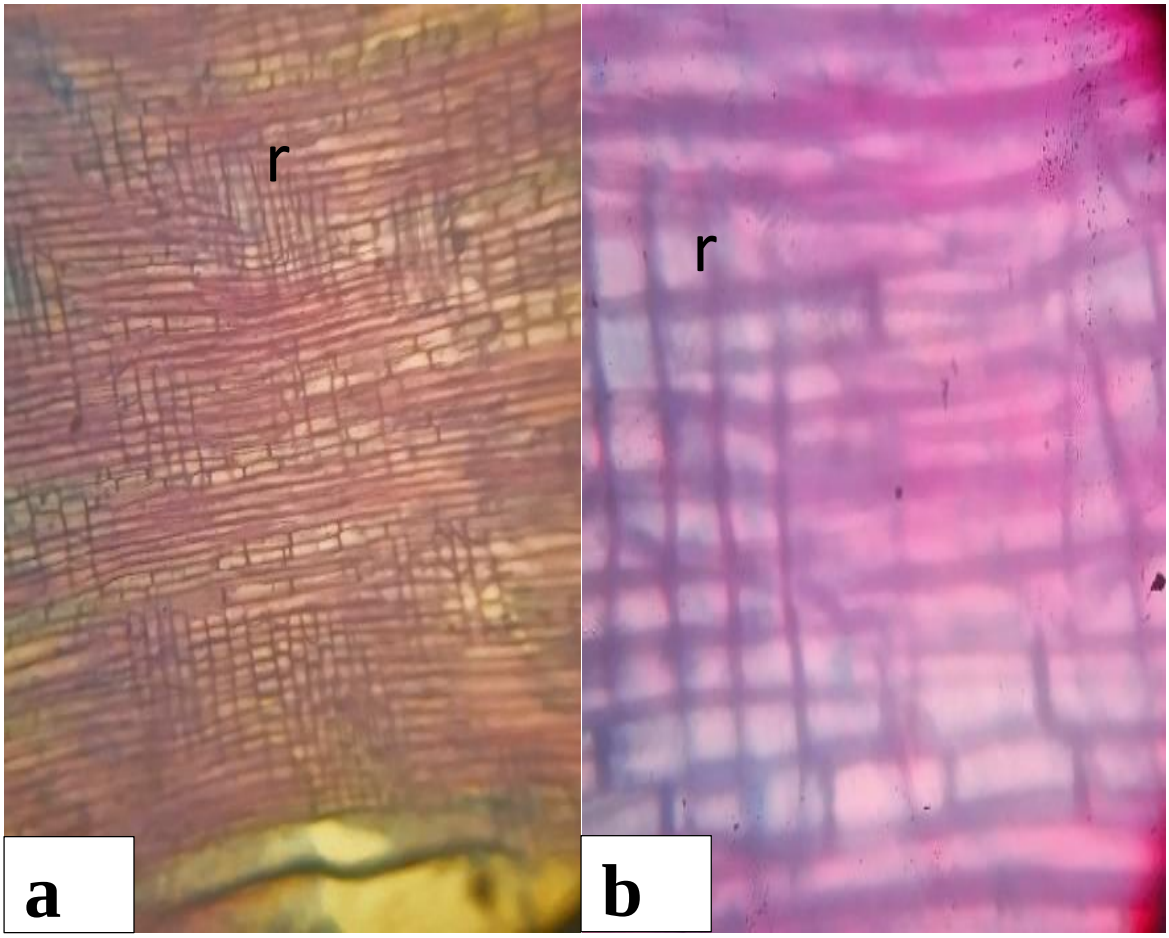


Figure 4.2.4.6 : Radial longitudinal Section (RLS) of *Alstonia congensis* stem wood.

{ a (x40) & b (x100)} r: rays (combination of square like and rectangular like rays)

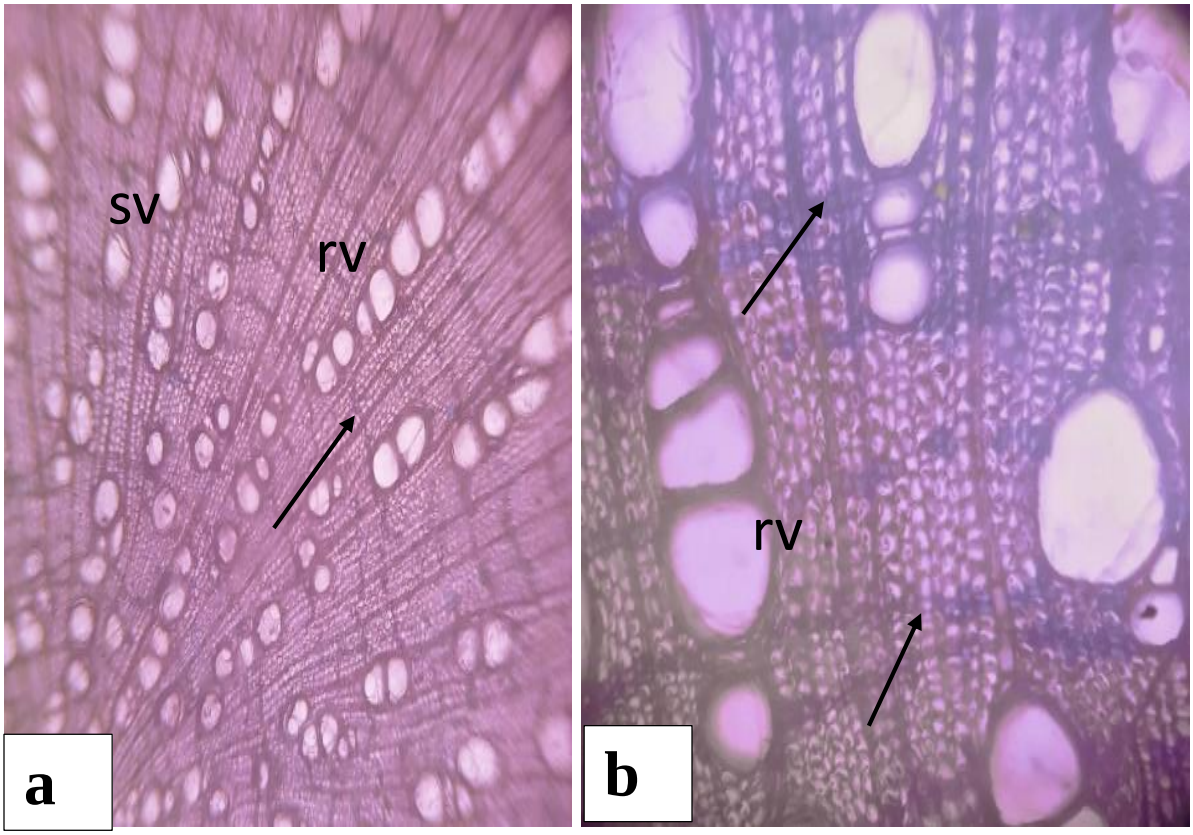


Figure 4.2.4.7: Transverse Section (TS) of *Alstonia boonei* root wood.
{a (x40) & b (x100)} Arrow shows ctp: continuous tangential band parenchyma, rv: radial multiple vessel, sv: solitary vessel

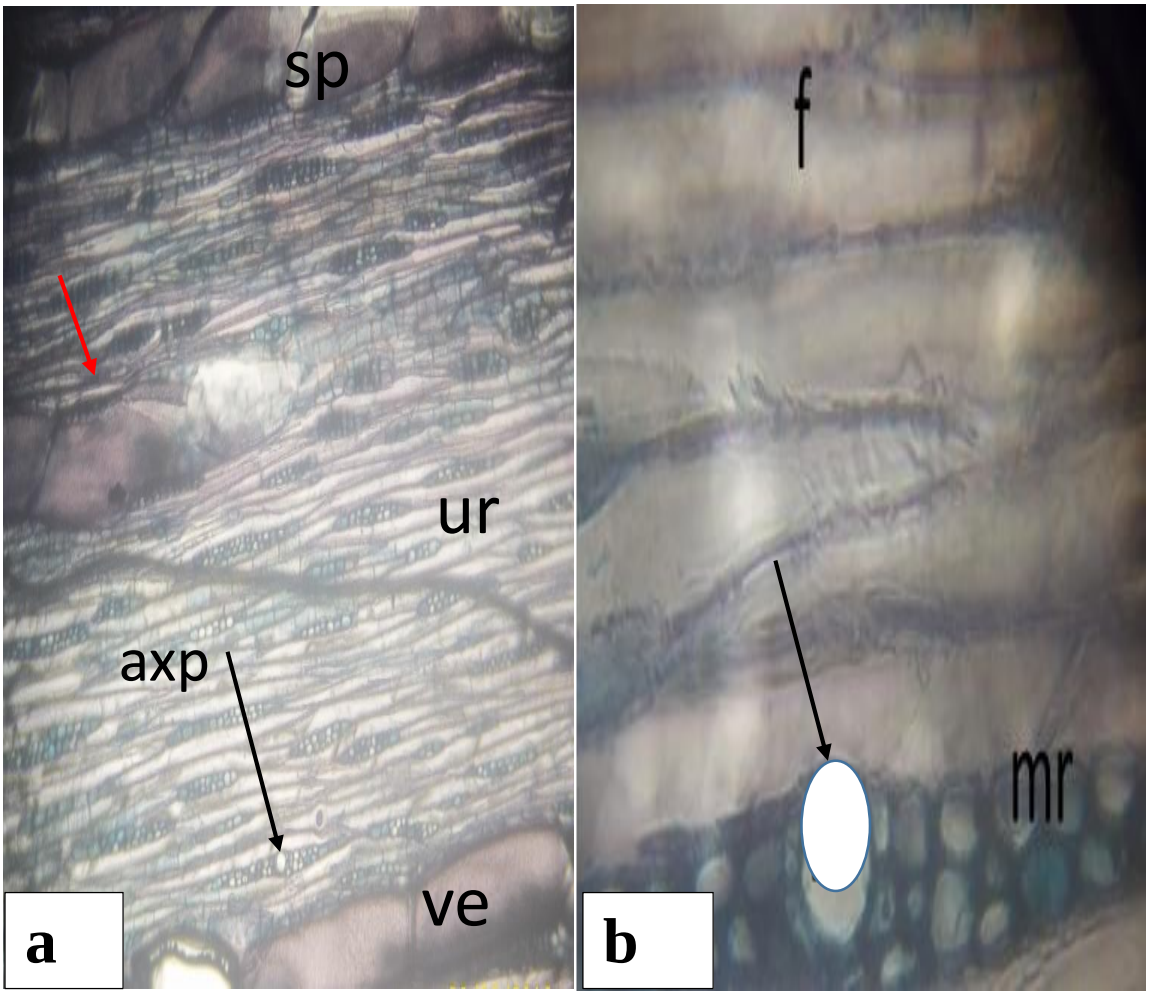


Figure. 4.2.4.8: Tangential Longitudinal Section (TLS) of *Alstonia boonei* root wood.

{ a (x40) & b (x400)} coloured arrow shows paratracheid parenchyma, axp: axial parenchyma, black arrow shows laticifer, f: fibre, mr: multiseriate ray, ur: uniseriate ray, sp:simple perforation

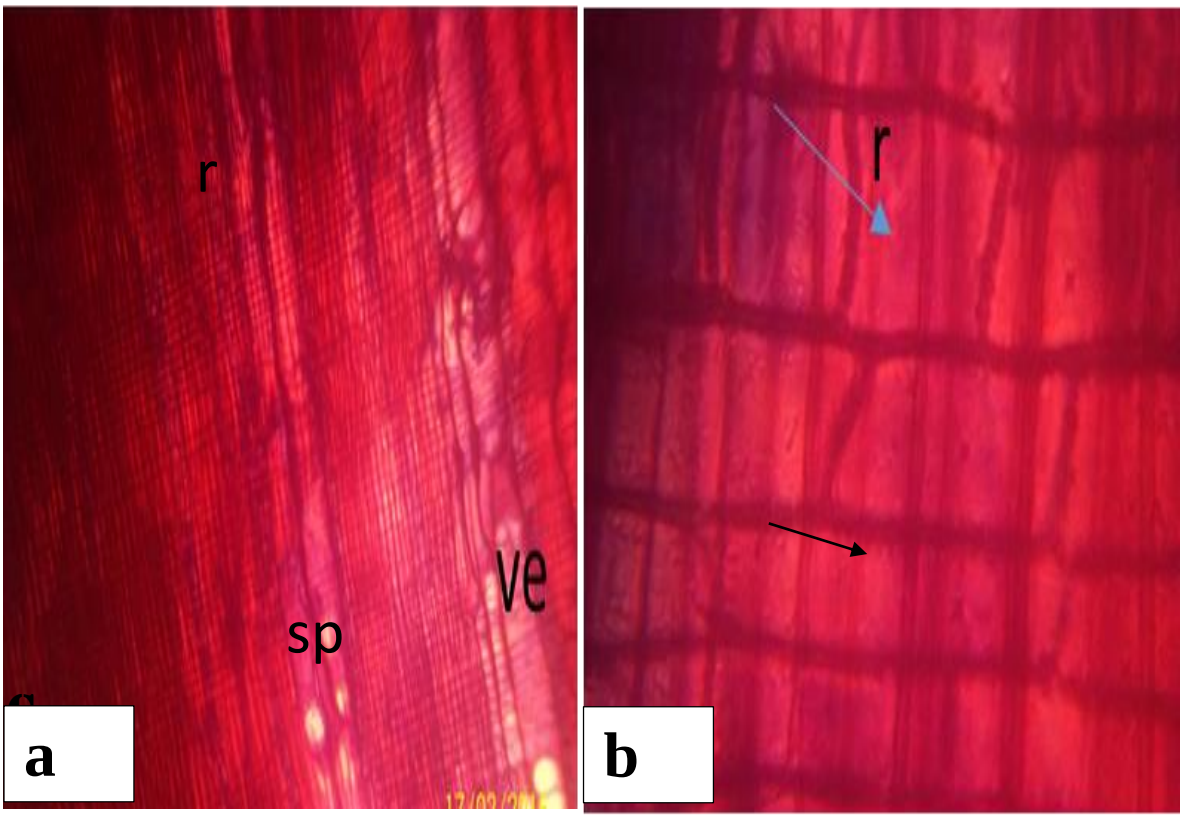


Figure 4.2.4.9: Radial longitudinal Section (RLS) of *Alstonia boonei* root wood.
{a (x40) & b (x400)}. Sp: Simple perforation, r: rays, ve: vessel, blue arrow showing rectangular shape ray, black arrowing showing square shape ray

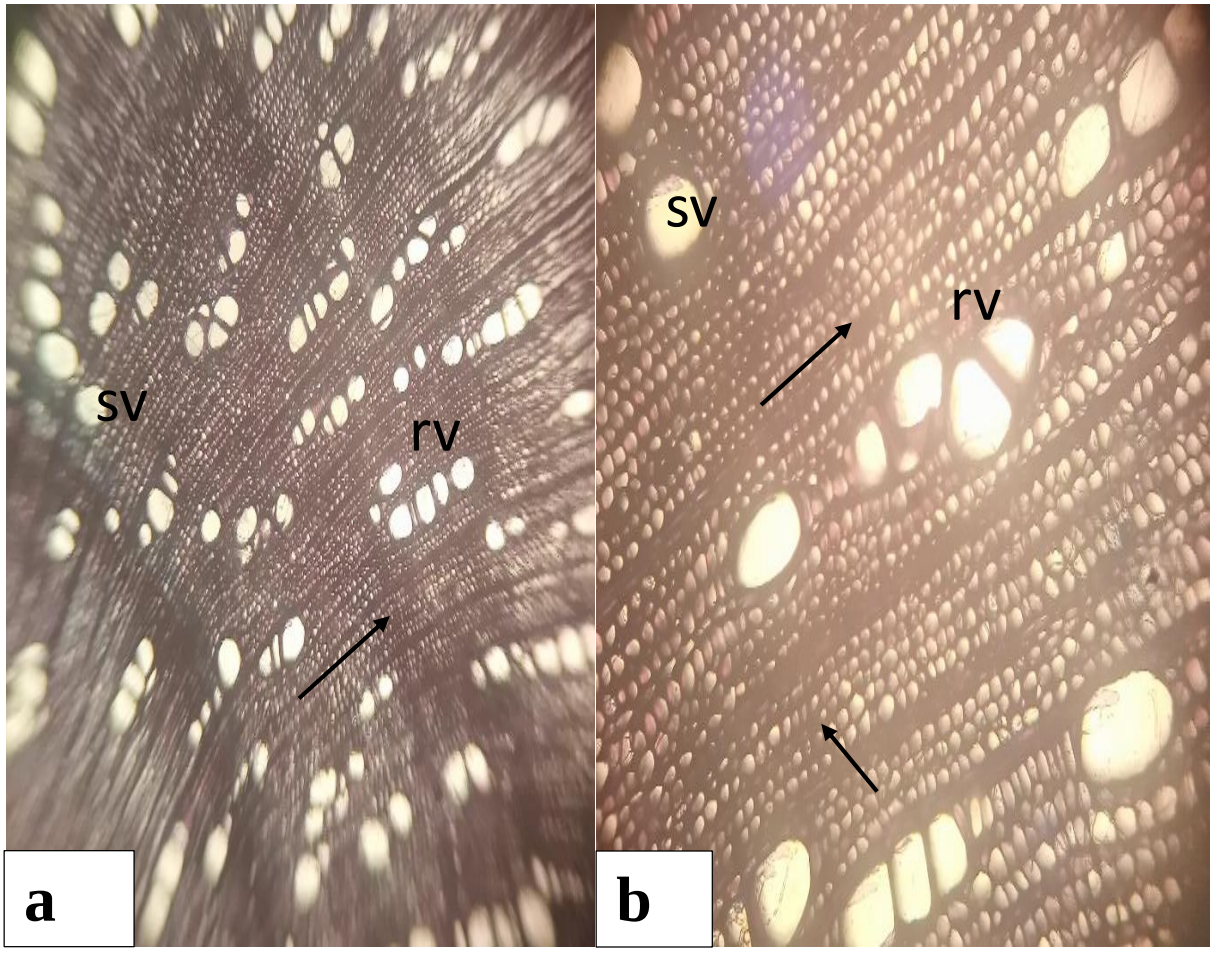


Figure 4.2.4.10: Transverse Section (TS) of *Alstonia congensis* root wood. Arrow shows ctp: continuous tangential band parenchyma, rv: radial multiple vessel, sv: solitary vessel

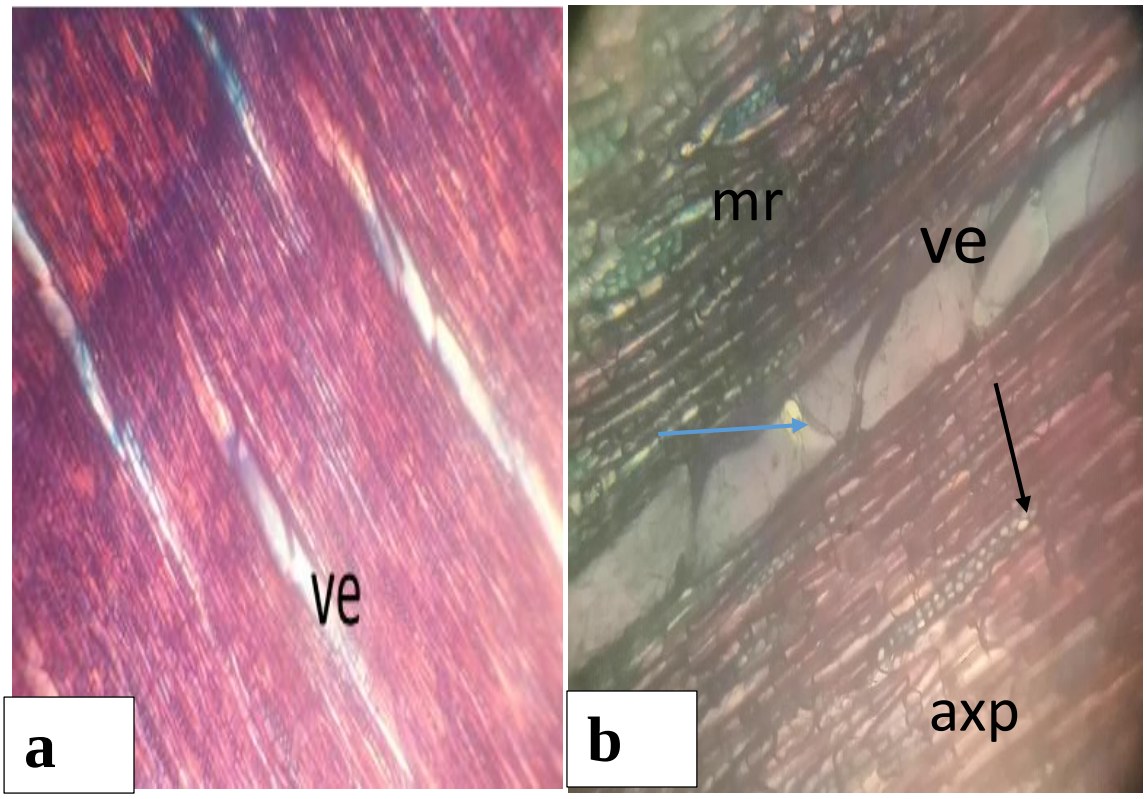


Figure 4.2.4.11: Tangential Longitudinal Section (TLS) of *Alstonia congensis* root wood. {a_i (x40) & b (x100)}. axp: axial parenchyma, mr: multiseriate ray, ve: vessel, ur: uniseriate ray;black arrow shows laticifer, blue arrow indicate simple perforation

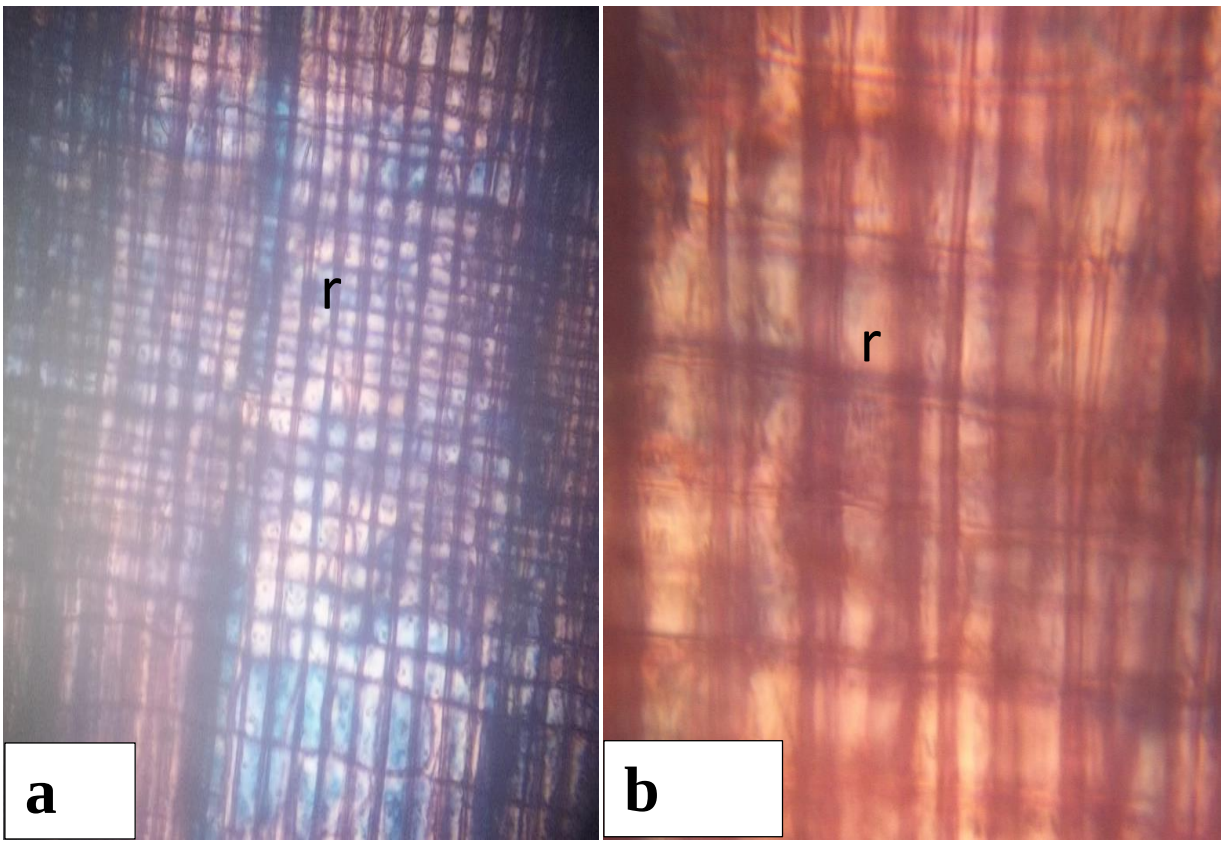


Figure 4.2.4.12: Radial longitudinal Section (RLS) of *Alstonia congensis* root.

{a (x40) & b (x400) r: rays

Table 4.2.4: *Qualitative* features of *Alstonia boonei* and *Alstonia congensis* stem-wood and root wood

Sample	Parenchyma	Fibre	Tracheid	Vessel type	Perforation plate	Porosity	Rays	Resin canal/ tylose
ABS	Apotracheal	Non-septate/ pitted	Short	Solitary/ radial multiple	Simple	Semi-ring	Homogenous	Absent
ABR	Apotracheal	Non-septate/ pinoid pit	Short	Soltary/ radial multiple	Simple and tailed	Semi-ring	Homogenous	Present
ACS	Apotracheal	Non septate/ Pitted	Long	Solitary/ radial multiple	Simple	Semi-ring	Homogenous	Absent
ACR	Diffuse apotrachael	Non septate/ pinoid pit	Long	Solitary/ radial multiple	Simple,short tailed	Semi-ring	Homogenous	Present

ABS- *A. boonei* stem; ABR- *A. boonei* root; ACS- *A. congensis* stem; ACR- *A. congensis* root

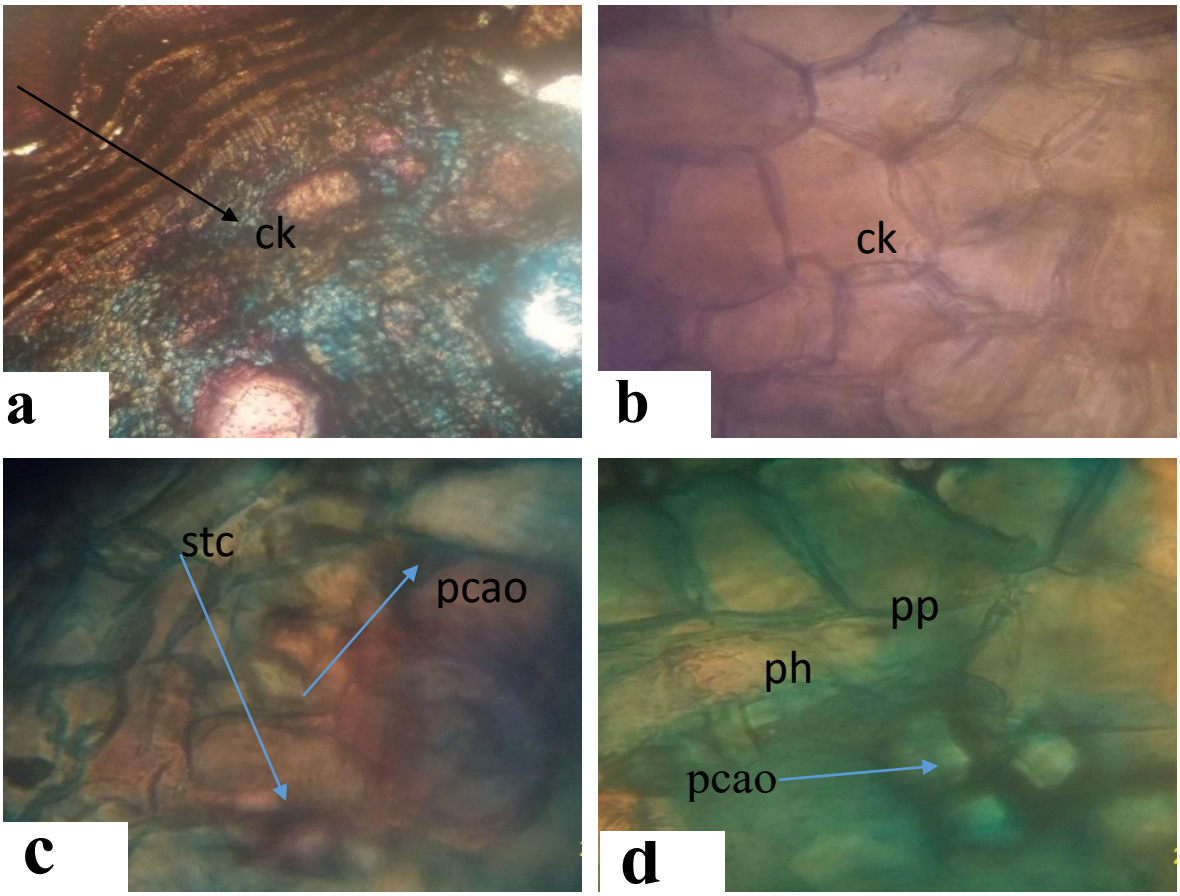


Figure 4.2.5.1**:** Transverse section (TS) of *Alstonia boonei* stem-bark.

{a_i (x40) & b, c, d (x100)} ck: cork, pcao: prismatic calcium oxalate crystal, ph: phloem, pp:phloem parenchyma, stc: stone cell.

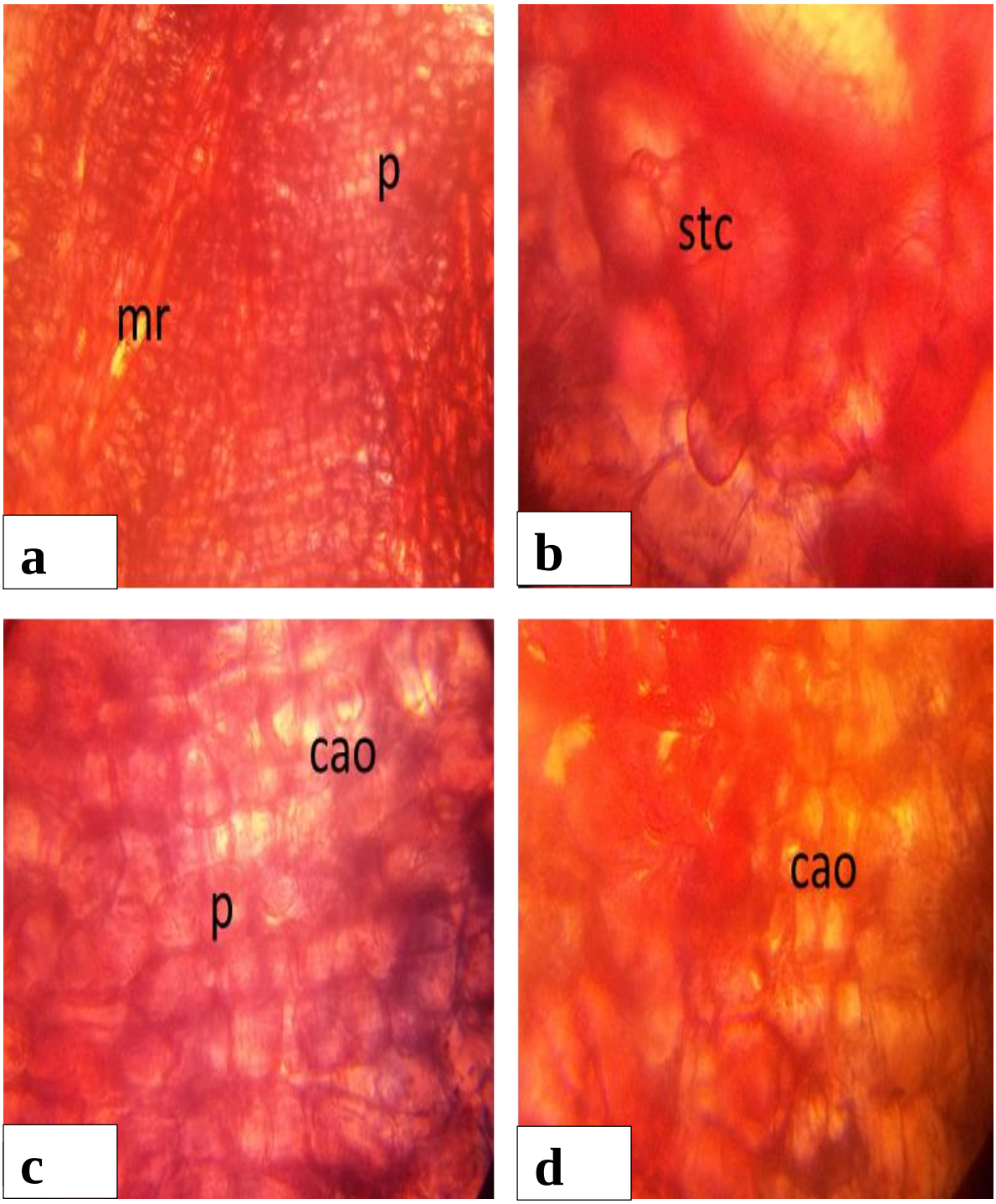


Figure 4.2.5.2**:** Transverse section (TS) of *Alstonia congensis* stem-bark.

{a (x40) & b, c, d (x100)} cao: calcium oxalate crystal, p: parenchyma, stc: stone cell.

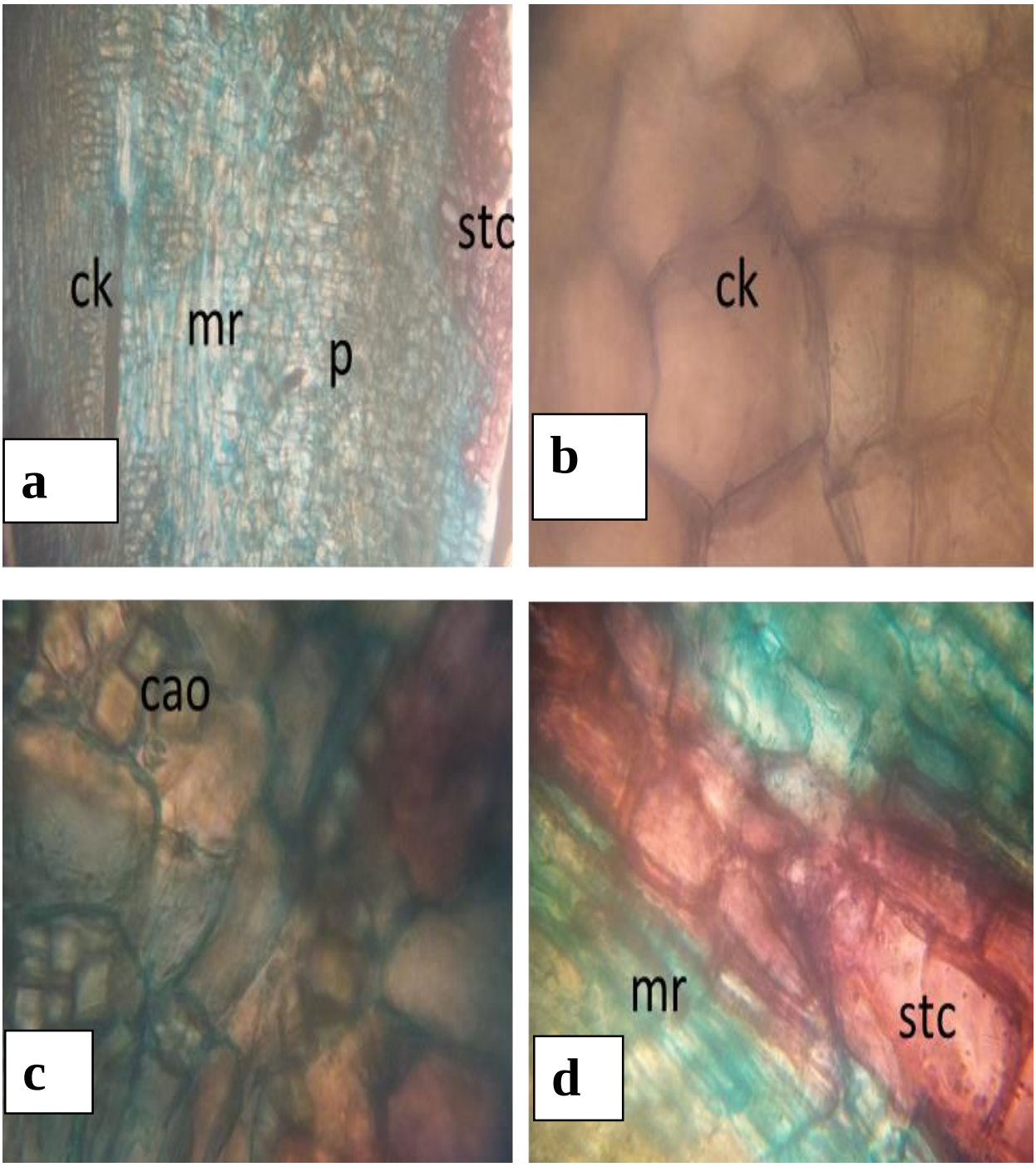


Figure 4.2.5.3: Transverse section (TS) of *Alstonia boonei* root-bark.

{a (x40) & b, c, d (x100). cao: calcium oxalate crystal, ck: cork cell, mr: medullary ray, p: parenchyma, sc:secretory cell, stc: stone cell

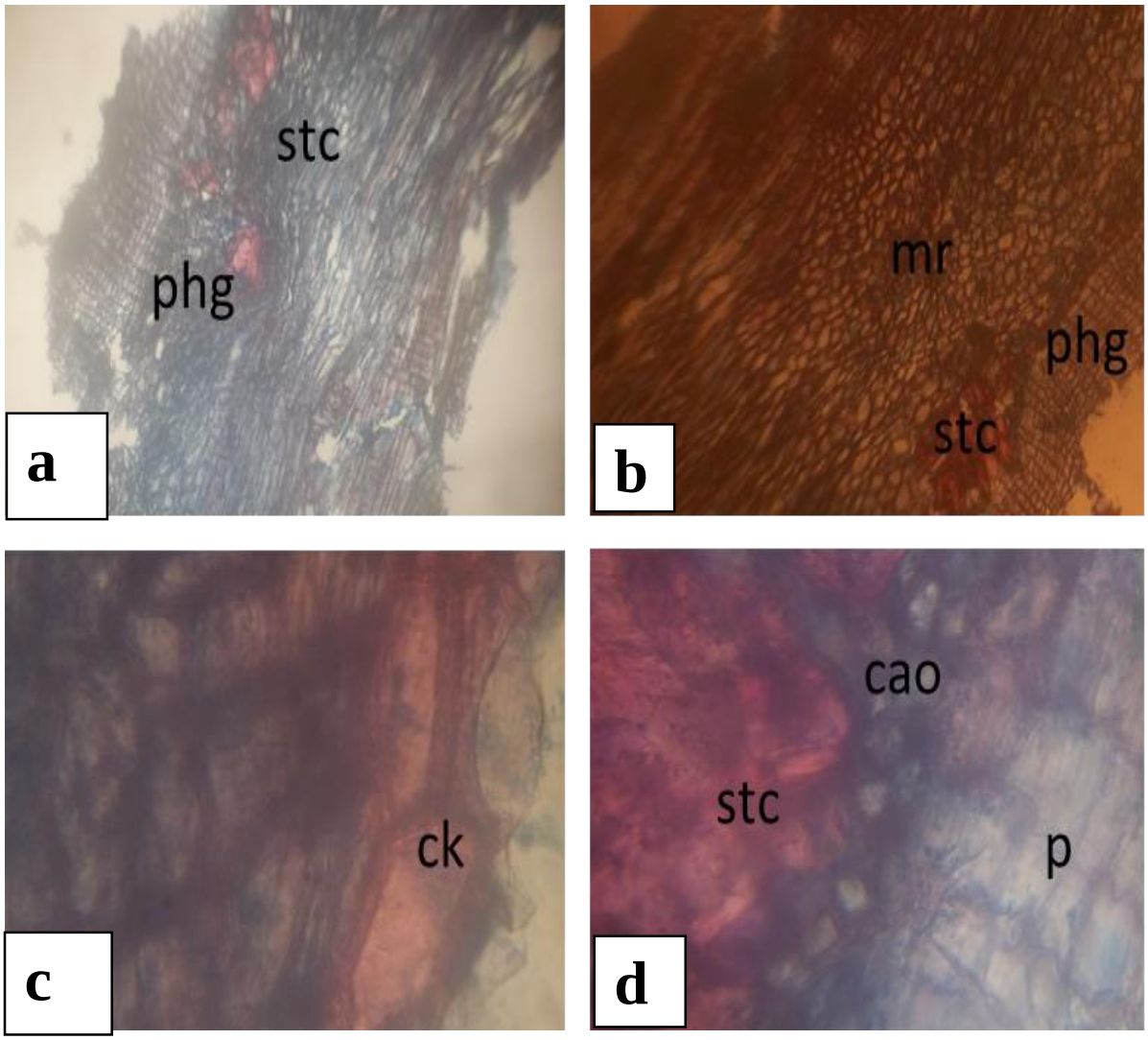


Figure 4.2.5.4: Transverse section (TS) of *Alstonia congensis* root-bark.

{a (x40) & b, c, d (x100). cao: calcium oxalate crystal, ck: cork cell, medullary ray, p: parenchyma, ph: phellogen,sc:secretory cell, stc: stone cells

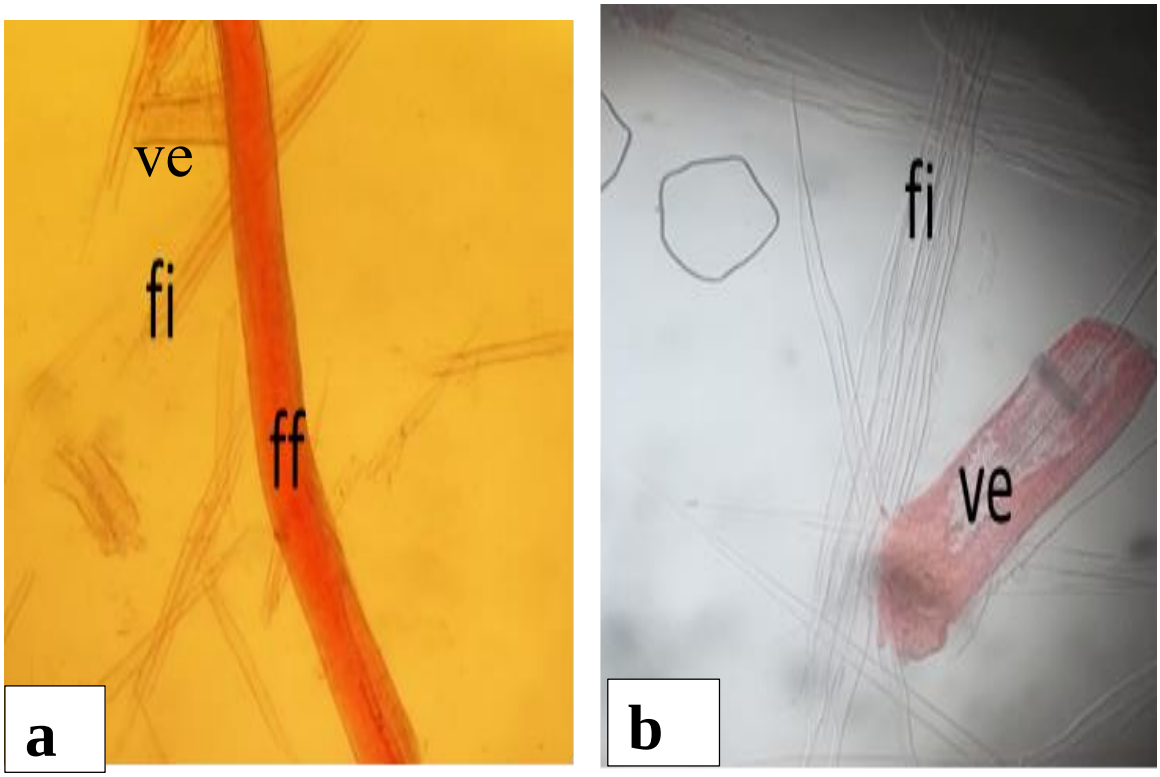


Figure 4.2.6.1: Stem-wood macerate of *Alstonia boonei* and *Alstonia congensis* (x100).ff: (a) *Alstonia boonei* and (b) *Alstonia congensis*, ff: fusiform fibre, fi fibre, r: ray, ve:vesssel element

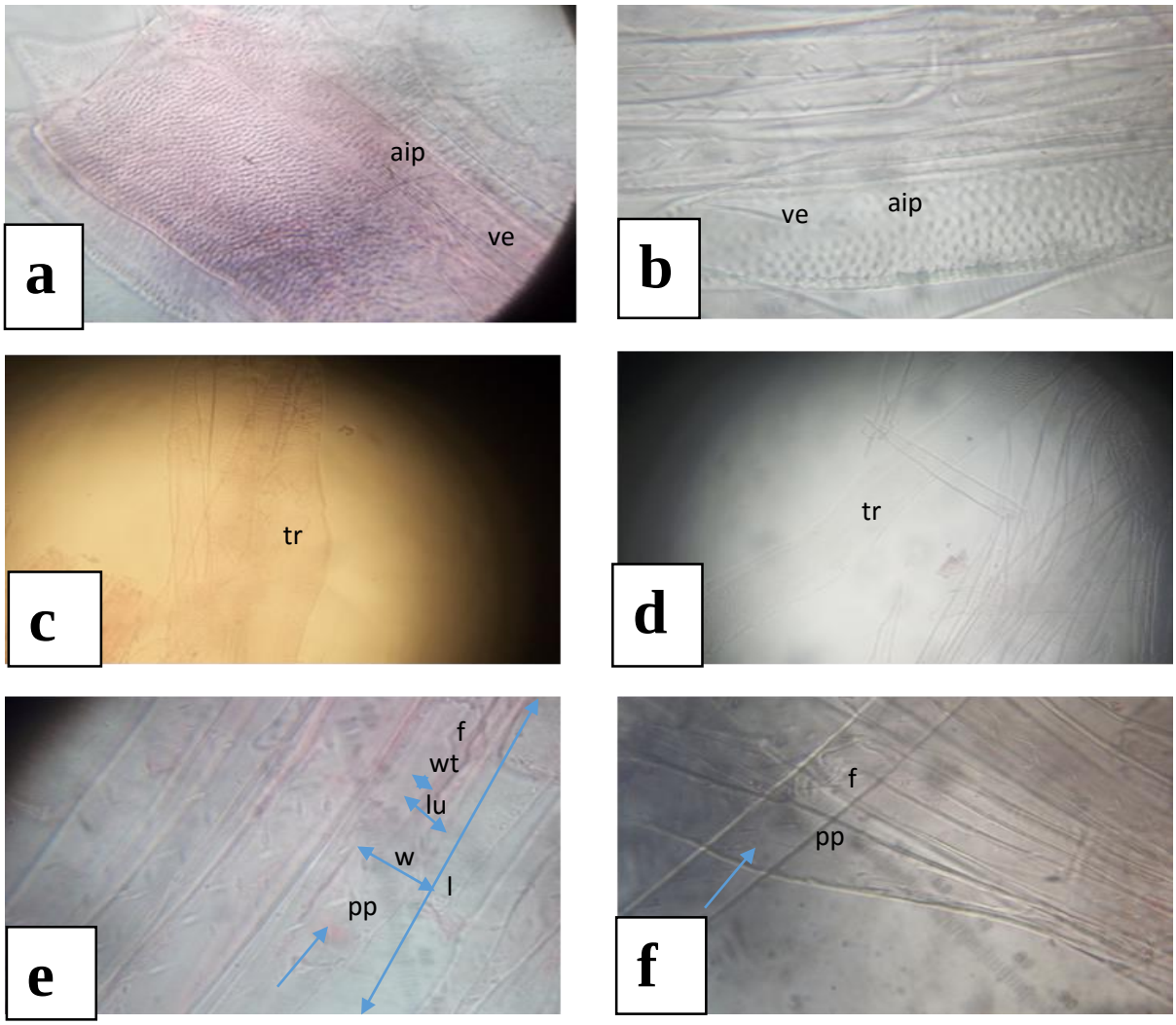


Figure 4.2.6.2: Root wood macerates *Alstonia boonei* and *Alstonia congensis*.(a, c & e) (x400, x100 &x100) *Alstonia boonei* macerates, (b, d &f) (x100) *Alstonia congensis* macerates

aip: alternate intervessel pit arrangement, l: length, lu: lumen, tr: tracheid, ve: vessel element; w: width, wt: wall thickness.

Table 4.2.6: Quantitative parameters of stem and root wood fibre and vessel of *Alstonia boonei* and *Alstonia congensis*

Sample	Fibre length	Fibre width	Wall thickness	Lumen	Vessel length	Vessel width
ABS	781.5±1.0	40.1±1.0	2.1±0.06	36.2±1.0	53.4±2.0	19.7±0.9
ACS	1044.0±2.0	37.5±1.0	2.0±0.06	33.7±0.9	61.5±2.0	14.8±0.5
ABR	1011.0±2.1	49.8±0.7	4.3±0.09	40.8±3.1	308.8±12.1	276.8±10.9
ACR	1108.0±4.5	38.75±0.5	4.75±0.09	30.0±0.5	147.3±1.7	115.8±1.7

*All measurements are in µm. n= 25. Mean ± SEM

ABS- *A. boonei* stem; ABR- *A. boonei* root; ACS- *A. congensis* stem; ACR- *A. congensis* root

4.3 Molecular evaluation of *Alstonia boonei* and *Alstonia congensis* accessions

4.3.1 Measurement of isolated DNA of *A. boonei* and *A. congensis* accessions

Table 4.3.1 presents the accessions of *Alstonia boonei* and *A. congensis* collected for DNA isolation and Table 4.3.2 presents the concentration and purity of DNA samples isolated from *A. boonei* and *A. congensis* leaf. Plate 4.3.1 shows the banding pattern and amplicon sizes range for nucleotide bases of PCR amplified DNA of *A. boonei* and *A. congensis* using ITS 1 and 4 in electrophoresis gel. Sequenced nucleotide bases obtained using ITS were above 600 bp.

4.3.2 Sequence alignment

An Unweighted Pair Group with Arithmetric Mean (UPGMA) dendogram showing the relationship among sequenced nucleotide compared with similar ITS data retrieved from GenBank is presented in Figure 4.3.2.

Table 4.3.1: Accessions of *Alstonia boonei* and *Alstonia congensis* collected for DNA isolation

S/N	Plant	Location of collection	Habitat
1	*Alstonia congensis* (Ac1)	Itokin, along Ikorodu Express way	Dryland
2	*Alstonia congensis* (Ac2)	Itokin, along Ikorodu Express (farmland)	Dryland
3	*Alstonia congensis*	Valentino, Ondo state	water
4	(Ac3) *Alstonia congensis* (Ac4)	Italuowo, Ondo state	Dryland
5	*Alstonia boonei* (Ab1)	Ekiti, Farmland	Dryland
6	*Alstonia boonei* (Ab2)	Opposite IITA	Dryland
7	*Alstonia boonei* (Ab3)	Valentino, Ondo state	Dryland
8	*Alstonia boonei* (Ab4)	Orogun junction, Ibadan	Dryland
9	*Alstonia boonei* (Ab5)	Behind department of Pharmacognosy	Dryland
10	*Plumeria alba* (Pl)	Behind Pharmacognosy lab., UI	Dry land

Table 4.3.2: Quantitative and qualitative parameters of DNA Isolates from *Alstonia boonei* and *Alstonia congensis*

Sample	Conc.(ng/µL)	A_{260}/A_{280}
Ac1	530.3±0.16	1.87± 0.01
Ac2	221.4±0.1.2	1.70 ± 0.05
Ac3	342.3±0.90	1.80 ± 0.01
Ac4	770.7± 0.03	2.03 ± 0.06
Ab1	589.7±0.9	1.90 ±0.04
Ab2	455.7 ±0.05	1.93 ±0.02
Ab3	648.3 ±0.05	1.90 ± 0.01
Ab4	449.8±0.8	1.85 ±0.01
Ab5	193.7±1.0	1.63±1.0
Pl	3514.8±0.002	2.13±0.002

Data are presented as mean ± SEM (n = 3). Ac- *Alstonia congensis* accessions (1-4); Ab- *Alstonia boonei* accessions (1-5); Pl-*Plumeria alba*

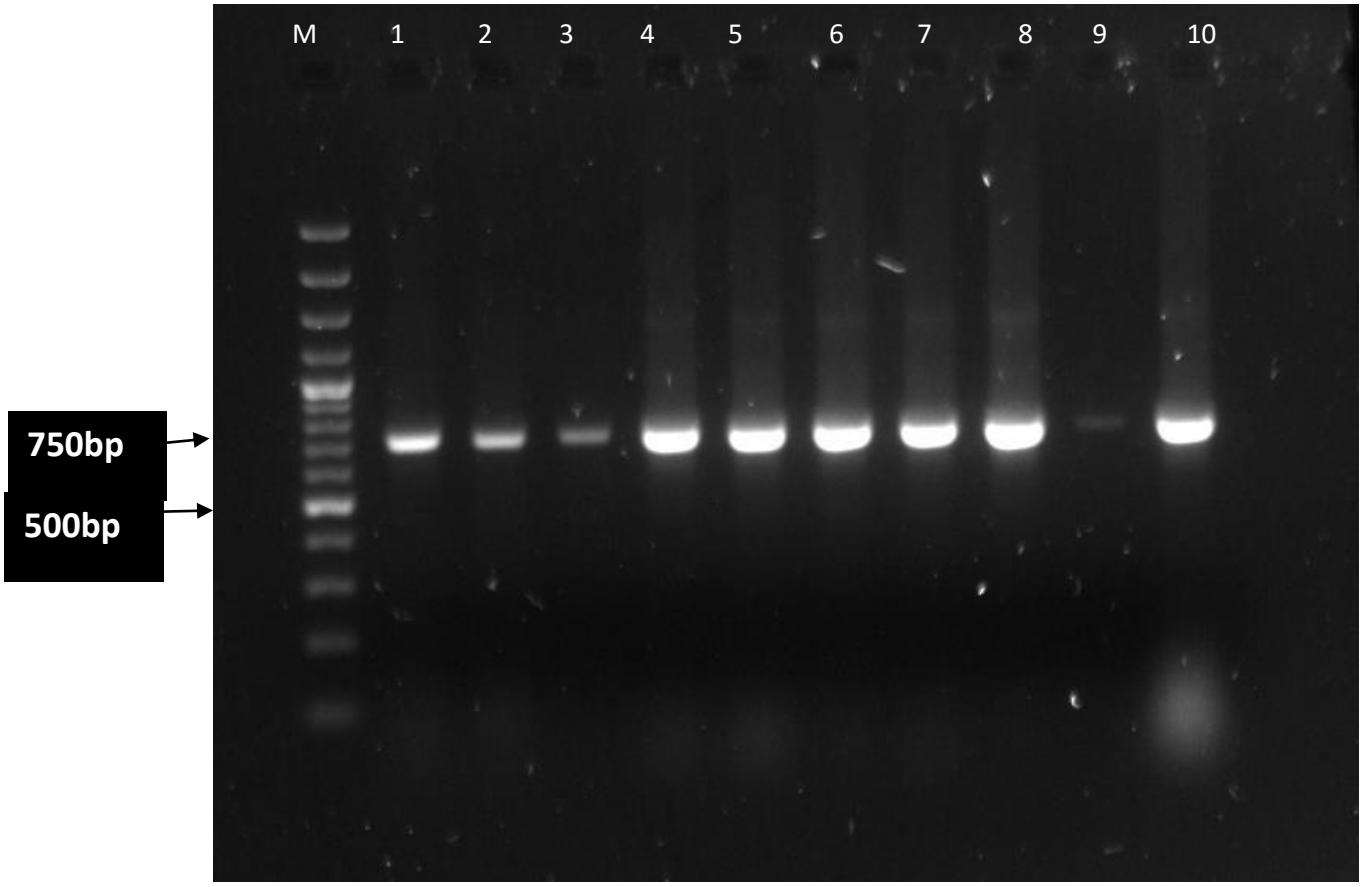


Plate 4.3.1: Agarose gel showing banding pattern of amplified DNA of *Alstonia* species with ITS primer. M- 100 bp DNA ladder; 1- 4=*A. congensis*; 5-9 = *A. boonei*., 10- *Plumeria alba* =outgroup

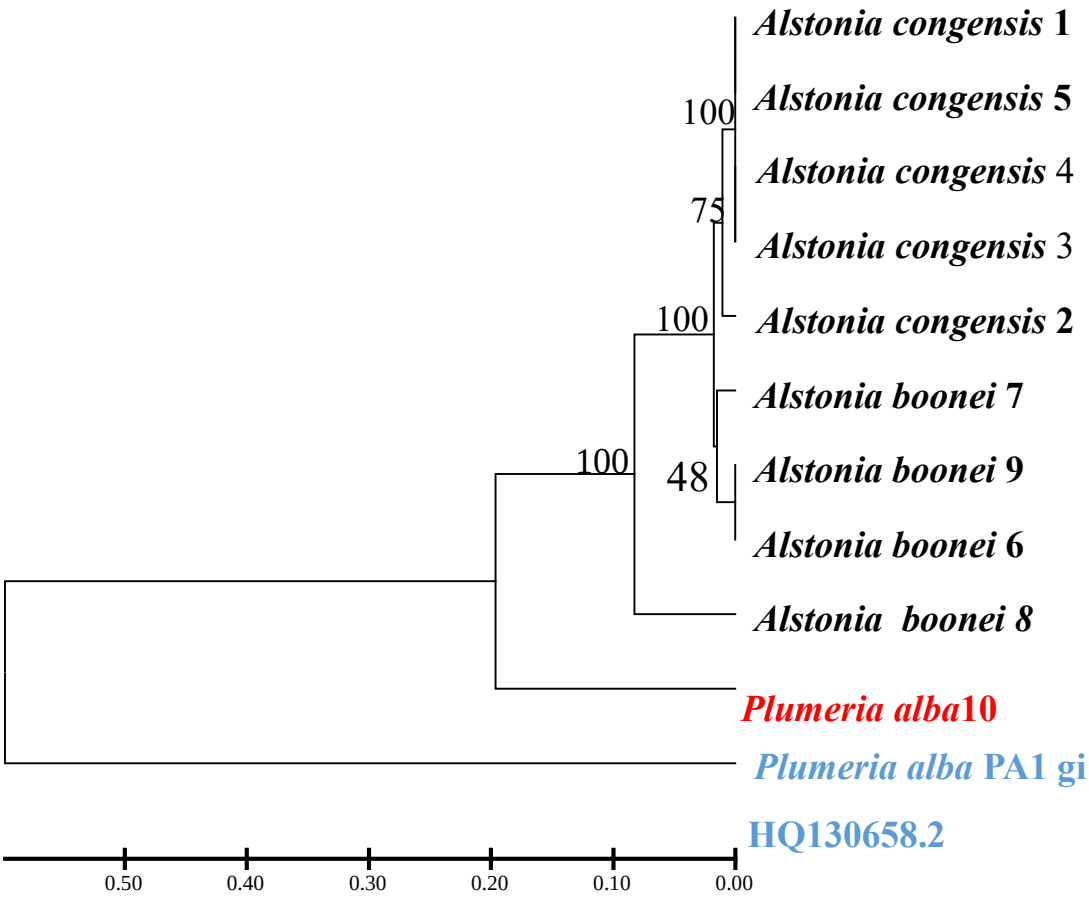


Figure 4.3.2: A UPGMA dendogram showing relationship among sequenced *Alstonia* spp., an outgroup; *Plumeria alba* and its reference ITS data retrieved from the Genbank (Mega 7.0.18)

4.4 Proximate and physico-chemical features of *Alstonia boonei* and *Alstonia congensis*

Table 4.4.1presents the proximate and physico-chemical analyses result of powdered leaf and stem-bark for *A. boonei* and *A. congensis,* which included total ash, acid insoluble ash, crude fibres moisture contents. The moisture contents of *A. boonei* and *A. congensis* leaf and stem-bark are (9.1±0.04, 6.4±0.006, 9.9±0.017, 7.2±0.004) %, respectively. The percentage ethanol extractive values of powdered leaf and stem-bark for *A. boonei* and *A. congensis* are 2.85 ±0.05, 2.5 ±1.0, 4.5 ±0.8 and 4.0±0.09, respectively (Table 4.4.2).

4.5 Elemental contents of leaves and stem-barks of *Alstonia boonei* and *Alstonia congensis*

Table 4.5 presents the micro, macro and toxic elements in the powdered samples of the leaves and stem-bark of *A. boo*nei and *A. congensis.* Calcium is the most dominant element in *A. boo*nei and *A. congensis* leaf and stem-bark, with leaves containing the highest concentration: *A. boonei* (68.2± 3.2 mg/ mL), *A. congensis* (65.7± 1.0 $mgmL^{-1}$), stem-barks have the following concentration; *A. boonei* (52.2± 1.2 $mgmL^{-1}$) and *A. congensis* (39.7± 1.0 $mgmL^{-1}$).

4.6 Phytochemical constituents of *A*lstonia *boonei* and *A*lstonia *congensis* leaf and stem-bark powdered samples

Table 4.6 presents the phytochemical constituents present in the powdered samples of leaves and stem-barks of *A. boonei* and *A. congensis* some of which include alkaloids, tannins, flavonoids.

4.7 Fluorescence characters of *A.lstonia boonei a*nd A*lstonia. congensis* leaves and stem-bark powdered samples

Table 4.7 presents the result of fluorescence analysis evaluated on the powdered samples of the leaf and stem-bark powder of *A. boo*nei and *A. congensis*. The powdered samples give different colour with the addition of various chemical reagents.

4.8 Yields of aqueous crude extracts and fractions of *Alstonia boonei* and *Alstonia congensis* leaf and stem-bark

Table 4.8.1 presents the percentage yield of aqueous crude extracts and Table 4.8.2 presents the yield of fractions of *A. boonei* and *A. congensis* leaf and stem-bark.

Table 4.4.1: Proximate content (%) of powdered leaf and stem-bark of *Alstonia boonei* and *Alstonia congensis*

Plant samples	Protein	Crude fat	Crude fibre	Carbohydrate	Moisture content	Total ash	Acid-insoluble ash
ABL	11.4±0.02	5.6±0.35	25.8±0.011	66.2±0.02	9.1±0.04	8.5±0.07	1.4±0.02
ABS	4.9±0.015	3.0±0.02	31.6±0.008	79.86±0.02	6.4±0.006	5.9±0.014	0.7±0.15
ACL	10.1±0.2	3.3±0.003	26.6±0.007	70.0±0.007	9.9±0.017	6.8±0.026	0.9±0.4
ACS	4.0±1.5	4.4±0.009	37.2±0.005	79.7±0.04	7.2±0.004	4.8±0.03	0.6±0.05

Data are presented as mean ± SEM (n = 6). ABL- *A. boonei* leaf; ABS- *A. boonei* stem-bark; ACL- *A. congensis* leaf; ACS- *A. congensis* stem-bark

Table 4.4.2: Extractive values of *Alstonia boonei* and *Alstonia congensis* leaves and stem-barks

Samples	Ethanol (%)	Water (%)
ABL	2.8±0.05	3.5±0.02
ACL	2.5±1.0	3.0±0.4
ABS	4.5±0.8	5.0±0.09
ACS	4.0±0.09	4.3±0.01

Data are presented as mean ± SEM (n = 6). ABL- *A. boonei* leaf; ACL- *A. congensis* leaf; ABS- *A. boonei* stem-bark; ACS- *A. congensis* stem-bark

Table 4.5: Concentration of mineral elements present in the leaves and stem barks of *Alstonia boonei* and *Alstonia congensis* (mg/g)

Sample	Ca	Na	K	Mg	Cd	Mo	Mn	Pb	Fe	Zn	Ni
ABL	68.2± 3.2	2.9±0.07	50.1 ± 2.0	0.9 ±0.3	0.4± 0.02	12.6± 3.0	0.1 ± 0.01	0.1 ±0.003	2.6±0.2	0.2±0.01	3.3± 0.5
ABS	52.2± 1.2	3.0 ±0.8	23.2 ± 1.5	0.9 ± 0.5	0.08± 0.05	14.8 ± 2.2	0.02 ±0.001	0. 1±0.01	0.2±0.12	0.3±0.1	2.3 ±1.0
ACL	65.7± 1.0	6.0± 0.04	50.0 ± 2.5	0.8 ± 0.5	0.1 ±0.08	15.3 ±1.4	0.09 ±0.004	0.14±0.01	1.4 ±0.1	0.3±0.2	2.4 ±0.3
ACS	39.7± 1.0	0.4 ±0.04	18.8 ± 1.2	0.05± 0.01	0.06 ± 0.02	16.7 ± 1.5	0.02±0.0 01	0.10 ±0.02	1.2±0.4	0.3±0.1	3.0±0.6

Data are presented as mean ± SEM (n = 3). AB- *A. boonei*; AC- *A. congensis*

Cu: not observed in any of the plant and their part

ABL: *A. boonei* leaf

ABS: *A. boonei* stem-bark

ACL: *A. congensis* leaf

ACS: *A. congensis* stem-bark

Table 4.6: Phytochemical constituents of *Alstonia boonei* and *Alstonia congensis*

Samples	Flavonoid	Tannins	Alkaloids	Anthraquinones	Saponins	Cardiac-glycoside
ABL	+	++	++	-	++	+
ABS	-	++	++	-	++	+
ACL	++	++	++	-	++	++
ACS	+	++	++	-	++	++

leaves and stem-barks

ABL: *A. boonei* leaf

ABS: *A. boonei* stem-bark

ACL: *A. congensis* leaf

ACS: *A. congensis* stem-bark

Key notes-: - = absent; + = present; ++ = highly present

Table 4. 7: Fluorescence characters of leaves and stem-barks of *Alstonia boonei* and*Alstonia congensis* powder

Treatment	ABL			ABS			ACL			ACS		
	Daylight	UV Light		Daylight	UV Light		Daylight	UV Light		Daylight	UV Light	
		254 nm	365 nm		254 nm	365 nm		254 nm	365 nm		254 nm	365 nm
1N HCl	Dark green	Purple	Purple	Brown	Yellow	Purple	Dark Green	Dark Brown	Purple	Brown	Yellow	Purple
1 N NaOH (aq)	Black	Black	Dark Purple	Amber Brown	Black	Deep brown	Black	Black	Purple	chocolate brown	Black	Deep brown
50% KOH	Brown	Deep brown	Brown	Amber Brown	Black	Deep Brown	Brown	Deep brown	Brown	Amber Brown	Black	Deep Brown
50% HNO_3	Brown	purple	purple	Reddish brown	Brown	Purple	Reddish Brown	Black	Dark purple	Reddish brown	Brown	Purple
Acetic acid	Brown	Black	Dark purple	Brownish	Brown	Purple	Deep brown	Black	Purple	Brownish	Brown	Purple
Iodine water	Black	Black	Purple	Reddish brown	Brown	Yellowish brown	Blue black	black	Deep blue	Reddish brown	Brown	Yellowish brown
Acetic acid	Blue black	black	black	Blue black	black	Deep blue	Black	Black	Purple	Blue black	black	Deep blue
$FeCl_3$	Dark green	Black	Purple	Deep brown	Black	Purple	Carton Brown	Dark green	Purple	Deep brown	Black	Deep purple
HNO_3 + NH_3	Dark green	Black	Purple	Deep brown	Black	Purple	Dark green	Black	Deep purple	Brown	Deep black	purple
Picric acid	Black	Black	Deep blue	Yellow	Orange	Orange	Black	Black	Deep blue	Yellow	Orange	Orange

ABL: *A. boonei* leaf

ABS: *A. boonei* stem-bark

ACL: *A. congensis* leaf

ACS: *A. congensis* stem-bark

Table 4.8.1: Percentage yields of *Alstonia boonei* and *Alstonia congensis* aqueous crude extracts

Taxa	Morphological Part	Weight (Kg)	Yield (g)	Percentage yield (%)
A. boonei	Leaf	4.0	140	3.5
A. boonei	Stem-bark	4.0	186	4.7
A. congensis	Leaf	4.0	130	3.0
A. congensis	Stem-bark	4.0	150	3.6

Table 4.8.2: Yields of *Alstonia boonei* and *Alstonia congensis* partitioned fractions from aqueous crude extracts

Samples	Sample weight (g)	DCM (g)	Ethyl acetate (g)	Aqueous (g)
A. boonei leaf	130.0	3.14	0.11	125.31
A. boonei stem-bark	176.0	7.64	0.32	147.43
A. congensis leaf	100.0	2.95	0.54	87.80
A. congensis stem-bark	100.0	8.32	0.79	80.04

Key

DCM: Dichloromethane

4.9 Antispasmodic assay of crude extracts and fractions of *Alstonia boonei* and *Alstonia congensis*

Table 4.9 presents the IC_{50} values of antispasmodic activities of leaf and stem-bark crude extracts (ABLC, ACLC, ABSC, ACSC) and fractions (ABSD, ABSE, ABSA, ACSD, ACSE and ABSA) of *A. boonei* and *A. congensis* on spontaneous and high potassium (K^+ 80 mM) induced contraction on isolated rat ileum. ABLC, ACLC, ABSC and ACSC showed antispasmodic activity on both spontaneous and high potassium induced contraction of isolated rat ileum within the concentration range of 0.003-10 $mgmL^{-1}$ (Figures 4.9.1, 4.9.2, 4.9.3, 4.9.4), respectively with IC_{50} values of (0.98± 0.3, 0.22±0.5), (1.68 ±0.98, 0.25±0.05), (1.15±0.1, 0.03± 0.2) and (1.05±0.80, 0.12±0.01), respectively (Table 4.9). Out of all the fractions, ABSD showed the highest relaxation activity (Figure 4.9.5) on both spontaneous and high potassium induced contraction on isolated rat Ileum with IC_{50} values (0.31± 0.02, 0.02±0.05) while ACSA showed spasmodic activity on spontaneous contraction on rat Ileum (Figure 4.9.10) (Appendices VIII-IX).

4.10 Antidiarrhoeal activities of *Alstonia boonei* and *Alstonia congensis* stem-bark crude extracts and DCM fractions

Table 4.10 presents the antidiarrhoeal activity of aqueous crude extracts and DCM fractions of *A. boonei* and *A. congensis* stem-bark. Both *A. boonei* and *A. congensis* crude extracts (ABSb and ACSb) showed dose dependent antidiarrhoeal activity as the activities increases with increase in dosing. ABSb at 200 mg/kg exhibited higher percentage diarrhoeal inhibition at 76.6% compared to ACSb 200 mg/kg which showed 70.0% diarrhoeal inhibition. The DCM fractions of *A. boonei* and *A. congensis* stem-bark also showed dose dependent diarrhoeal inhibition activity as shown in figure 4.10.

4.11 Formulated Herbal Preparation

Plate 4.11 shows the samples of formulated antidiarrhoeal remedy produced from *Alstonia boonei* stem-bark. An adult patient would be expected to take between 1.5 g and 2.0 g (DIASTIL powder) once or twice daily while children above 5 years would take between 5 mL - 10 mL (0.25-0.50 g) (DIASTIL Syrup) once or twice daily.

Table 4.9: Antispasmodic effect of *Alstonia boonei* and *Alstonia congensis* Crude extracts and fractions

Extracts	IC_{50} ($mgmL^{-1}$)	
	Spontaneous	$[K^+]$ (80 mM)
ABLC	0.98± 0.31	0.22±0.5
ABLD	0.36 ± 0.16	0.03 ± 0.01
ABLE	1.68 ± 0.73	0.8±0.05
ABLA	2.57 ± 1.44	1.00± 0.2
ABSC	1.15±0.1	0.03± 0.2
ABSD	0.31± 0.02	0.02±0.05
ABSE	0.35 ± 0.03	0.03 ± 0.14
ABSA	2.38±0.65	0.90 ±0.06
ACLC	1.68 ±0.98	0.25±0.05
ACLD	0.39 ±0.24	0.04± 0.001
ACLA	8.61 ±2.91	2.76 ±0.10
ACLE	0.42± 0.02	0.03±0.01
ACSC	1.05± 0.80	0.12±0.01
ACSD	0.34±0.13	0.05±0.01
ACSE	2.84± 0.12	0.52± 0.09
ACSA	-	-

ABLC: *A. boonei* leaf crude extract; ABLD: *A. boonei* leaf DCM fraction; ABLE: *A. boonei* leaf ethyl acetate fraction; ABLA: *A. boonei* leaf aqueous fraction; ABSC: *A. boonei* stem-bark crude extract; ABSD: *A. boonei* stem-bark DCM fraction; ABSE: *A. boonei* stem-bark ethyl acetate fraction; ABSA: *A. boonei* stem-bark aqueous fraction; ACLC: *A. congensis* leaf crude extract; ACLD: *A. congensis* leaf DCM fraction; ACLE: *A. congensis* leaf ethyl acetate fraction; ACLA: *A. congensis* leaf aqueous fraction; ACSC:*A. congensis* stem-bark crude extract; ACSD: *A. congensis* stem-bark DCM fraction; ACSE: *A. congensis* stem-bark ethyl acetate fraction; ACSA: *A. congensis* stem-bark aqueous fraction.

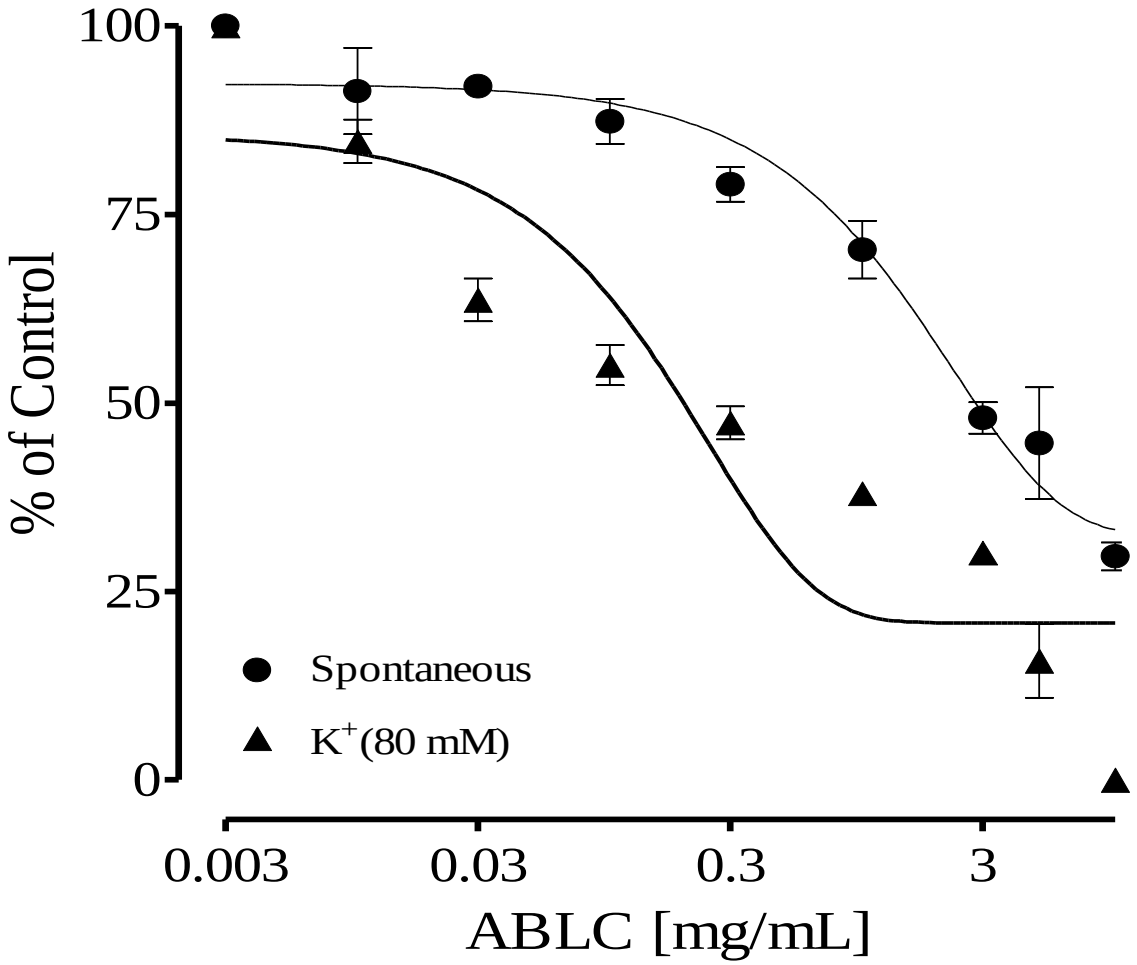


Figure 4.9.1: Antispasmodic effects of crude aqueous extract of *Alstonia boonei* leaf

Values: Mean ± SEM, n= 3. ABLC showed increased antispasmodic (relaxation) activity.

Note: the 100 % is the initial contraction before treatment (-ve control)

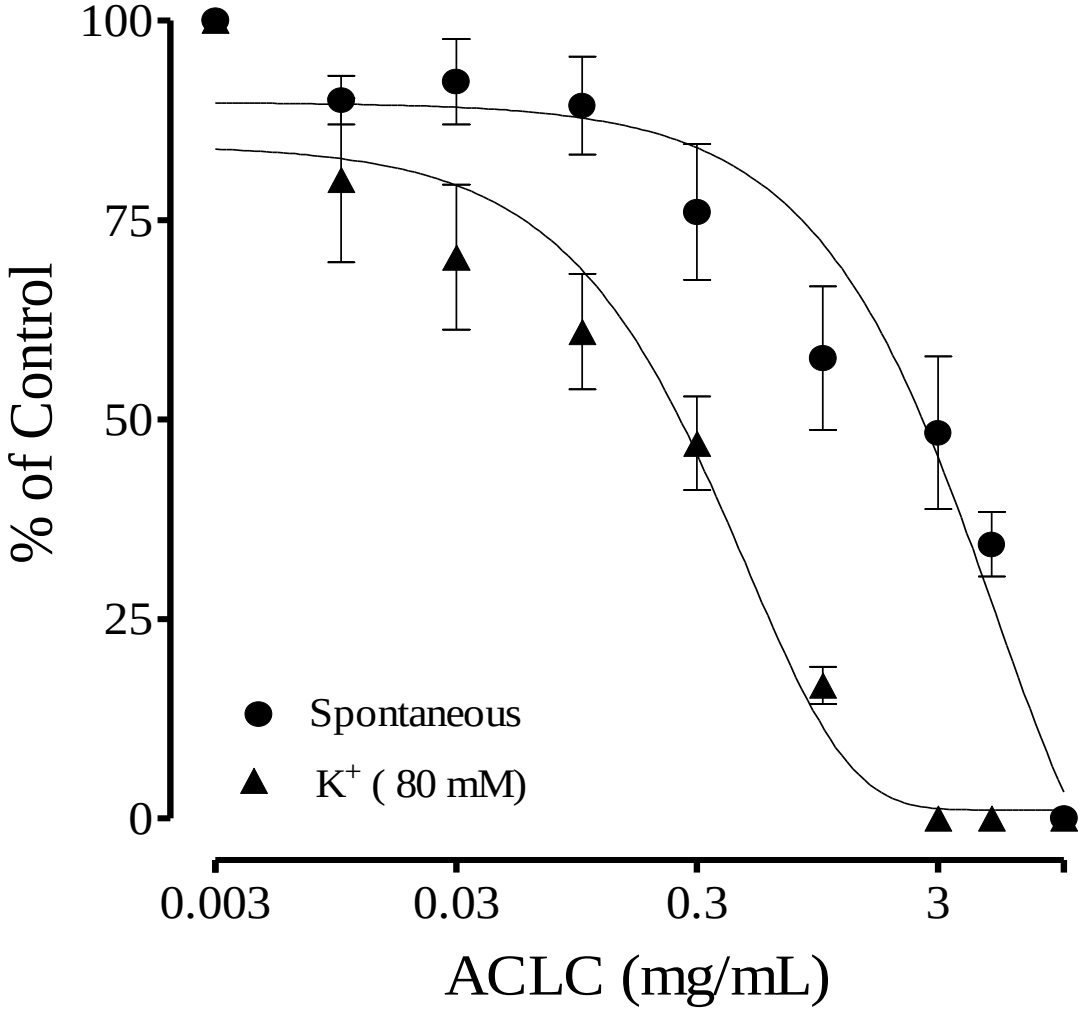


Figure 4.9.2: Antispasmodic effects of aqueous extract of *Alstonia congensis* leaf

Values: Mean ± SEM, n= 3 for each. ACLC showed increased antispasmodic (relaxation) activity.

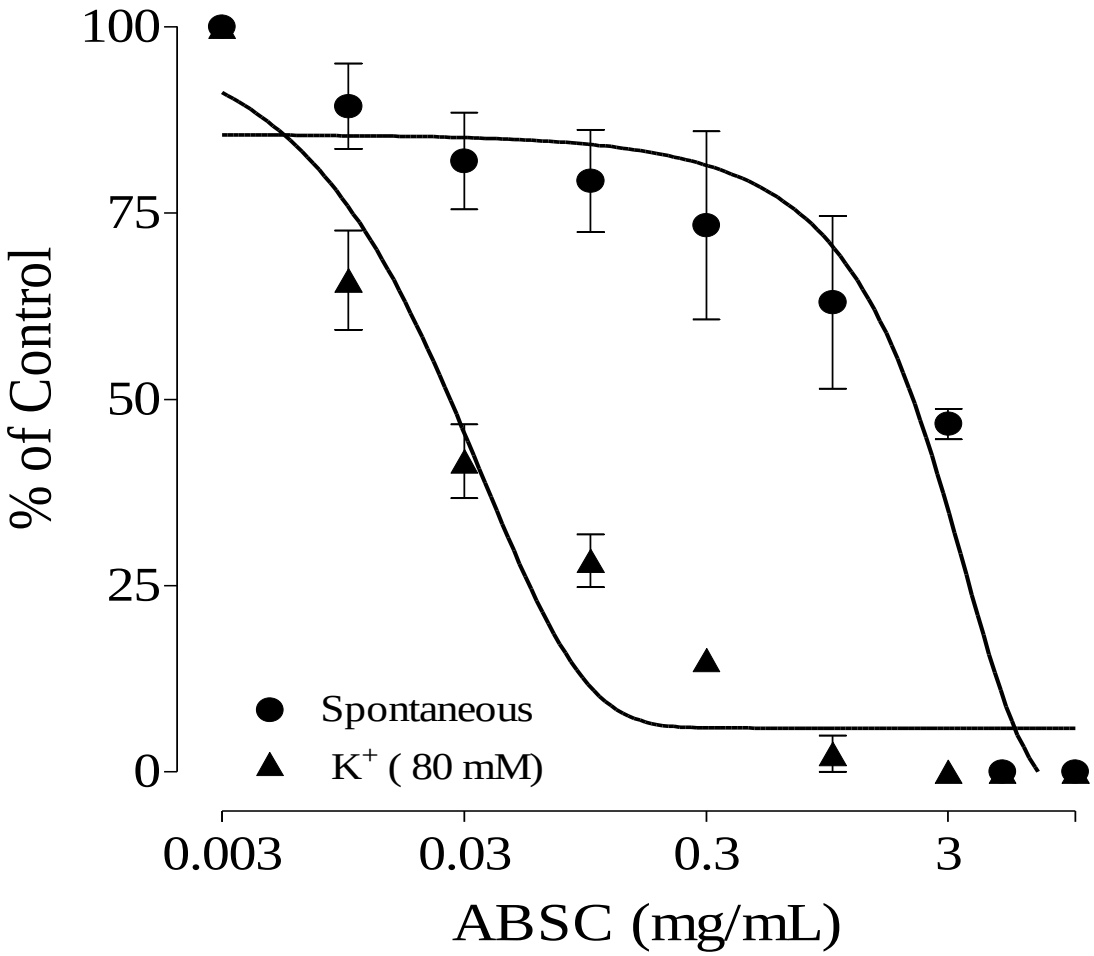


Figure 4.9.3: Antispasmodic effects of aqueous extract of *Alstonia boonei* stem-bark. Values: Mean ± SEM, n= 3. ABSC showed increased antispasmodic activity.

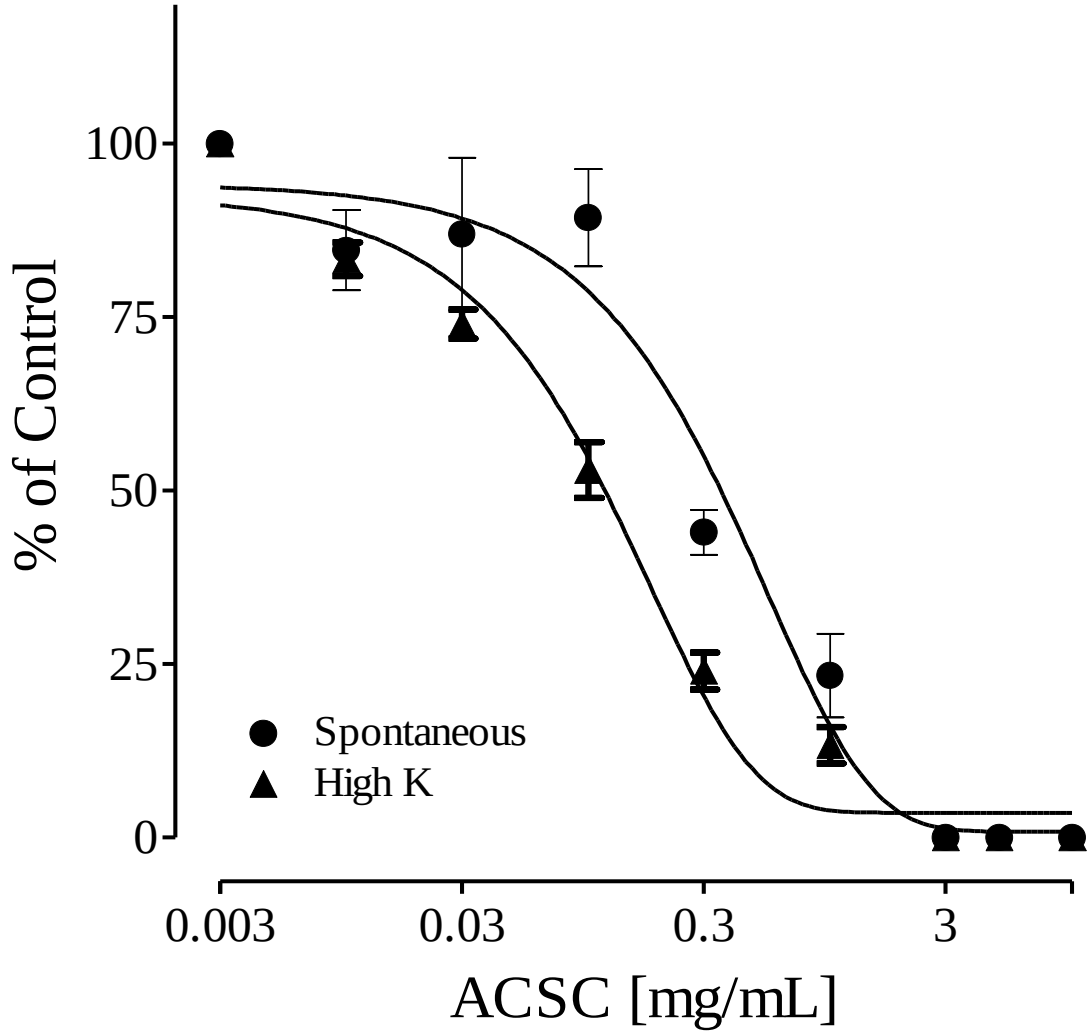


Figure 4.9.4: Antispasmodic effects of aqueous crude extract of *Alstonia congensis* stem-bark. Values: Mean ± SEM, n= 3. ACSC showed increased antispasmodic activity

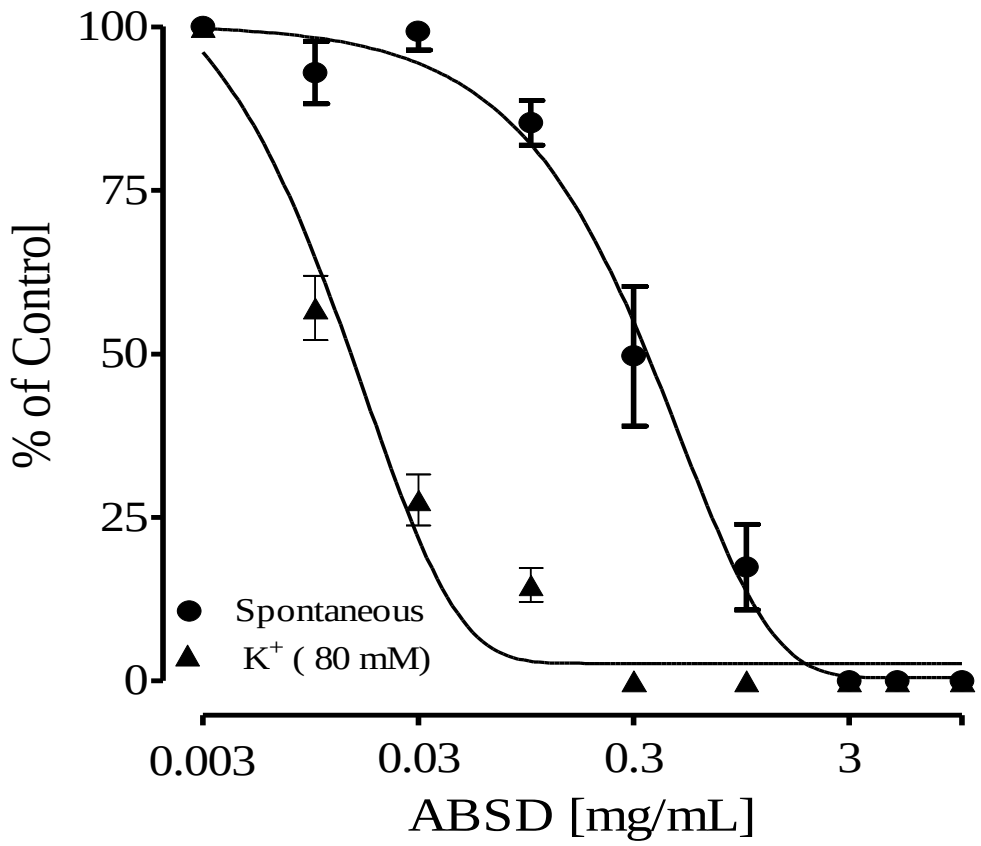


Figure 4.9.5: Antispasmodic effects of dichloromethane fraction of *Alstonia boonei* stem-bark. Values: Mean ± SEM, n= 3 for each. ABSD showed increased antispasmodic activity

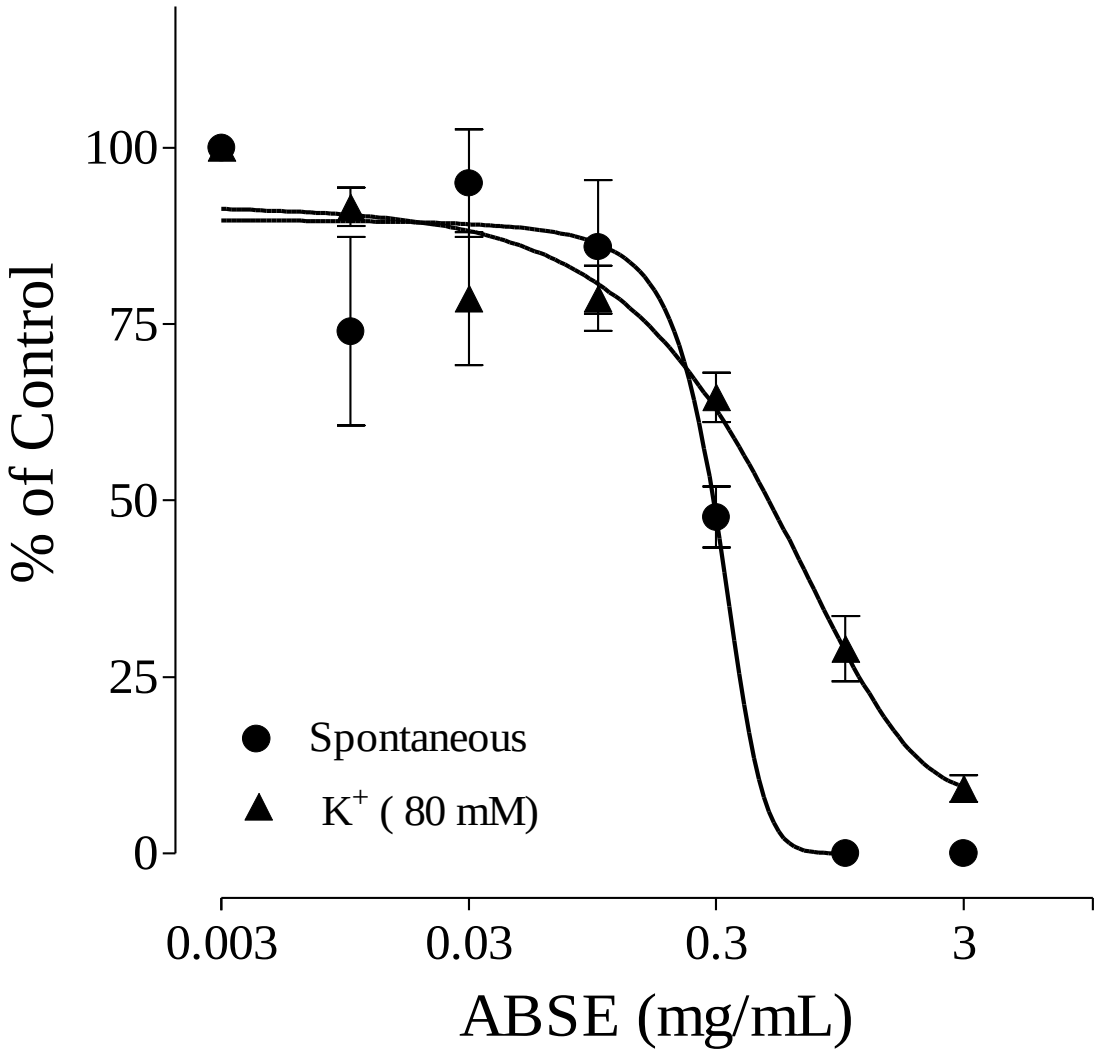


Figure 4.9.6: Antispasmodic effects of ethyl acetate fraction of *Alstonia boonei* stem-bark. Values for each: Mean ± SEM, n= 3. ABSE showed increased antispasmodic activity

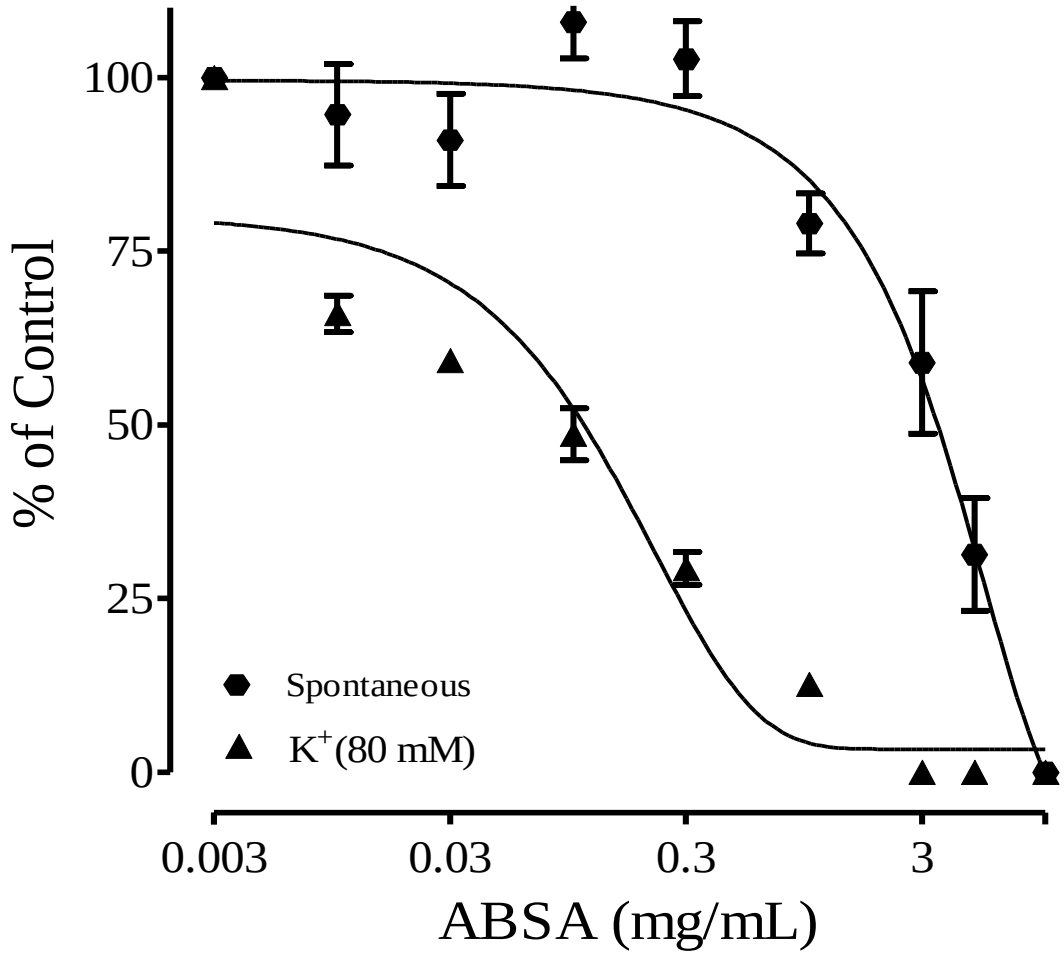


Figure 4.9.7: Antispasmodic effects of aqueous fraction of *Alstonia boonei* stem-bark.

Values: Mean ± SEM, n= 3 for each.

ABSA showed slight spasmodic (contraction at 0.01-0.3 $mgmL^{-1}$) and later showed antispasmodic activity

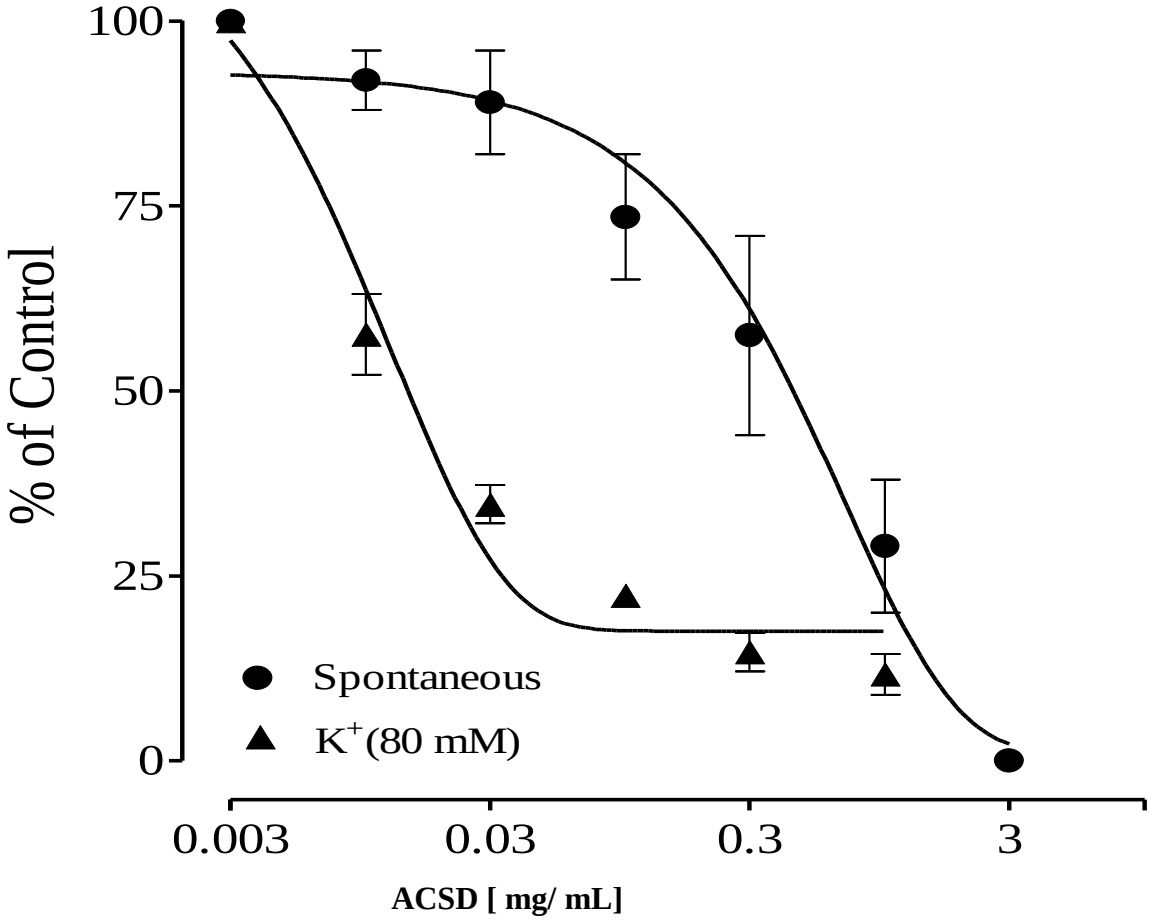


Figure 4.9.8: Antispasmodic effects of dichloromethane fraction of *Alstonia. congensis* stem-bark.

Values: Mean ± SEM, n= 3.

ACSD showed increased antispasmodic activity

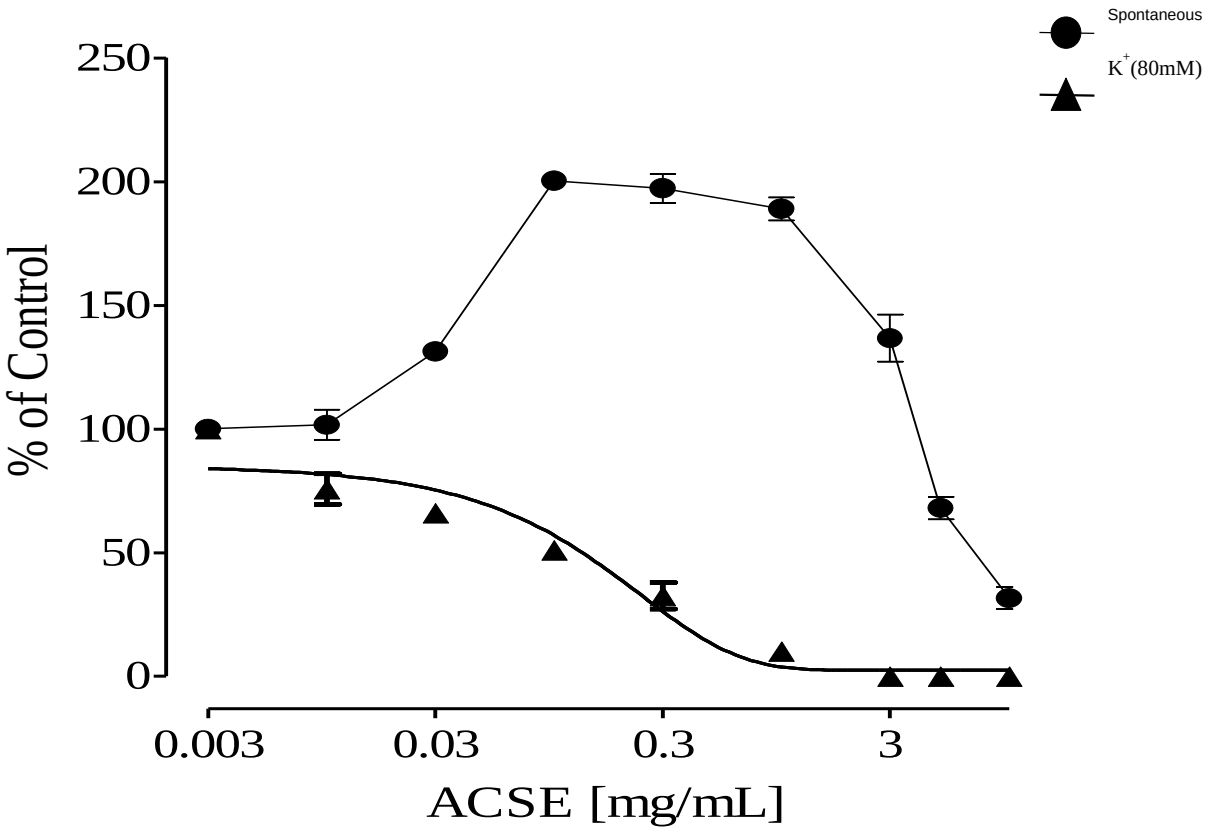


Figure 4.9.9: Antispasmodic effects of ethyl acetate fraction of *Alstonia congensis* stem-bark

Values: Mean ± SEM, n= 3.

ACSE showed spasmodic activity at (0.001-0.3 $mgmL^{-1}$) and antispasmodic activity at 0.1-10 $mgmL^{-1}$

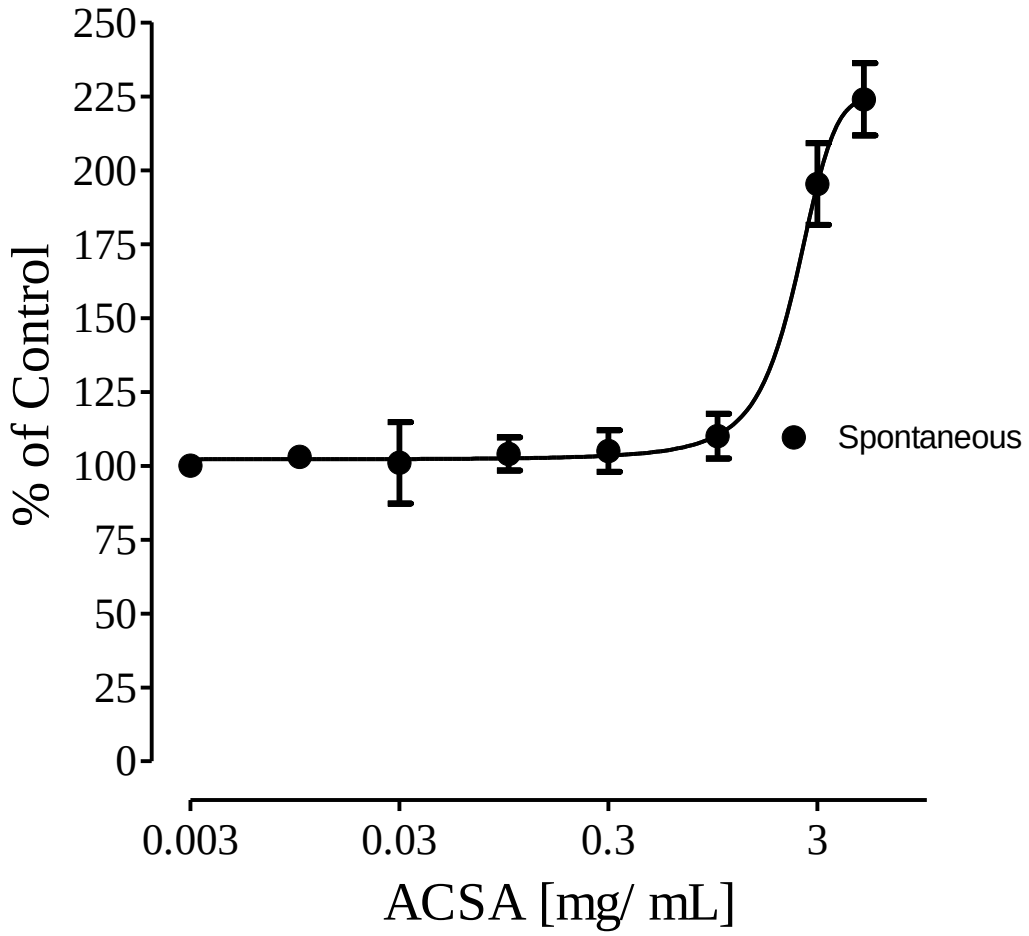


Figure 4.9.10: Spasmodic effects of aqueous fraction of *Alstonia congensis* stem-bark

Values: Mean ± SEM, n= 3.

ACSA showed no activity 0.003-0.3 $mgmL^{-1}$ and showed spasmodic activity from 1-10 $mgmL^{-1}$

Table 4.10: *A*ntidiarrhoeal activities of *Alstonia boonei* and *Alstonia congensis* stem-bark crude extracts and DCM fractions on castor oil induced diarrhoeal mice

Dose (mg/ kg)	Onset time of diarrhoea (min)	Total defaecation	Number of wet faeces	% Inhibition of diarrhoea
Negative control	27.7 ± 2.8	11.8 ±2.15	11.0 ± 1.8	0.0
Loperamide 5	73.2 ± 5.4***	3.6 ± 0.5***	1.4 ± 0.4	87.5
ABSb 100	44. 7 ± 2.1	8.6 ± 1.6	5.6 ± 1.2	49.1
ABSb 200	65.1 ± 3.5***	4.8 ± 1.0***	2.6 ± 0.6	76.3
ACSb 100	44.1 ±2.0	8.4 ± 2.0	6.0 ± 1.6	45.5
ACSb 200	66.7 ± 1.0***	4.4± 0.9***	3.2 ± 0.4	70.9
ABSD 50	45.0 ± 2.8	7.8 ±0.8	5.0 ± 0.6	54.5
ABSD 100	57.0 ± 2.1*	4.0 ± 1.2*	3.4 ± 0.2	69.1
ABSD 200	64. 9 ± 5.0***	3.6 ± 1.2***	1.4 ± 1.2	87.3
ACSD 50	45.6 ± 4.9	8.4± 1.2	5.6 ± 0.8	49.1
ACSD 100	59.1 ±2.0*	6.6 ± 0.5*	3.0 ± 0.9	73.0
ACSD 200	68.6 ± 1.0***	4.0 ± 0.7***	2.1 ± 0.8	81.0

Data are presented as mean ± SEM (n = 5). *P < 0.05, **P < 0.001, ***P < 0.0001, compared with negative control group and evaluated with one-way ANOVA followed by a Tukey multiple comparison post hoc test). ABSb: *Alstonia boonei* stem-bark aqueous crude extract; ACSb: *Alstonia congensis* stem-bark aqueous crude extract; ABSD: *Alstonia boonei* stem-bark dichloromethane fraction; ACSD: *Alstonia congensis* stem-bark dichloromethane fraction

Table 4.11:Antidiarrhoeal effects of *Alstonia boonei* stem-bark and *Picralima nitida* seed on castor oil induced diarrhoeal mice.

Dose (mg/ kg)	Total defaecation	Number of wet faeces	% Inhibition of diarrhoea
Negative control	13.0 ±3.1	13.0 ± 3.1	0.0
Loperamide 5	5.7 ± 0.5***	2.5 ± 0.4	80.7
AP (100)	7.0±1.2	7.3±0.5	44.0
AP (200)	6.3±0.7**	4.9±0.02	62.3
AP (400)	5.7±1.2***	3.2 ± 1.2	75.3
AP (500)	5.0±0.5***	2.5 ± 0.6	80.0
AP2 (100)	6.5±0.8*	7.0 ± 1.6	46.2
AP2 (200)	6.0±1.3**	4.4 ± 0.4	66.2
AP 2(400)	5.4±2.1***	3.0 ± 0.02	77.0
AP2 (500)	5.0±1.5***	1.4 ± 0.6	89.0
AP 3 (100)	6.7±0.90*	6.0 ± 0.1	51.5
AP 3 (200)	5.5.±0.10***	3.6 ± 1.2	72.3
AP 3 (400)	5.6±1.20***	2.6 ± 0.01	80.0
AP 3(500)	4.8 ± 0.02***	1.2 ± 0.02	91.1

Data are presented as mean ± SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, compared with negative group (one-way ANOVA followed by a Tukey multiple comparison post hoc test)

Plate 4.11: Formulated and packaged DIASTIL Syrup for children and DIASTIL Powder for adults containing only *A. boonei* stem-bark

4.12 Isolation of compounds from DCM fraction of *Alstonia boonei* stem-bark

Table 4.12 presents the column chromatographic fractions collected during isolation of compounds from the DCM fraction of *A. boonei* stem-bark while Plate 4.12 shows the TLC profile of pure compounds A and B isolated from the DCM fractions of *A. boonei* stem-bark.

4.13 Antispasmodic assay of isolated compounds A and B from *Alstonia boonei* DCM fraction

Figures 4.13.1 and 4.13.2 show the antispasmodic activities of compounds A and B isolated from the DCM fraction of *A. boonei* stem-bark on spontaneous and high potassium (80 mM) induced contraction on isolated rat ileum, Compound A showed concentration dependent antispasmodic activities at concentrations 0.003-10.0 μgmL^{-1} on spontaneous contraction with IC_{50} value of 0.29 ±0.05 while Compound B showed spasmodic activities at concentrations 0.003-5 μgmL^{-1} and also showed antispasmodic effect at 10 μgmL^{-1} on spontaneous contraction with IC_{50} value of 2.2 ±0.7. Table 4.13 presents the IC_{50} values of antispasmodic activities of compounds A and B isolated from the DCM fraction of *A. boonei* stem-bark on spontaneous and high potassium (80 mM) induced contraction on isolated rat ileum.

4.14 Characterisation and structure elucidation of isolated compounds A and B from DCM fraction of *Alstonia boonei* stem-bark

Table 4.14.1 presents the ^{1}HNMR and ^{13}CNMR chemical shifts of compound A, while Table 4.14.2 presents the ^{1}HNMR and ^{13}CNMR chemical shifts of compound B. Figures 4.14.1- 4.14.9 show the mass spectra, ^{1}HNMR, ^{13}NMR and 2-DNMR spectra of compound A while Figures 4.14.10- 4.14.15 show the mass spectra, ^{1}HNMR, ^{13}NMR and 2-DNMR spectra of compound B.

Table 4.12: Fractions from the column chromatography of *Alstonia boonei* stem-bark Dichloromethane fraction

S/N	Eluting solvents				Pooled fractions	number for pooled fraction	Weight (mg)
	N-Hex	DCM	ETOAC	MeOH			
1-10	100	-	-	-	1-10	i	10.0
11-20	80	20	-	-			
21-30	60	40	-	-	11-20	ii	50.0
31-40	40	60	-	-			
41-50	20	80	-	-	21-40	iii	150.0
51-60	-	100	-	-	41-60	iv	15.0
61-70	-	80	20	-	61-80	v	80.0
71-80	-	60	40	-	81-90	vi	2.0
81-90	-	40	60	-			
91-100	-	20	80	-	91-110	vii	3.0
101-110	-	-	100	-	-		
111-120	-	-	80	20	111-130	viii	8.0
121-130	-	-	60	40	-		
131-140	-	-	40	60	131-140	ix	5.0
141-150	-	-	20	80	141-150	x	5.0

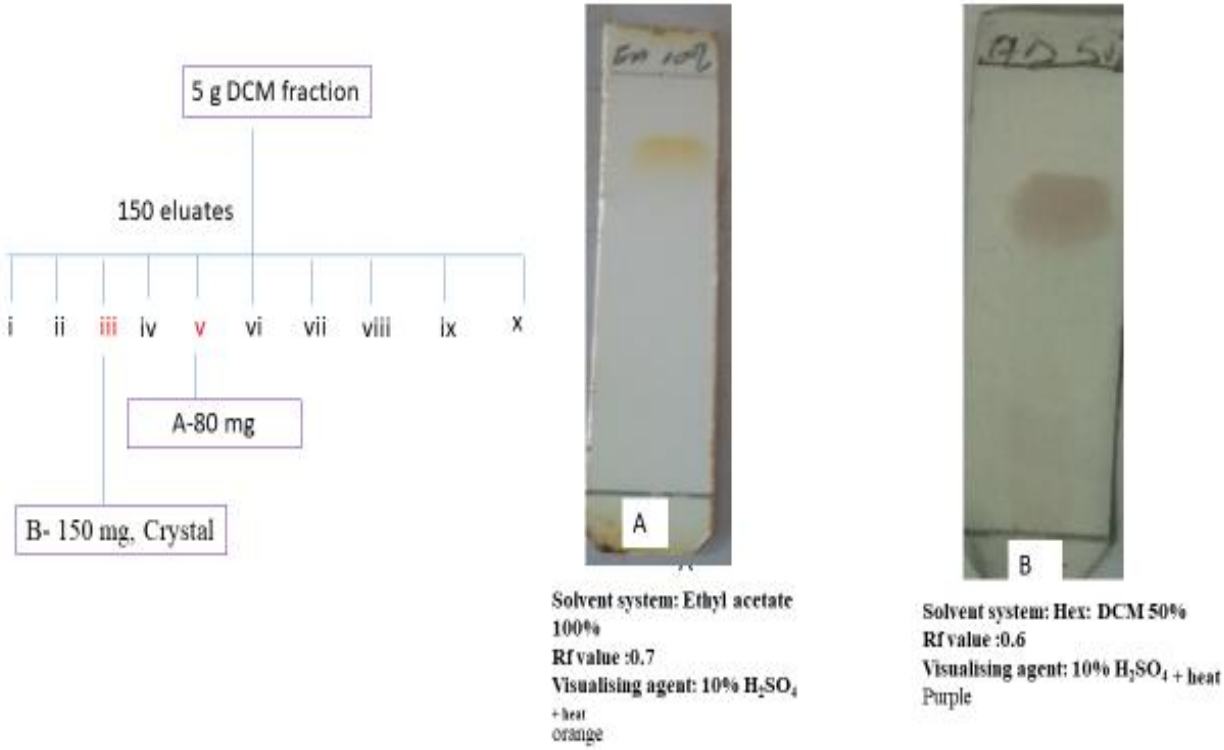


Plate 4.12: Thin layer chromatography of isolated compounds A and B from DCM fraction of *Alstonia boonei* stem-bark

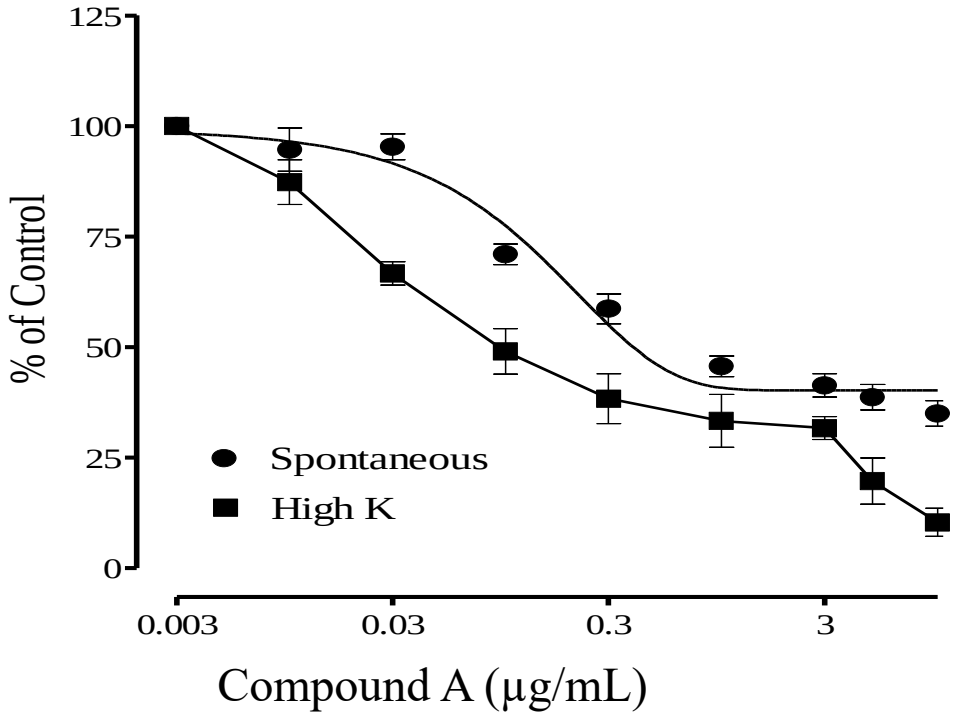


Figure 4.12.1: Effects of Compound A isolated from *Alstonia boonei* stem-bark on contraction of isolated rat ileum. Compound A showed increase total relaxation activities on both spontaneous and high potassium induced contraction

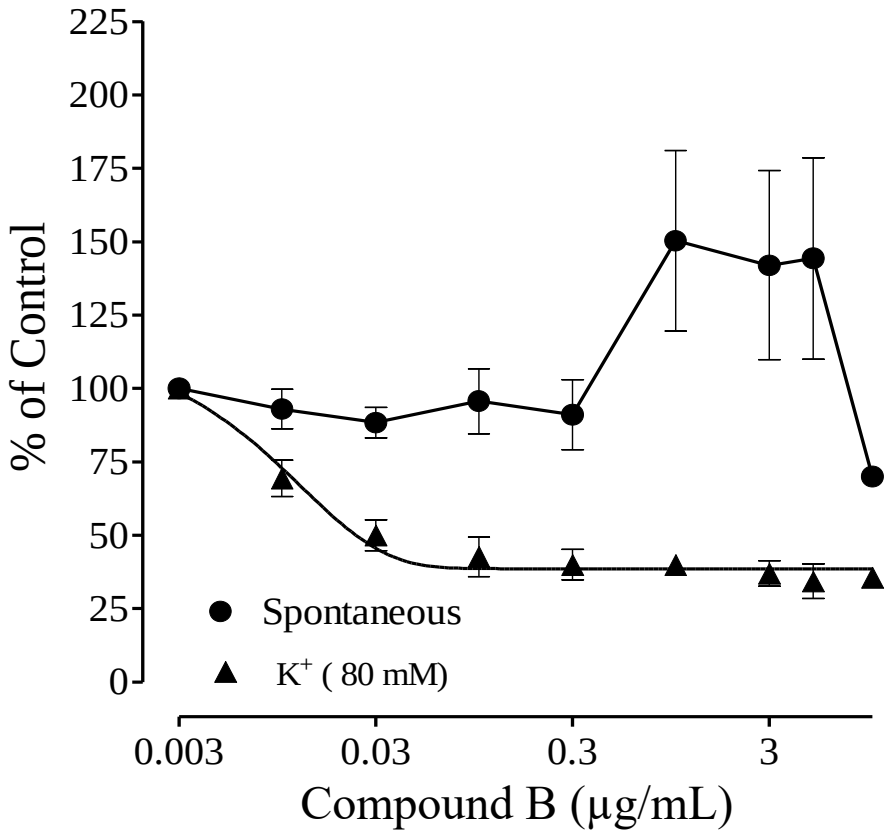


Figure 4.12.2: Effects of Compound B isolated from *Alstonia boonei* stem-bark on contraction of isolated rat ileum. Compound B showed spasmodic (contraction) activities at concentrations 0.003-5 μgmL^{-1} and showed antispasmodic (relaxing) effect at 10 μgmL^{-1}

Table 4.13: Effects of compounds A and B isolated from the dichloromethane fraction of *Alstonia boonei* stem-bark on isolated rat ileum

Compounds	IC_{50} (µg/mL)	
	Spontaneous	K^+ (80 mM)
A	0.29 ±0.05	0.09 ±0.01
B	2.2 ±0.7	0.9 ±0.1
Verapamil	0.22 ±0.01	0.04 ±0.06

Data are presented as mean ± SEM (n = 3) and evaluated with non-Linear regression curve.

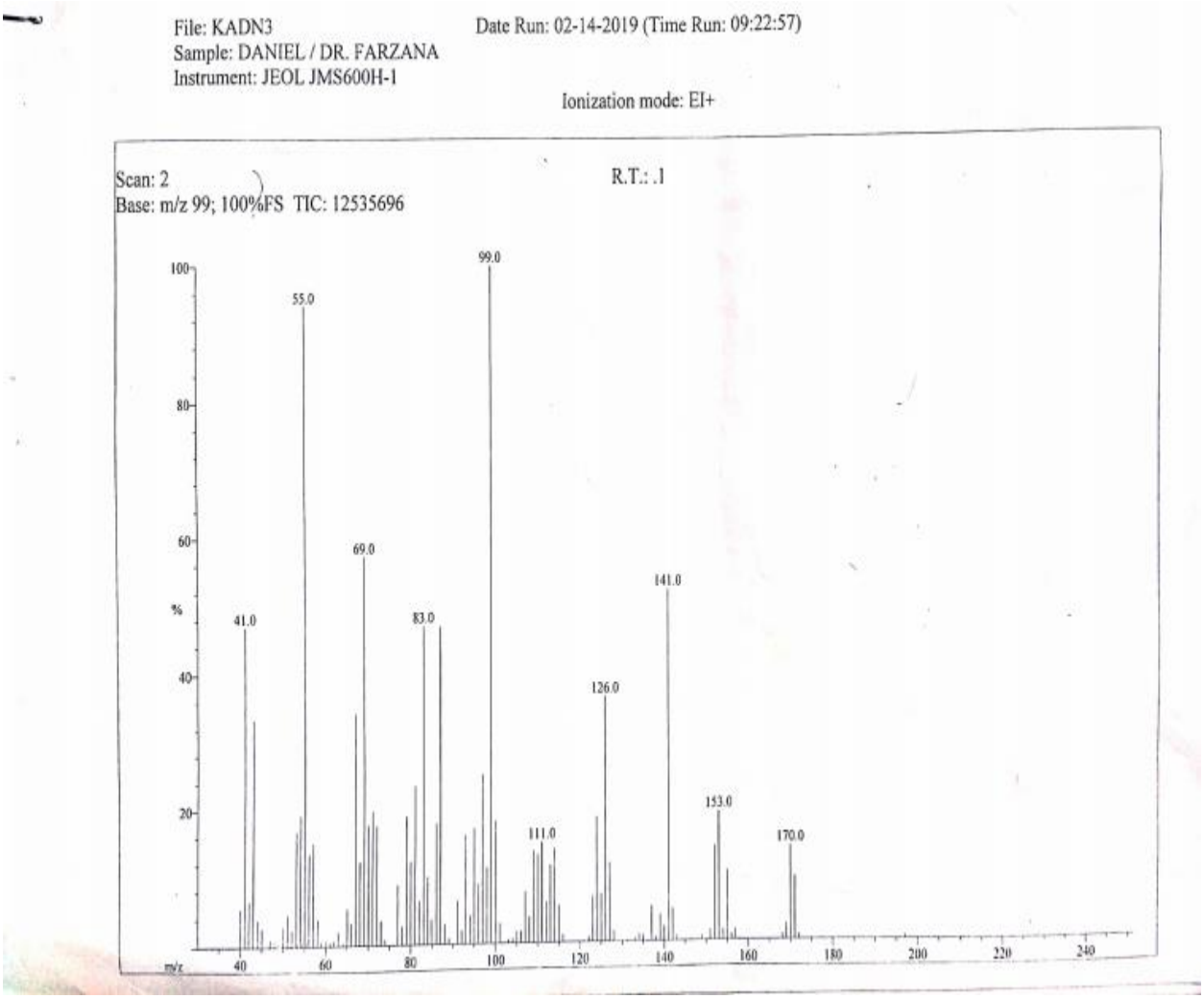


Figure 4.14.1: Mass Spectrum of Compound A isolated from DCM fraction of *Alstonia boonei* stem-bark

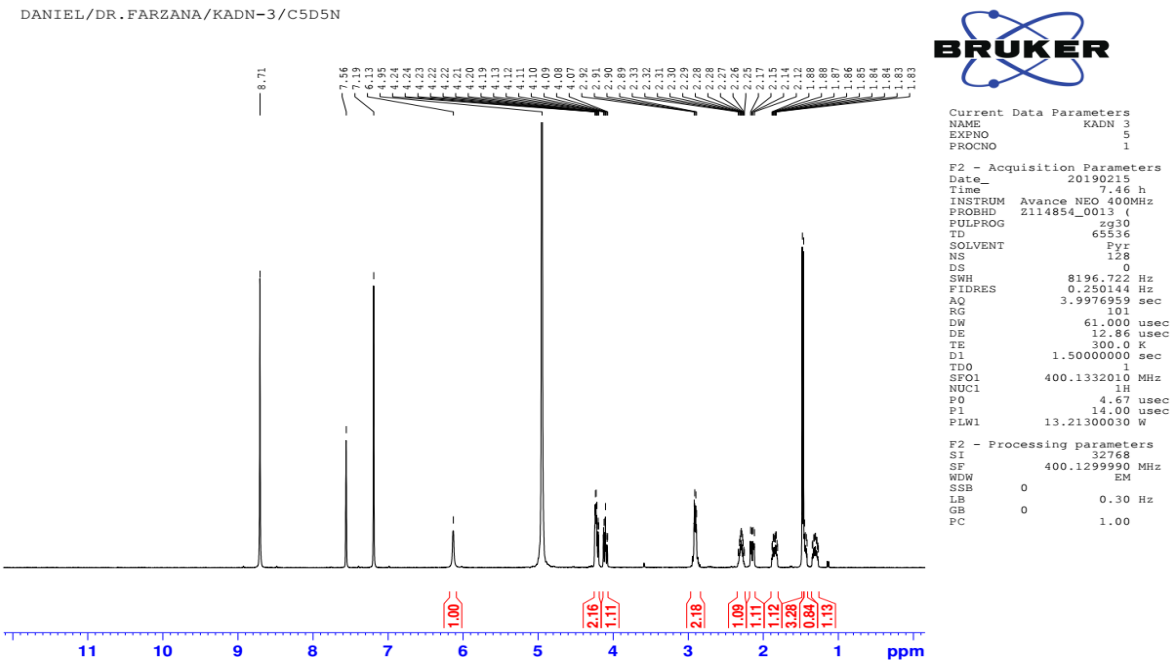


Figure 4.14.2: Proton (^{1}H) NMR Spectroscopy of compound A isolated from DCM fraction of *Alstonia boonei* stem-bark

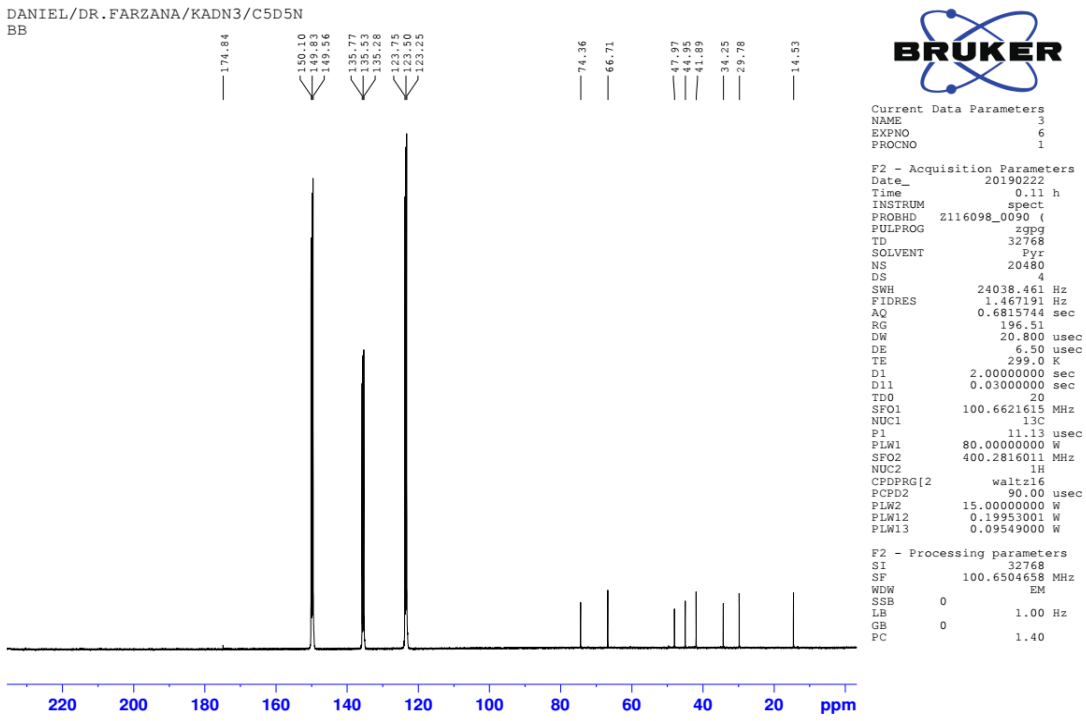


Figure 4.14.3: Carbon 13 (^{13}C) NMR Spectroscopy of compound A isolated from DCM fraction of *Alstonia boonei* stem-bark

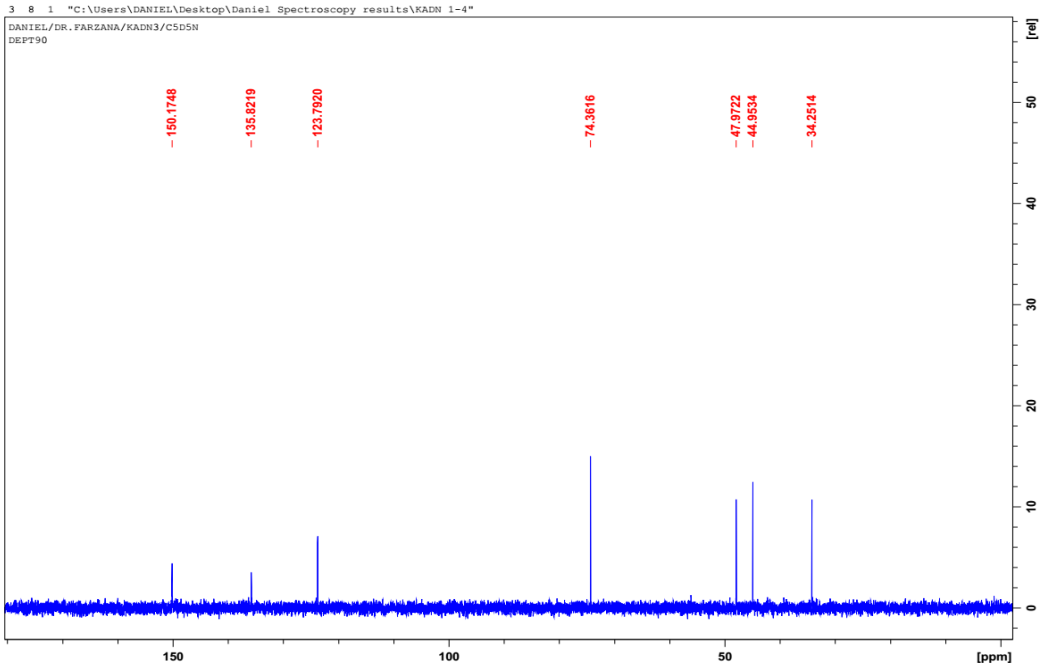


Figure 4.14.4: DEPT 90 (Distortionless Enhancement by Polarisation Transfer) Spectrum of compound A isolated from DCM fraction of *Alstonia boonei* stem-bark

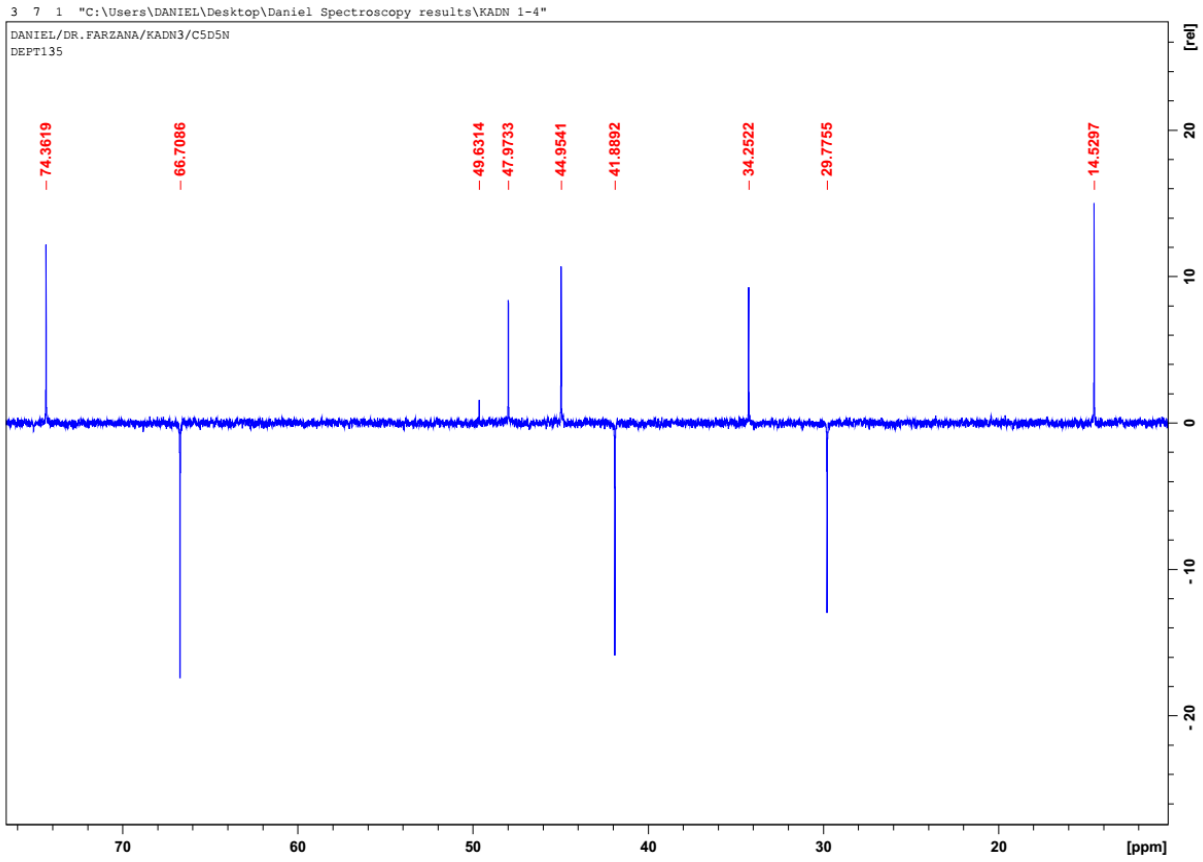


Figure 4.14.5: DEPT 135 (Distortionless Enhancement by Polarisation Transfer) Spectrum of compound A isolated from DCM fraction of *Alstonia boonei* stem-bark

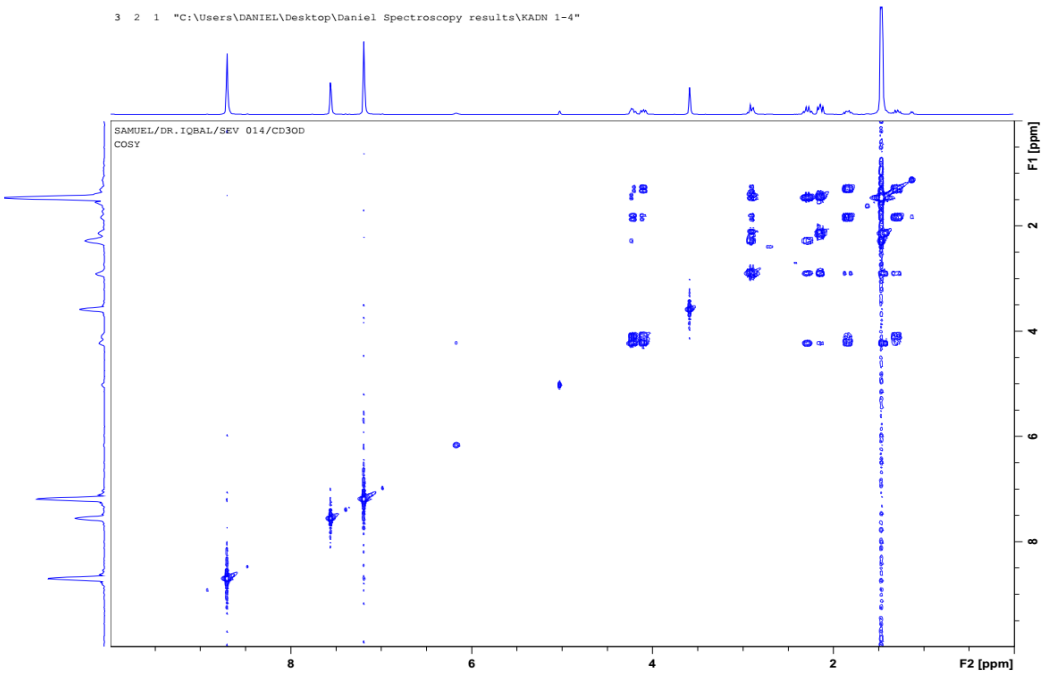


Figure 4.14.6: COSY Spectrum of Compound A isolated from DCM fraction of *Alstonia boonei* stem-bark

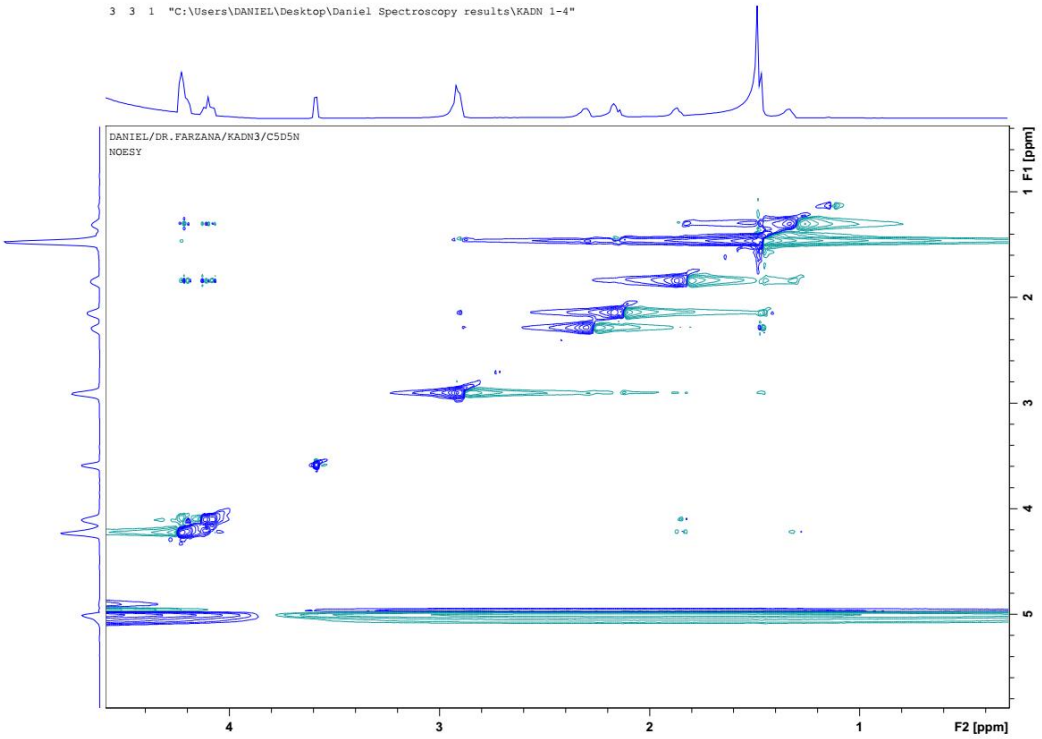


Figure 4.14.7: NOESY spectrum of Compound A isolated from DCM fraction of *Alstonia boonei* stem-bark

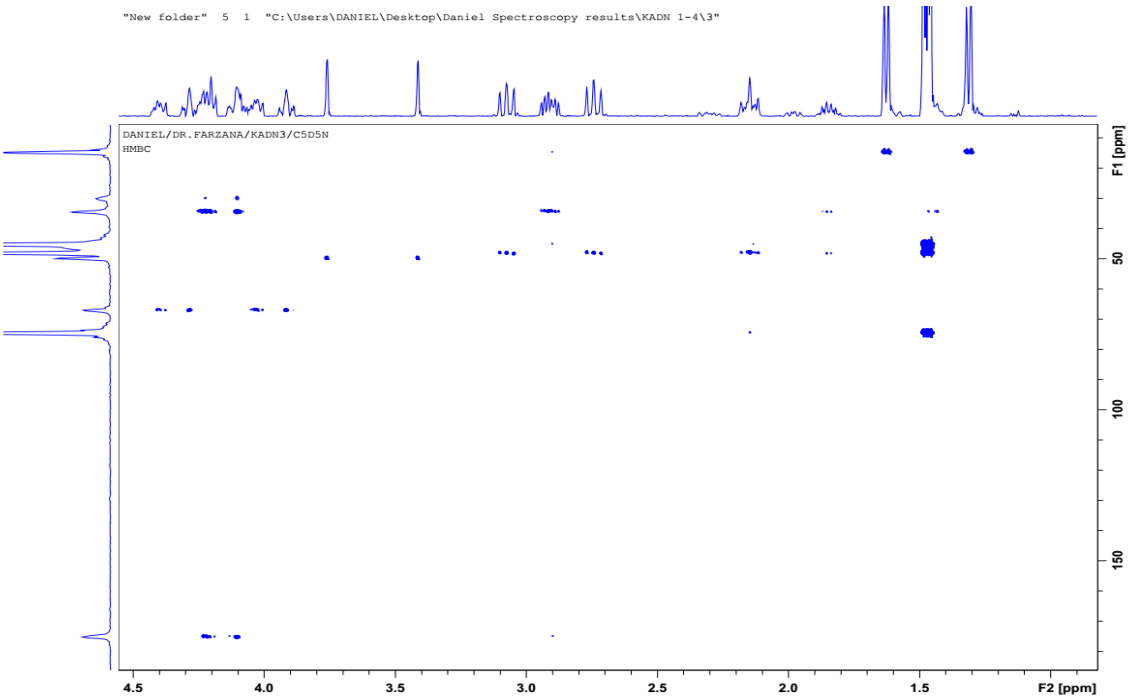


Figure 4.14.8: Heteronuclear Multiple Bond Correlation Spectrum of Compound A isolated from DCM fraction of *Alstonia boonei* stem-bark

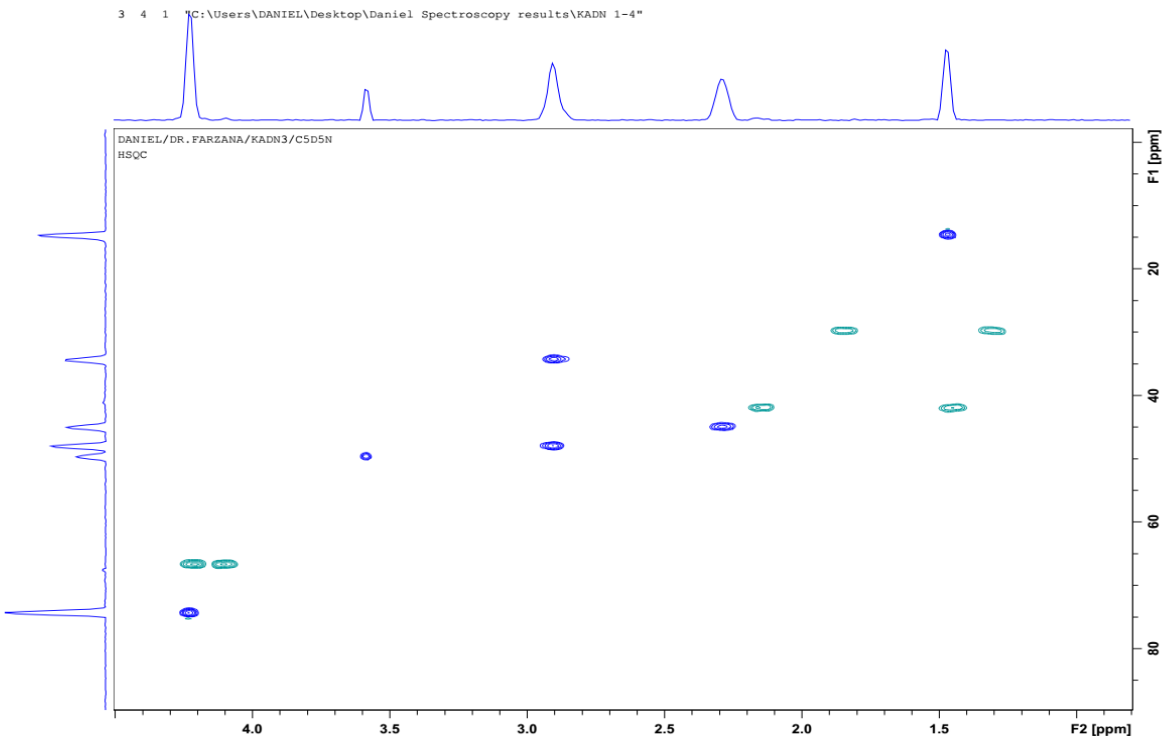


Figure 4.14.9: Heteronuclear Single Quantum Coherence spectrum of Compound A isolated from DCM fraction of *Alstonia boonei* stem-bark

Mass Spectrum, 1D and 2D- NMR of compound B isolated from DCM fraction of *Alstonia boonei* stem-bark

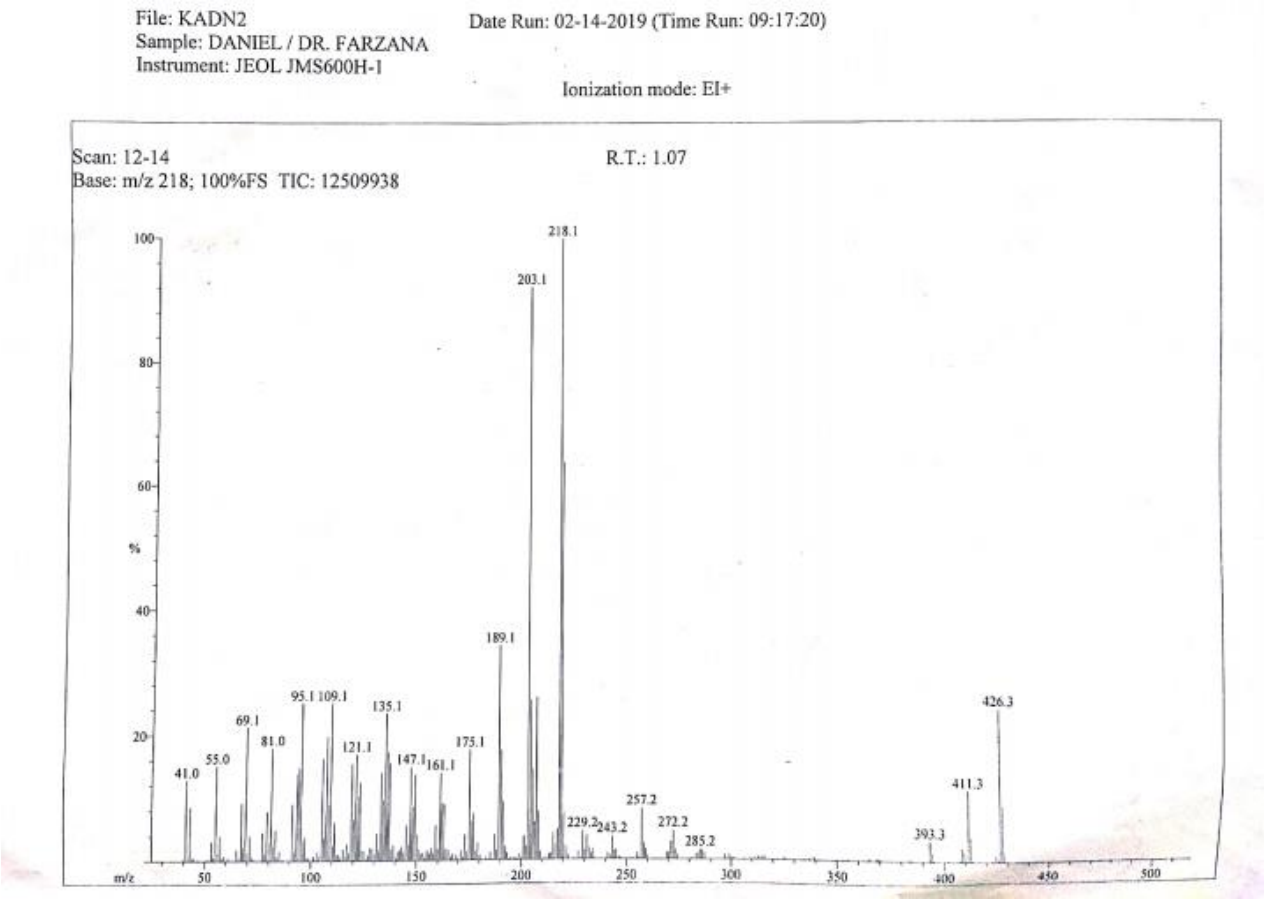


Figure 4.14.10: Mass spectrum of Compound B isolated from DCM fraction of *Alstonia boonei* stem-bark

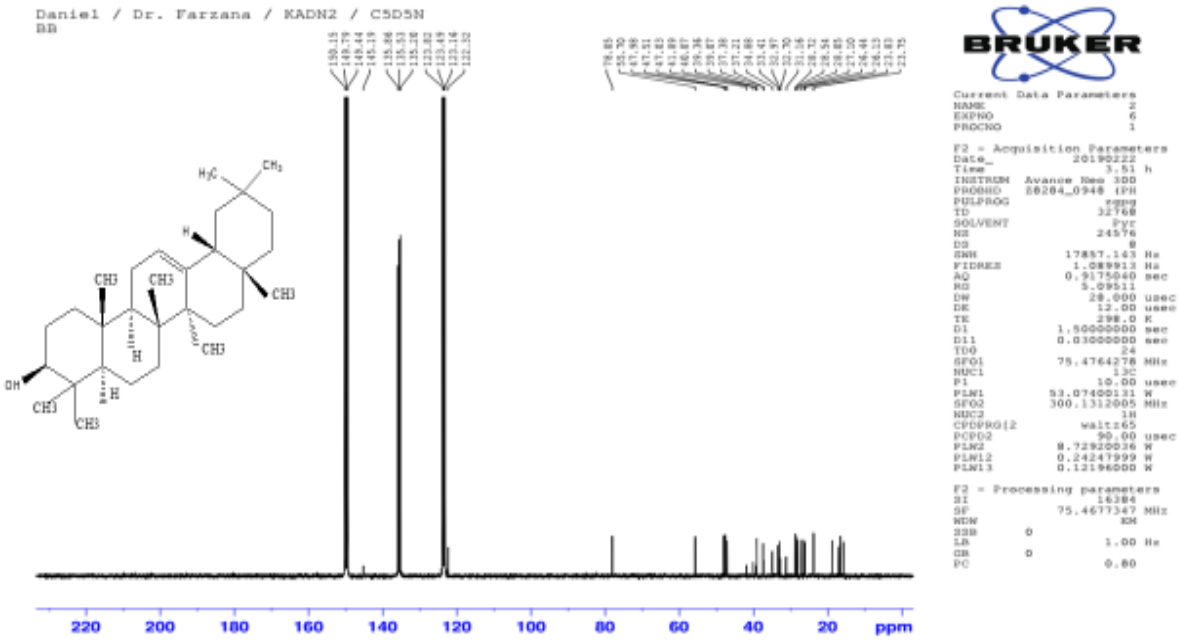


Figure 4.14.11: Carbon 13 (^{13}C) NMR Spectroscopy of Compound B isolated from DCM fraction of *Alstonia boonei* stem-bark

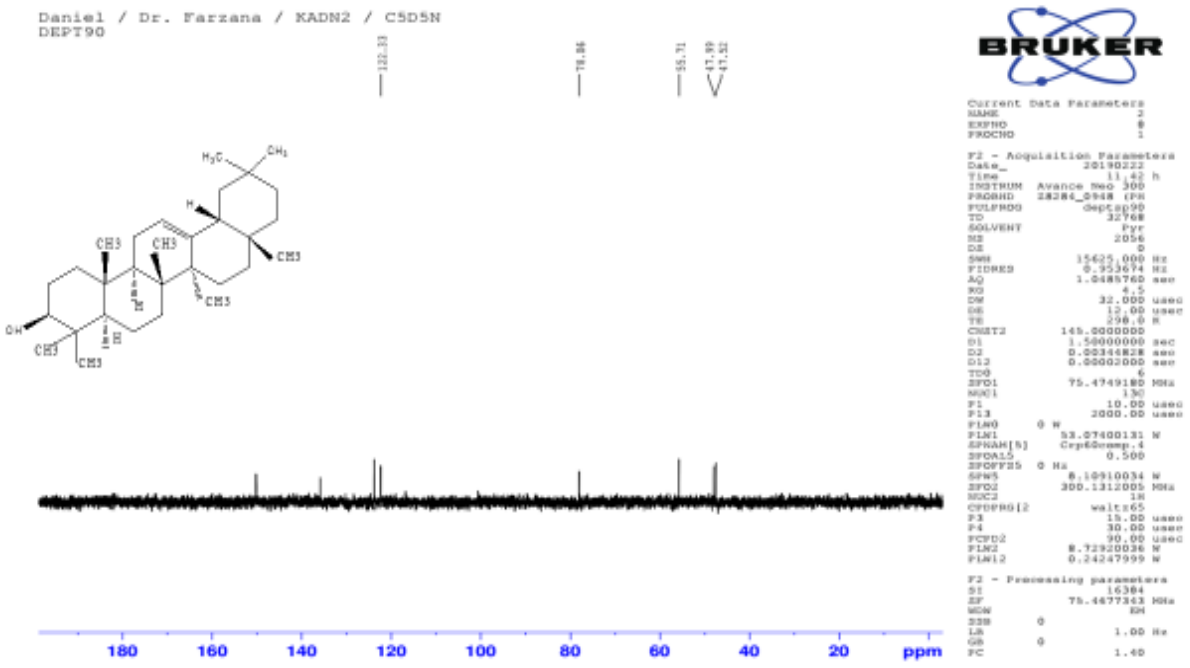


Figure 4.14.12: DEPT 90 (Distortionless Enhancement by Polarisation Transfer) Spectrum of Compound B isolated from DCM fraction of *Alstonia boonei* stem-bark

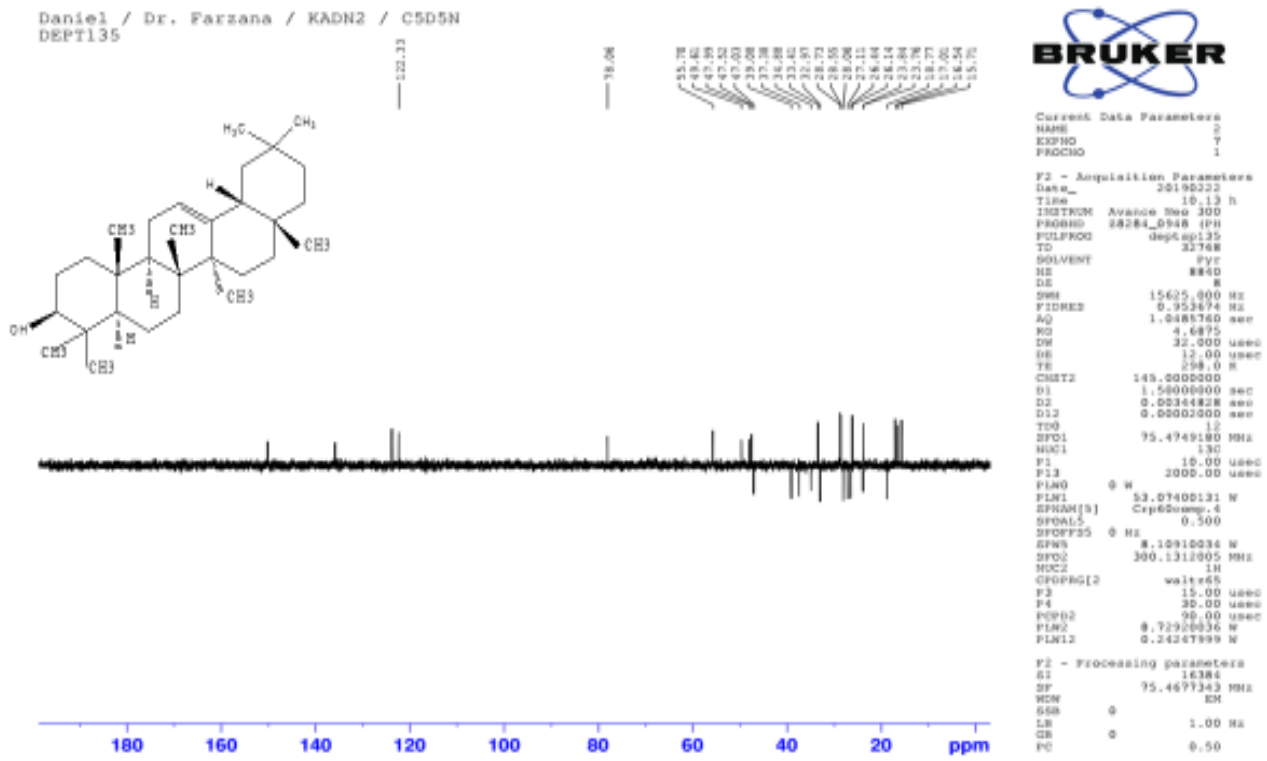


Figure 4.14.13: DEPT 135 (Distortionless Enhancement by Polarisation Transfer) Spectrum of Compound B isolated from DCM fraction of *Alstonia boonei* stem-bark

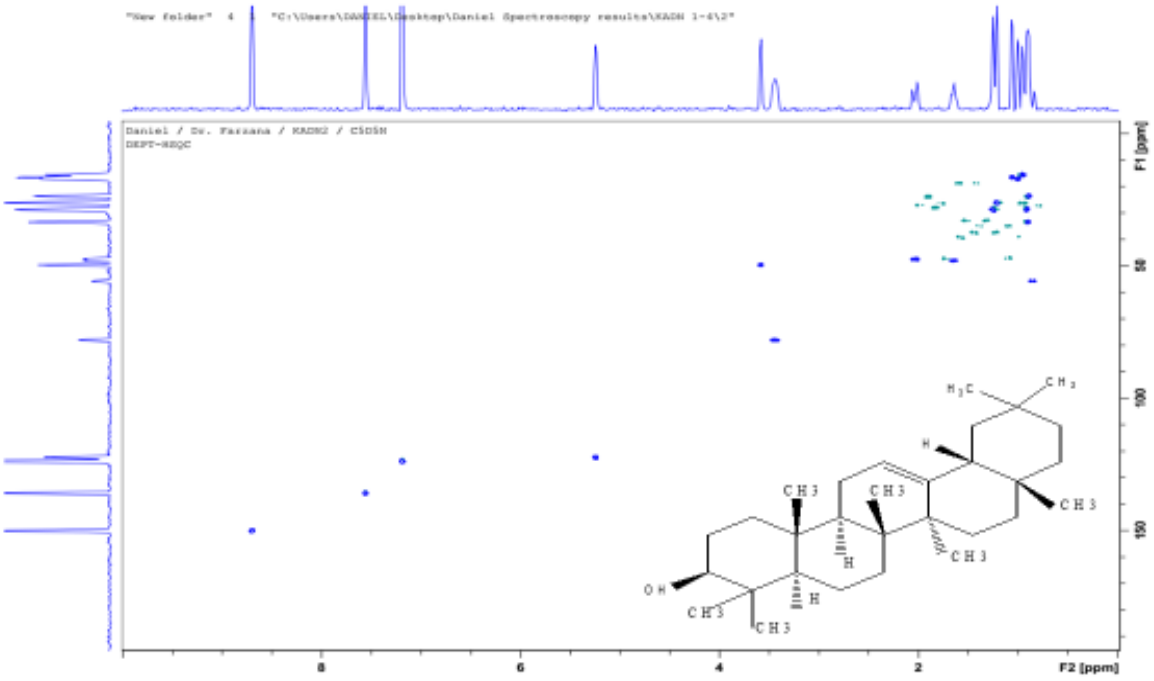


Figure 4.14.14: Heteronuclear Single QuantumCoherence Spectroscopy of Compound B

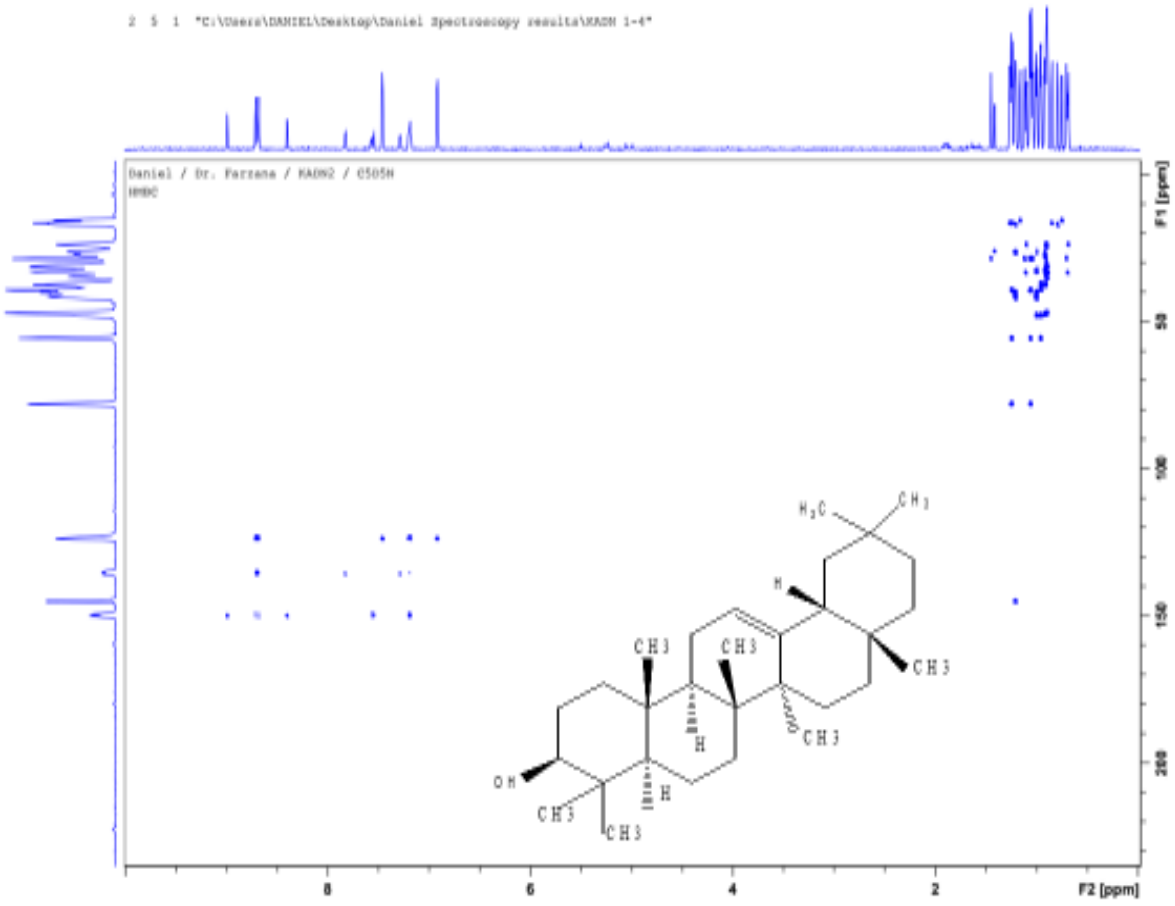


Figure 4.14.15: HMBC Spectroscopy of Compound B isolated from DCM fraction of *Alstonia boonei* stem-bark

Table 4.14A: HNMR and ^{13}C- NMR (C_5D_5N) Chemical Shifts of Compound A

Position	∂_H (ppm) Obs (C_5D_5N,400Hz)	∂_c (ppm)	HMBC	H-H	NOESY	∂_H (ppm	∂_c (ppm) Marini-bettolo *et al*., 1983)
1	-	178.9				-	175
2	-	-				-	
3	4.21 (2H, m)	66.7	5,4	H-7		4.5-4.1 (3H, m)	66.9
4	1.84 (2H, m)	29.8	5, 9			-	29.4
5	2.92(1H,dd, J_{ax}=2.4, 8.6)	34.3				2.00 (1 H, bd, J_{ax}=6 Hz)	33.3
6	1.34 (2H, m)	41.9		H-8		-	40.7
7	4.21 (1H, m)	74.4		H-3		4.5-4.1 (3H, m)	74.4
8	2.28 (1H, m)	44.9	5, 9	H-6	H-6	-	43.8
9	2.92(1H, dd, J_{ax}=2.4, 8.6	47.9	5, 8, 10			-	46.8
-CH_3	1.47 (3H, d, 6.8 Hz)	14.3	5, 7, 8,9			1.2 (3H, d, J= 7Hz)	13.3
-OH	6.13 (br.s, 1H)						

dd-double doublet, m- multiplet. br.s-broad singlet

Table 4.14.2: ^{1}H-NMR and ^{13}C- NMR (C_5D_5N) Chemical Shifts of Compound B

Position	∂_H (ppm) Obs (C5D5N,400Hz)	∂_c (ppm)	∂_H (ppm) Reported (Okoye *et al*.,2014)	∂c (ppm)
1	1.60 (2H, m)	39.0	1.55	38.8
2	-	27.1	1.52	27.4
3	3.44 (H, m)	78.0	3.20	79.2
4	-	39.3	-	39.0
5	-	55.7	0.71	55.4
6	1.65 (2H, m)	18.7	1.53	18.6
7	1.37 (2H, m)	32.9		32.9
8		40.1		40.2
9		47.5	1.95	47.4
10		37.1		37.2
11		23.7	1.84	23.8
12	5.26 (H, t, 3,2, 3.6)	122.3	5.16 t	121.9
13	-	145.1	-	145.4
14	-	41.8	-	41.9
15		26.4		26.4
16	1.83 (2H, m)	27.1		27.1
17		32.7	-	32.7
18		47.9	1.89	47.8
19	2.02 (2H, m)	47.0	1.59	47.0
20		31.1	-	31.3
21		37.0	1.66	37.4
22	1.09 (2H, m)	34.9		34.9
23	0.96 (3H, s)	15.7	0.76	15.7
24	1.25 (3H, s)	28.5	0.98	28.3
25	1.06 (3H, s)	16.5	0.92	
26	1.01 (3H, s)	17.0	0.94	
27	1.21 (3H, s)	26.1	1.11	
28	0.92 (3H, s)	28.6	0.81	
29	0.90 (3H, s)	33.4	0.85	
30	0.91 (3H, s)	23.8	0.85	

dd-double doublet, m- multiplet, t: triplet

CHAPTER FIVE

DISCUSSION, CONCLUSION, RECOMMENDATIONS AND CONTRIBUTIONS TO KNOWLEDGE

5.1 Discussion

5.2 Macroscopic and organoleptic evaluation of *Alstonia boone*i and *Alstonia congensis* leaves and stem-barks

Macroscopic evaluation is valuable in standardisation and identification of medicinal plants. The procedure is easy and costless to achieve, requiring no particular scientific equipment. Evaluation of macroscopic, microscopic and physical properties of medicinal plants can give important, simple, quick and requisite information for ascertaining purity and quality of crude drugs. The morphological or macroscopic, and anatomical or microscopic description of a medicinal plant is the initial step for establishing its identity. The study on the morphology of the leaves of *A. boonei* and *A. congensis* showed that their leaves are simple, arranged in whorls, latex copious, prominent midrib, dark and light green on the upper and lower surfaces respectively with margins entire which are the striking similarities that causes the misidentification between the two species. The only striking difference was observed in the leaf apex shape of these two species, *A. boonei* showed acute or acuminate or emarginate shape while *Alstonia congensis* has mostly acuminate leaf apex shape. This observation was similar to the observation made by Orwa *et al*. (2009) on macroscopic evaluation of *A. boonei* and *A. congensis*. The whorled arrangement of leaves and latex production are the major characteristics of plants in the genus *Alstonia* and Family Apocynaceae.

5.3 Microscopic evaluation of *Alstonia boonei* and *Alstonia congensis*

Microscopic characters of the leaf are used as taxonomic markers to aid the correct identification of the plant species. For instance, anatomical characteristics of leaf epidermises (such as stomata, trichomes and other characters) are useful identification tools. Thus, microscopic assessment is an essential means for identification of medicinal plants and is one of the most indispensable standards in monographs found in herbal pharmacopoeias and adequately used in this Thesis.

5.3.1 Qualitative description of epidermises

The adaxial epidermises of *A. boonei* and *A.congensis* are apostomatic with thick anticlinal wall which is polygonal in shape and this conforms to the previous work done on other species of *Alstonia* which are *A. scholaris* and *A. venatata* (Khayde and Vaikos, 2009, Sutha *et al*., 2011).The abaxial layers of both *A. boonei* and *A. congensis* are characterised with coastal cells, wax and papillae with thin, polygonal, straight anticlinal wall epidermal cells, which are smaller than their adaxial counter-parts; trichomes are absent on the abaxial surfaces of the two species and stomata are present on the lower surfaces of the species. This is similar to the study of Khayde and Vaikos (2009) and Sutha *et al*., (2011) who reported the presence of stomata, papillose on the abaxial layers of *A. scholaris* and *A. venatata*. According to Stace (1984), the distribution and frequency of stomata are useful in solving systematics problems. Kadiri *et al*. (2006) also averred that the presence or absence of stomata in the epidermis of leaf is useful in delineating taxa at both species and generic levels. Stomatal study of the *A. boonei* and *A. congensis* leaves showed that the leaves are hypostamatic, that is, the stomata are located only on the abaxial layer and composed of anomocytic (the stoma encircled by different sizes of subsidiary cells with no special arrangement) and paracytic (stomata surrounded by two subsidiary cells, parallel to the pore) type, around the lower epidermis, exhibiting shared characteristics with the stomata type found in *Alstonia venatata, Carissa caranduas* and *Alstonia scholaris* (Sutha *et al*., 2011; Rijhwani, 2013), as well as with members of Apocynaceae. Generally, stomata in the Apocynaceae are primarily anomocytic or paracytic, and they occur only on the abaxial surface. *A. boonei* presented significantly higher stomata frequency compared to *A.*

congensis. The frequency of stomata is high when the epidermal cells are small while the frequency is low when epidermal cells are big. (Salisbury, 1927, Rijhwani, 2013)

5.3.2 Transverse sections of *Alstonia boone*i and *Alstonia congensis* leaves

5.3.2.1 Mid-ribs of *A. boonei* and *A. congensis*

The vascular bundles of *A. boonei* and *A. congensis* are bicollateral and surrounded by sclerenchyma sheaths, this aligned with the report of Khayde and Vaikos (2009) and Mohan *et al*., (2011) on the vascular bundles type present in *A. Scholaris* and *A. venatata* to be bicollateral. Bicollateral vascular bundles are diagnostic features of plants in Apocynaceae family (Carvallo *et al*., 2017). Phloem replication in the bicollateral bundles may be attributed to rise in physiological need for the supplementation of the conductor system area (Carvallo *et al*., 2017). Five to six layers of collenchyma cells were observed at the lower epidermis of *A. boonei*, while *A. congensis* have 4-5 layers of collenchyma cells. Laticifer was observed in parenchyma cell of *A. boonei*, calcium oxalate crystals and laticifers were observed in parenchyma cells of *A. congensis.*

5.3.2.2 Lamina of *Alstonia boonei* and *Alstonia congensis*

Leaves of *A. boonei* and *A. congensis* are dorsiventral. Similarly, *A. boonei* and *A. congensis* presented dorsiventral mesophyll, with one to two layers of palisade parenchyma cells located along the adaxial plane and spongy parenchyma covering 60-70% of the mesophyll on the abaxial plane of the leaf. Dorsiventral mesophylls are diagnostic features of plants in Apocynaceae family with the exception of some species, such as *Aspidosperma quebracho* Griseb, with iso-bilateral mesophyll (Carvallo *et al*., 2017). Both species have lateral veins lying in the region between the palisade and spongy parenchyma. They are formed of phloem and xylem vessels, and contain prismatic calcium oxalate. This is similar to the study of Abdalla *et al.,* (2016) who reported the presence of lateral veins in between the palisade parenchyma cells and spongy mesophyll of *Nerium oleander* L. and Catharanthus *roseus* (L). G. Don. Such veins could be a diagnostic feature for the Apocynaceae family.

5.3.3 Venation pattern of *Alstonia boonei* and *Alstonia congensis*

The venation pattern of *A. boonei* and *A. congensis* leaves conformed to pinnate camptodromous type with festooned brochidodromous secondaries. *Alstonia scholaris* was reported to show pinnate brochidodromous venation (Rijhwani, 2013). The secondary veins are produced on both sides of the primary vein alternately, which further divide to tertiary veins. The vein terminals are dendritic in nature. Vein-islet of the two species are imperfect with large number of veinlets. Both species lack areoles. This is in accordance with the report on *A. scholaris* (Mohan and Imhadar, 1982). A striking difference was observed between the venation patterns of these morphologically-similar species quantitatively. The vein-islet number of *A. boonei* (9.0± 0.7) is slightly higher than that of *A. congensis* (8.8±1.8). According to the report by Khayde and Vaikos (2009) the vein-islet number of *A. scholaris* was reported to be 10.0. The vein islets of the two species are obliquely spherical in shape. Vein terminations are distinct, mostly unbranched. Parallel positioning of the primary and secondary veins with reticulate veinlets is characteristic of the genus *Alstonia* (Sutha *et al*., 2011).

5.3.4 Microscopic evaluation of stem and root woods of *Alstonia. boonei* and *Alstonia congensis*

Alstonia boonei and *Alstonia congensis* have semi ring pore (that is the earlywood pores are larger than late wood pores, with a gradual transition in pore size through the growth). According to a previous study by Metcalf and Chalk (1989), semi ring pores is a diagnostic feature of plants in the family Apocynaceae. Both *A. boonei* and *A. congensis* woods have solitary and radial multiple vessels, simple vessel perforations, alternating inter-vessel pits and similar vessel ray pits, stem fibres of the two species are pitted and non-septate; the root fibres of the two species are non-septate with pinoid pit. The wood of both species showed uni-seriate and multi-seriate rays. The root wood of *A. boonei* and *A. congensis* are characterised with fusiform ray with resin canal but these were not observed in the stem wood of both *A. boonei* and *A. congensis.*

5.3.5 Microscopic evaluation of stem and root barks of *Alstonia boonei* and *Alstonia congensis*

The stem-barks and root-barks of *A. boonei* and *A. congensis* contain stone cells, cork, phloem and prismatic calcium oxalate. While *A. boonei* stem and root barks contain both brachysclereids and osteosclereid stone cells, *A. congensis* has brachysclereid type.

5.4 Molecular authentication of *Alstonia species* accessions through ITS DNA barcode

The delineation of *A. boonei* and *A. congensis* species was done at molecular level using ITS universal primer. The purity of the DNA samples isolated was within limit of A_{260}/A_{280} (1.7-2.0); this is within the ratio of 1.8-2.0 required limit documented by Matasyoh *et al*. (2008) for good quality DNA. The single DNA band of the *Alstonia* species and the outgroup *Plumeria alba* was effectively amplified by the universal primers (ITS1 and ITS4) Plate 4.3.1). This result is supported by Kim *et al*. (2016) who demonstrated that ITS primer was the most suitable for DNA barcode of *Paenonia* spp.

The DNA marker (100 bp) showed that the DNA sizes of the amplified products by the universal primers is within 700 bp and this conforms with the conserved base pair region of the universal primers usually used in DNA barcode often within 400-800 bp (Yang *et al*., 2012). The sizes of the sequenced based pair (600-680 bp) were in line with the regions of base pair observed on the agarose gel electrophoresis. The ITS barcode has 100% amplification with *Alstonia* spp. and the outgroup (*Plumeria alba*). The sequences were grouped into three major clades by the UPGMA dendogram which are: clade 1 grouped accessions 1-5 (*A. congensis*) together with more than 50% closeness; clade 2 has *A. boonei* (accessions 6-9) grouped together showing more than 50% bootstrap; and the third group was an outgroup with its reference from Genbank (*Plumeria alba*) (Figure 4.3.2). The nrDNA ITS sequence exists extensively in plant tissues; such as leaves, stem, roots, and seeds compared to molecular markers in chloroplast which are commonly located in leaves. Thus, ITS sequences reveals extensively broadened prospect in the authentication of medicinal plants. The delineation of *A. boonei and A. congensis* at molecular level based on ITS sequence showed it is an extremely sensitive and stable method; it is consistent, and not affected by the physical or physiological factors of the plant samples.

The ITS (1 and 4) markers successfully delineated the accessions of *A. boonei* and *A. congensis* collected from different locations from each other thereby confirming these two species as being different from one another. One of the accessions collected as *A. boonei* (Acession 5) using the morphological features was grouped with *Alstonia congensis* after its DNA sequencing with ITS primer by the UPGMA dendogram. Though morphological characters of closely related species may be similar to the extent of misidentifying and confusing them for one another but genetic study reveals the genetic make up of individual and since no two closely related species can have the same DNA composition, hence its advantages over other approaches in delineating species of organism that are closely similar. Thus, ITS was able to resolve the mis-identification issues of *A. boonei* and *A. congensis* and this indicates that it is a good DNA barcode candidate for the authentication and delineation of *Alstonia* species.

5.5 Physico-chemical analysis

Physico-chemical evaluation of medicinal plants is important in assessing the quality, nutritional significance and health effects of medicinal plants (Taiga, 2009) as well as setting standards since the parameters are always constant in plants (Alam and Najum, 2015).

(a) Ash Values: Ash value reveals the presence and quantum of impurities (such as inorganic composition) in plant drugs as well as herbal raw materials. It also enhances the assessment of quality and purity of the crude drugs (Prasad *et al*., 2012).

In this study, the total ash values of the leaves and stem-bark are 8.5% and 5.9%, respectively for *A. boonei;* and 6.8% and 4.8%, respectively for *A. congensis*. For both *Alstonia* species, leaves possessed higher total ash values (8.5% and 6.8%) than those of the stem-barks (5.9% and 4.8%). *Alstonia boonei* leaf and stem-bark gave higher total ash values (8.5%, 5.9%) than those of *A. congensis* (6.8%, 4.8%) which can be used to differentiate between the two species and set standards for them. The report here reveals that total ash content varies within wide limits for various taxa (as previously maintained by Prasad *et al*., 2012). The acid-insoluble ash of the leaves and stem-bark powder of 1.40% and 0.7% were obtained, respectively, for *A. boonei*; and 0.9% and 0.6%, respectively, for *A. congensis*. The acid-insoluble ash obtained in this study signifies that the plant samples

are in excellent physiological state with little extraneous matter such as sand, silica and soil. An increase in ash value would be indicative of contamination, substitution, adulteration or careless handling in crude drug preparations of herbal raw materials. This result is in accordance with those reported for other *Alstonia* species. For instance, the total ash value of *A. venetata* leaf was 5.3%, and the acid insoluble ash is 1.01% (Sutha *et al*., 2011).

(b) Moisture content: The leaf and stem-bark gave 9.1±0.04% and 6.4± 0.0006%, respectively for *A. boonei*, while those of *A. congensis* are 9.9± 0.017%, 7.2± 0.004%, respectively. Previous study by Khayade and Vaikos, 2009) on the moisture content of *A. scholaris* leaf, reported it had 5.35%. Moisture values in crude drug according to British Herbal Pharmacopoeia should not be more than 14% (Ambi *et al*., 2018). Thus, the values obtained in this study are within the acceptable limits for most vegetable drugs. The leaves of both species gave high moisture contents, without any significant difference. Meanwhile, the stem-barks of both *A. boonei* (6.4%) and *A. congensis* (7.2%), produced low moisture contents compared to those of their leaves which are significantly different. Both the leaf and stem-bark of *A. congensis* gave moisture contents that are within the acceptable limits according to BHP (1990). The moisture content of crude drug has a direct role in its stability. The lower the moisture content, the lesser the chances of deterioration or spoilage, the higher the stability level, the lower the possibility of microbial growth and the longer the shelf life of the herbal raw materials or finished products (Okhale *et al*., 2010). Low moisture content reduces the hydrolytic enzymatic activities on plants' active constituents and can also minimise inaccuracy in the quantitative analysis of drug products (Alam *et al*., 2016).

(c) Solvent-extractive values: Water soluble extractive values of both leaves and stem-barks of *A. boonei* and *A. congensis* are higher than those of the ethanol soluble extractives. This indicates that the plant samples contain more of water-soluble constituents than alcohol soluble constituents. This finding is in tandem with the findings of Sutha *et al*. (2011) who reported that the water-soluble extractive value of *A. venetata,* was higher than the alcohol-soluble extractive value. Soluble extractive values do help to detect adulterated, substituted or already exhausted drug.

Food nutrient contents

The leaves and stem-barks of *A. boonei* and *A. congensis* were found to contain large amounts of protein, carbohydrate, crude fibre, crude fat; but the relative contents of protein, fat and moisture in the leaves of both species are significantly higher than in their stem-barks, which in turn possessed higher percentage of carbohydrate and crude fibre than the leaves. Carbohydrate constitutes the highest percentage in their leaves and stem-barks which is suggestive of good energy source potentials, capable of supplying energy to human brain and muscle cells (Shemishere *et al*., 2018). The presence of protein in both plant species will be a plus to their therapeutic efficacy since protein is an essential component of human diet needed for the replacement of dead tissues, hormone, enzyme formation and strong immunity (Igile *et al*., 2013). The high content of crude fibre may indicate beneficial effects in the digestive tracts, absorbing water from the gut aiding bulk stool, lowering cholesterol with triglycerides, preventing cancer and other digestive disorders, as well as reducing weight gains as suggestive in the content of low crude fat if incorporated into weight-reducing diets (Shemishere *et al*., 2018).

5.6 Elemental analysis of *A. boonei* and *A. congensis*

The present study shows that *A. boonei* and *A. congensis* leaves and stem barks could serve as good sources of mineral elements especially calcium. The leaves of the two species were richer than the stem-barks in mineral elements. Calcium is the most abundant mineral in the leaves of *A. boonei* and *A. congensis*. The calcium content in *A. boonei* leaves and stem barks were (68.2 and 52.2) mgg^{-1}, respectively, while the content in *A. congensis* leaves and stem barks were (65.7 and 39.7) mgg^{-1}, respectively. The high concentration of calcium contained in these two species can be of high therapeutic value in reduction of diarrheoaic episodes in both infants and adults. According to the study carried out by Fraebel *et al*. (2018), reduction in calcium ion leads to occurrence of diarrhoea in a malnourished and hypocalcemia infant which stopped after the replacement of calcium therapy. The concentration of sodium in the leaves of *A. boonei* and *A. congensis* were (2.9 and 6.0) mgg^{-1} while the concentrations in the stem barks of *A. boonei* and *A. congensis* were (3.0 and 0.4) mgg^{-1}, respectively. Sodium enhances the osmotic equilibrium between the extra cellular fluid and the tissue cells in order to maintain the pH of blood (Ayo, 2013).

Potassium concentrations in the leaves of *A. boonei* and *A. congensis* were (50.09 and 50.04) mgg^{-1}, respectively, while the concentrations in the stem barks of *A. boonei* and *A. congensis* were (23.24 and 5.79) mgg^{-1}, respectively. Potassium aids the reduction of blood pressure and maintenance of cardiac rhythm (Ayo, 2013). The proportion of sodium to potassium (Na^+/K^+ ratio) found were (0.06) in *A. boonei* leaves, (0.12) in *A. congensis* leaves, (0.13) in *A. boonei* stem-barks and (0.07) in *A. congensis* stem-barks. These ratios are less than the recommended ratio of 0.6 (Akinyeye *et al*., 2010). The plants will be suitable for use in health situations where sodium or potassium is contraindicated as in the case of hypertension.

Magnesium concentration found were (0.9 and 0.9) mgg^{-1} for *A. boonei* leaves and stem-barks, respectively, and (0.8 and 0.05) mgg^{-1} for *A. congensis* leaves and stem-barks, respectively. These values in the leaves and stem barks of the two species showed low accumulation of magnesium when compared to values obtained from previous reports on medicinal plants (Ozcan and Akbulut, 2008). Its low accumulation in the two plants could aid or synergise with other components for diarrhoeal control, or to make them perform some of the validated pharmacological activities reported. Concentration of iron (Fe) in the leaves and stem barks of *A. boonei* were 2.6 and 0.2 mg/g, respectively, and for *A. congensis*, the concentrations were 1.40 mgg^{-1} in leaves and 1.18 mgg^{-1} in stem barks. In this study, accumulated Fe in both species was above the 20 ppm permissible limit in edible plants (FAO/WHO, 1984). The concentrations of lead (Pb) were found to be 0.1 mgg^{-1} in the leaves of both *A. boonei* and *A. congensis*, and the same value (0.1 mgg^{-1}) was found for the stem barks of both species. According to the WHO (1998), the WHO permissible limit recommended for herbal medicine is $10mgkg^{-1}$. Thus, lead concentrations in both leaves and stem-barks were quite low (below the permissible limit) and therefore safe for human use. Excess lead intake has been reported to be associated with impaired enzymatic function and mutilated neurophysiological processes in children (Otto *et al*., 1985).

The concentration of Zinc (Zn) was found to be 0.2 and 0.3 mgg^{-1} in leaves of *A. boonei* and *A. congensis*, respectively, and 0.3 mgg^{-1} in the stem-barks of both species. According to the WHO (1998), the permissible limit recommended for daily intake of Zinc is 15 $mgkg^{-1}$. Hence, the Zn concentrations obtained in the leaves and stem-barks of *A. boonei* and *A. congensis* are below limit. The values of manganese in the leaves (0.1 and 0.09) $mgg^{-1}$for

A. boonei and *A. congensis*, respectively, and the stem-barks, (0.02) mgg^{-1}) was recorded for both. Manganese assists the body in metabolising protein and carbohydrates. The concentrations found in this study are low when compared to the 2.5–3.0 mg/day (Hunt and Nielsen, 2009) Recommended Dietary Allowance (RDA). The concentrations of cadmium (Cd) in the leaves of *A. boonei* and *A. congensis* were (0.4 and 0.1) mgg^{-1} and in stem-barks (0.08 and 0.06) mgg^{-1}, respectively. The permissible limit of Cd by WHO (2007) in herbal medicinal products is 0.3 $mgkg^{-1}$. Cadmium is a trace element with vague direct functions in both plants and humans. Accumulation of Cd damages the kidneys and liver. However, the cadmium concentration obtained in samples of the present study is below the official maximum limit, thereby making both species safe for consumption. The Nickel (Ni) concentrations were (3.3 and 2.4) mgg^{-1} in the leaves, and (2.3 and 3.0) mgg^{-1} in the stem-barks of *A. boonei* and *A. congensis*, respectively. Nickel is required in diminutive quantity for normal production of insulin in the pancreas. The deficiency of Nickel causes lowered activities of different dehydrogenase and transaminase enzymes, alpha-amylase, and its deficiency mainly alters carbohydrate metabolism (Kabata-Pendias and Pendias, 1992). According to the Environmental Protection Agency (EPA), daily Nickel intake should be less than 1mg to avoid toxicity (McGrath and Smith, 1990). The concentrations of Nickel in the present study could be used to determine the safe dose in the human subject within the official limit.

The values of molybdenum (Mo) in the leaves of *A. boonei* and *A. congensis* were (12.6 and 15.3) mgg^{-1}, respectively, while the concentrations in the stem barks of *A. boonei* and *A. congensis* were (14.8 and 16.9) mgg^{-1}, respectively. Molybdenum is a vital trace element critical for animal survival. Paucity in the biosynthesis of molybdenum co-factor (Moco) promotes pleitropic failure of Mo-enzyme activities and early childhood death, but excessive intake has also been reported to be associated with foetal deformities (Schwarz and Belaidi, 2013). The Recommended Dietary Allowance (RDA) for adults is 45$\mu gday^{-1}$ according to National Academy of Science, (2001) which should guide any therapy. Chromium and copper were not detected in the leaves nor in the stem-barks of *A. boonei* and *A. congensis*.

5.7 Phytochemical analysis of *A. boonei* and *A. congensis*

Secondary metabolites found in the leaves and stem-barks of the two species were terpenes, alkaloids, saponins, flavonoids and cardiac glycosides. Khayade *et al*. (2009) and Sutha *et al*. (2011) also reported the existence of these secondary metabolites in *A. scholaris* and *A. venatata* leaves, respectively. Thus, part of the plants' secondary metabolites, singly or working synergistically, may have been responsible for the observed *in vitro* antispasmodic and *in vivo* anti-diarrhoeal effects exhibited by the extracts of *A. boonei* and *A. congensis* leaves and stem-barks, thus justifying their biological activities.

5.8 Fluorescence characters of *Alstonia boonei a*nd *Alstonia congensis* Powder

The fluorescence evaluation is also an important pharmacognostic technique for detecting unofficial admixtures in adulterated samples. Different colour changes were observed in the daylight and under ultraviolet lamp at 254 nm and 365 nm in the presence of diverse chemical reagents added to the powdered samples of the leaf and stem-bark. Sutha *et al*. (2011) also reported the fluoresce character of *A. venatata* powdered leaf samples when reacted with different chemical reagent and viewed under ultra violet rays at different wavelength. Alam and Saqib (2014) reported that the distinctive fluorescence characters exhibited by various plants could be used as a standard in the identification and authentication of medicinal plants.

5.9 Biological Experiments with crude extracts, fractions and isolated compounds

5.9.1 Effects of *Alstonia boonei* and *Alstonia congensis* leaf and stem-bark crude extract on spontaneous contraction of isolated rat ileum

The aqueous leaf crude extract of *A. boonei* **(**ABLC), aqueous leaf crude extract of *A. congensis* (ACLC), aqueous crude extract of *A. boonei* stem-bark (ABSC) and aqueous crude extract of *A. congensis* stem-bark (ACSC), caused concentration-dependent antispasmodic effects between the range of 0.003-10.00 $mgmL^{-1}$ (for the leaf) and 0.003-3.00 $mgmL^{-1}$ (for the stem-bark); as presented in Figures 4.9.1-4.94 with IC_{50} values of 0.98 ± 0.51 $mgmL^{-1}$, 1.68 ± 0.73 $mgmL^{-1}$, 1.15±0.72 $mgmL^{-1}$, and 1.05±0. $mgmL^{-1}$, respectively on spontaneous contraction of isolated rat ileum. The ABLC showed the highest

antispasmodic activity on spontaneous contraction of isolated rat ileum. The antispasmodic activities were revocable by rinsing the tissue with Tyrode's solution.

5.9.2 Effects of *Alstonia boonei* and *Alstonia congensis* leaf and stem-bark crude extracts on K^+ (80 mM) induced contraction of isolated rat ileum

ABLC, ACLC, ABSC and ACSC showed concentration-dependent antispasmodic activities on K^+ (80 mM) induced contraction within a concentration range of 0.003-3.0 $mgmL^{-1}$, 0.003-5.0 $mgmL^{-1}$, 0.003-0.3 $mgmL^{-1}$and 0.003-3.0 $mgmL^{-1}$ (Figures 4.9.1- 4.94), respectively with corresponding IC_{50} values of 0.22±0.5, 0.25±0.05, 0.03±0.1 and 0.12±0.8. The isolated rat ileum preparations on exposure to high [K^+] (80 mM) produced a constant contraction by depolarisation of the tissues. The application of the extracts to the tissue bath in cummulative manner demonstrated a complete relaxation effect (antispasmodic action) on the isolated tissue. However, ABSC and ACSC had a more significant antispasmodic effect on K^+ (80 mM) induced contraction than ABLC and ACLC.

Medicinal plants contain mixtures of several secondary metabolites that can function as active substances for different pharmacological activities including the observed antispasmodic.

The antispasmodic activities of aqueous crude extracts of *A. boonei* and *A. congensis* leaves on spontaneous and high [K^+] (80 mM) induced contraction of isolated rat ileum also aligned with the study of Aziz *et al*., (2012) which reported that aqueous crude extract of *Anthemis mauritiana* Maire & Sennen flowers exhibited revocable dose-dependent relaxation effect on spontaneous contraction of isolated rabbit jejunum smooth muscle with IC_{50} value of 1.48 ± 0.02 $mgmL^{-1}$ and had similar antispasmodic effect on high [K^+] (75 mM) induced contraction of isolated rat jejunum smooth muscle with IC_{50} value of 0.48 ± 0.09 $mgmL^{-1}$.

5.9.3 Antispasmodic effect of *Alstonia boonei* and *Alstonia congensis* leaf and stem-bark fractions on spontaneous contraction of isolated rat ileum

The antispasmodic effects of dichloromethane, ethyl acetate and aqueous fractions of leaves and stem-barks of *A. boonei* (ABLD, ABLE, ABLA, ABSD, ABSE, ABSA) and *A.*

congensis (ACLD, ACLE, ACLA, ACSD, ACSE and ACSA) aqueous crude extracts were evaluated. The ABLD and ACLD showed concentration dependent antispasmodic activities in a cumulative manner from 0.003 to 3.0 $mgmL^{-1}$ and 0.003-1.0 $mgmL^{-1}$, respectively with IC_{50} values of 0.36±0.16 $mgmL^{-1}$ and 0.39±0.24 $mgmL^{-1}$, respectively on spontaneous contraction of isolated rat ileum (Appendix VII) with no significant difference. The ABLE showed concentration dependent spasmodic activity between 0.003-1.0 $mgmL^{-1}$ in an increasing manner and showed total relaxation of the tissue at 3 $mgmL^{-1}$ (Appendix XI), with IC_{50} value of 1.68±0.73 $mgmL^{-1}$ (Table 4.9) while ACLE showed concentration dependent significant antispasmodic activity between the range of 0.003-5 $mgmL^{-1}$ (Appendix XIII), with IC_{50} value of 0.42 ± 0.02 $mgmL^{-1}$ on the spontaneous contraction of isolated rat ileum.

The ABSD and ACSD exhibited concentration dependent significant antispasmodic activities in dose cumulative manner on spontaneous contraction of isolated rat ileum within the range of 0.003-1 $mgmL^{-1}$ and 0.003-3 $mgmL^{-1}$ (Appendix IX and Appendix XVII), respectively with IC_{50} values of 0.31±0.02 $mgmL^{-1}$and 0.34±0.13 $mgmL^{-1}$, respectively. ABSE exhibited concentration dependent antispasmodic activity in a cumulative manner on spontaneous contraction of isolated rat ileum within the range 0.003-0.1 $mgmL^{-1}$, with IC_{50} value of 0.24 ±0.14 (Appendix XIII) and ACSE exhibited both spasmodic and antispasmodic activities within the range of 0.003-1.00 $mgmL^{-1}$and 3.00-10.00 $mgmL^{-1}$ (Appendix XXI), respectively, with IC_{50} value of 2.84±0.12 $mgmL^{-1}$. ABSA showed a slight spasmodic activity within the range of 0.001-0.1 $mgmL^{-1}$and showed antispasmodic activity within the range of 0.3-10 $mgmL^{-1}$ (Figure 4.9.7) with IC_{50} of 2.38±0.65 $mgmL^{-1}$ while ACSA lacked any antispasmodic activity within the range of 0.001-0.01 $mgmL^{-1}$ but showed spasmodic activity within the range of 0.3-10 $mgmL^{-1}$ in a cumulative manner on spontaneous contraction of isolated rat ileum (Figure 4.9.10).

5.9.4 Effect of *Alstonia boonei* and *Alstonia congensis* leaf and stem-bark fractions on high $[K^+]$ induced contraction of isolated rat ileum

ABLD exhibited a total relaxation activity on high $[K^+]$ induced contraction on isolated tissue within the range of 0.001-0.3 $mgmL^{-1}$with IC_{50} value of 0.03 ±0.01, ACLD showed its antispasmodic activity within the range of 0.001-1.0 $mgmL^{-1}$, also ABLE (0.001-1.0)

$mgmL^{-1}$ and ACLE (0.001-0.3) $mgmL^{-1}$ (Appendix IXX). ABSD displayed antispasmodic activity within the range of 0.001-1.0 $mgmL^{-1}$ with IC_{50} value of 0.02± 0.5 $mgmL^{-1}$while ACSD displayed relaxation activity within the range of 0.003-1.0 $mgmL^{-1}$, also ABSE (0.001-0.3) $mgmL^{-1}$, ACSE (0.001-3) $mgmL^{-1}$ and ABSA (0.001-3) $mgmL^{-1}$; they all showed concentration dependent antispasmodic activities on K^+ (80 mM) induced contractions with IC_{50} values of 0.05±0.01 $mgmL^{-1}$, 0.31±0.03 $mgmL^{-1}$, 0.09 ±0.02 $mgmL^{-1}$, 0.90 ± 0.6 $mgmL^{-1}$, respectively. The isolated rat ileum preparations on exposure to high [K^+] (80 mM) produced a persistent contraction by depolarisation of the tissues. The application of the extracts to the tissue bath in cummulative manner demonstrated a complete relaxation effect on the isolated tissue. However, ABLD and ABSD exhibited significant antispasmodic activities with IC_{50} values of 0.03 ±0.01 and 0.02 ± 0.05 $mgmL^{-1}$, respectively.

A. boonei and *A. congensis* leaves and stem-barks fractions have been proven to possess *ex vivo* antispasmodic potentials but *A. boonei* stem-bark DCM fraction showed higher potency than *A. congensis*. When purified through solvent-partitioning, the partitioned fractions exhibited both antispasmodic and spasmodic activities. The antispasmodic effect was concentrated in the organic fractions, more predominantly in the less polar fractions and this was also reported in previous study by Makrane *et al*., (2018) on *Origanum majorana* L. The DCM fractions of the two plants showed highly significant antispasmodic activities on both spontaneous and high potassium induced contractions on the isolated rat ileum, this was also supported by previous study by Bashir *et al*., (2006) on DCM fraction of *Calendula officinalis* on on both spontaneous and high potassium induced contraction of isolated rabbit jejunum. The ethyl acetate fraction of *Calendula officinalis* also exhibited both antispasmodic and spasmodic activities at different concentrations, the aqueous fraction of *A. congensis* stem-barks was reported to have spasmodic effect and the same was reported for aqueous fraction of *Calendula officinalis* (Bashir *et al*., 2006). Perhaps, the reported abortifacient property of the genus *Alstonia* (Taiwo *et al*., 1998) could be attributed to the observed spasmodic action of the polar constituents in this partitioned fraction.

The relaxation potential of the crude extracts and partitioned fractions of *A. boonei* and *A. congensis* on spontaneous movements of rat ileum could be because of their interference either with Ca^{2+}influx through Voltage Dependent of Calcium Channels (VDCs) from the

intercellular medium or Ca^{2+} release from sarcoplasmic reticulum (Karaki and Weiss, 1988). To substantiate the interaction of ABC, ACC and their fractions with VDC, the tissue was pre-treated with 80 mM [K^+]. A depolarisation of the membrane occurred, and therefore the VDC opened to allow the penetration of Ca^{2+} towards cytoplasm. The extract or fractions that countered contraction induced by KCl is said to be a VDCs blocker (Godfraind *et al*., 1986), hence all crude extracts, fractions except ACSA are VDCs blockers.

5.10 Antidiarrhoeal activities of *Alstonia boonei* and *Alstonia congensis* stem-bark aqueous crude extracts and dichloromethane (DCM) fractions

In vivo model was carried out to test the extracts and DCM fractions of *A. boonei and A. congensis* for possible effect on inhibition of gut motility in regards to antidiarrhoeal activity. The aqueous crude extracts of both *A. boonei* and *A. congensis* stem-barks (ABSb and ACSb), respectively, showed dose-dependent marked anti-diarrhoeal effects on diarrhoeic mice. ABSb and ACSb at 200 $mgkg^{-1}$ significantly decreased the total number of faeces and the total number of wet faeces upon administration of castor oil compared to the negative control; they gave diarrhoea- inhibitory values of 76.3 and 70.9 %, respectively, close to the anti-diarrhoeal potency of reference loperamide (87 %) at 5 $mgkg^{-1}$. Furthermore, ABSb and ACSb at 200 $mgkg^{-1}$ body weight significantly prolonged the onset of diarrhoea (following castor oil administration) with ACSb having longer onset (66.7 min) than ABSb (65.1 min) which is comparable to the effect of Loperamide (73.2 min) (Table 4.10)

The DCM fractions of *A. boonei* and *A. congensis* stem-bark; ABD at 200 $mgkg^{-1}$ exhibited a stronger diarrhoea- inhibitory effect of (87.3) % when compared with ACD at the same dose value (81.0) %, which is similar to the effect of Loperamide at 5 $mgkg^{-1}$ *b.w* (87.5) %. However, at the same dose, ACD gave a longer diarrhoea onset of 68.6 min than ABD which delayed diarrhoea for 64.9 min at the same concentration following castor oil administration; this property is analogous to that of the reference drug (loperamide at 5 $mgkg^{-1}$, with 73.2 min. diarrhoea onset). Diarrhoea is generally considered a result of altered gut motility and fluid accretion within the intestinal tract.

Castor oil hydrolysis leads to the formation of recinoleic acid (Ali *et al*., 2014), that causes changes in the movement of water and electrolytes thereby leading to hypersecretion and

spasm of the intestine. The use of medicinal plant preparations by Africans in the treatment of diarrhoeal diseases is very common in traditional medical practice (Ali *et al*., 2014). Many of such medicinal plants with antidiarrhoeal activities act by plummeting the gastrointestinal motility (antispasmodic action) or decreasing tissue secretion, inherent action against microbial infections or as anti-inflammatory agents (Sabiu and Ashafa, 2016). Without prejudice to other possible mechanisms, the present study has shown that one of the ways by which *Alstonia* species inhibit diarrhoea was by inhibiting tissue motility, thereby producing antispasmodic effects.

5.11 Antidiarrhoeal effects of herbal preparation of mixtures of *Alstonia boonei* stem-bark and *Picralima nitida* seed *decoction* on castor oil induced diarrhoea in mice

The three herbal mixture ratios showed dose-dependent antidiarrhoeal activities on castor oil induced diarrhoeic mice. Individually, each of the herbal mixtures (AP1, AP2, AP3) exhibited the highest diarrhoea-inhibitory activity at the highest dose of 500 $mgkg^{-1}$. *b.w* of 80.0, 89.0 and 91.1 %, respectively. Of the three herbal preparations, AP3 (2AB+1PN) contained the highest content of *A. boonei* stem-bark (twice *Picralima nitida*) and gave the highest diarrhoea-inhibitory activity of 91.1 %. However, when the AP3 potency was compared with the aqueous crude extract of *A. boonei* (AB) alone at the same dose of 200 $mgkg^{-1}$ *b.w*., ABC exhibited higher diarrhoea-inhibitory activity (76.3) % as against 72.3% by AP3, denoting that the antidiarrhoeal activity of *A. boonei* alone could be statistically equivalent to AP3 (mixture of *Alstonia boonei* and *Picralima nitida*)

5.12 Antispasmodic activity of compounds A and isolated from DCM fraction of *Alstonia boonei* stem-bark

The DCM fractions of *A. boonei* stem-bark showed highly significant antispasmodic activities both on spontaneous and induced contractions of the isolated rat ileum. It was therefore subjected to column chromatography. Compounds A and B were isolated as pure entitiesfrom the fraction and these were evaluated for antispasmodic activities on spontaneous and induced contractions on isolated rat ileum.

5.12.1 Effects of Compounds A and B isolated from DCM fraction of *Alstonia boonei* on spontaneous contraction of isolated rat ileum

Compound A exhibited antispasmodic activities from 0.001 to 10 μgmL^{-1} (Figure 4.13.1, Appendix XX) with IC_{50} of 0.29±0.05 μgmL^{-1} on spontaneous contraction of isolated tissue. Compound B exhibited spasmodic activity in a concentration- dependent manner from 0.001 to 5 μgmL^{-1} and showed a partial relaxation effect or partial antispasmodic activity at 10 μgmL^{-1} with IC_{50} value 3.5±0.98 μgmL^{-1} on spontaneous contraction of isolated tissue (Figure 4.13.2, Appendix XXII). In the present study, Compound A has shown a more significant antispasmodic activity than compound B. Athough the two compounds possessed biological activities on the smooth muscles of gastrointestinal tract. Based on the high antispasmodic effect on the isolated rat ileum shown by compound A, it could be an active antidiarrhoeal agent.

5.12.2 Effects of Compounds A and B isolated from DCM fraction of *Alstonia boonei* on high K^+ (80 mM)-induced contraction of isolated rat ileum

The active antispasmodic substance (s) in the DCM fraction of *A. boonei* stem-bark aqueous crude extract were isolated. Such phytochemical venture can lead to single-compound formulation as in conventional pharmaceutical or orthodox therapy practice. Formulation of pure compounds carry many advantages namely: exclusion of unwanted additional constituents, opportunity for different dosage-forms (including parenteral solutions), ease of analysis for quality control, ease of monograph compilation for the finished product, monitoring of deterioration in storage, etc. Isolation, characterisation and identification of pure active compounds can provide an easy or evidence-based analysis as well as standardisation of medicinal plants, and also in the detection of adulteration and different chemotypes in commercial samples of the particular medicinal plants

Compound A significantly relaxed high $[K^+]$ induced contraction of isolated rat ileum within the range of 0.01-5 μgmL^{-1} (Figure 4.13.1, Appendix XXI) with IC_{50} value of 0.09 μgmL^{-1}, ten times more potent as antispasmodic than Compound B which possessed only a partial relaxation effect on the isolated rat ileum.

Compound B exhibited a weak antispasmodic effect on high [K^+] induced contraction of isolated rat ileum within the range of 0.01-10 µgmL^{-1} with IC_{50} value of 0.9 µgmL^{-1} (Figure4.13.2, Appendix XXIII).

Compound A isolated from *A. boonei* showed significant antispasmodic effect on spontaneous and high [K^+]-induced contractions and was ten times active than compound B. It could be one of the synergistic constituents with echitamidine previously reported in *A. boonei* as antispasmodic on guinea pig ileum (Ojewole, 1984). Compound B, on the other hand, had exhibited a biphasic property, being both spasmodic and antispasmodic in action. Isolation of compounds therefore can help to identify the specific constituent(s) responsible for a particular bioactivity alone, or in synergy with other constituents as may be the case in the present study.

The isolated pure compounds from *A. boonei* stem-bark DCM fraction on spontaneous movements of rat ileum could be because of their interference either with Ca^{2+}influx through Voltage Dependent of Calcium Channels (VDCs) from the intercellular medium or Ca^{2+} release from sarcoplasmic reticulum (Karaki and Weiss, 1988). To validate the interaction of the isolated compounds from ABC with VDC, the tissue was pre-treated with 80 mM [K^+]. A depolarisation of the membrane occurred, and therefore the VDC opened to allow the penetration of Ca^{2+} towards cytoplasm. Any substance that countered contraction induced by KCl is said to be a VDCs blocker (Godfraind *et al*., 1986), hence compound A is a VDCs blocker.

The inhibitory effect of crude aqueous extracts, the organic fractions and isolated compounds on spontaneous and high [K^+] induced contractions of rat ileum may appear to be due to a Calcium Channel Blocker (CCB) effect and may be liable for the antidiarrhoeal effect of these samples. This suggests the presence of Ca^{2+} antagonists in the extracts and that Compound A isolated from the DCM fraction of *A. boonei* is one of many constituents of *A. boonei* extract with Ca^{2+} antagonists and hence, its usefulness in treating the tissue hyperactivity in diarrhoea. These results can help to provide a better understanding of the antispasmodic mechanisms in reducing intestinal motility which can be used in non-infectious diarrhoeas.

5.13 Identification of isolated compounds using spectroscopic techniques

5.13.1 Structural characterisation and Identification of Compound A (Boonein)

Compound A was obtained as a needle like crystal and when viewed under Ultraviolet light at 254 nm and 365 nm wavelength gave no fluorescence this is due to the absence of conjugated bond in the compound but it gave orange colouration with chromogenic reagent 10% sulphuric acid and heat at 100^{o}C. Three markers were contained in the compound, these include a carbonyl group at C-1, a methyl group at C-8 and an hydroxyl group at C-7. The proton NMR showed the existence of fourteen protons in all. An hydroxyl group at δ_H 6.13 ppm which integrated as singlet with broad band and attached to C-7, a methyl proton (C-8) at δ_H 1.47 ppm which integrated as doublet, 3 methylene protons at δ_H 4.21 ppm which integrated as multiplets, at δ_H 1.84 ppm which integrated as multiplets and at δ_H 1.34 ppm all are found at position 3, 4 and 6, respectively and 4 methine proton at δ_H 2.92 ppm (dd), δ_H 4.21 ppm (C-7), δ_H 2.28 ppm and δ_H 2.92 ppm. The protons on C-3 and on the hydroxyl group appeared at δ_H 4.21 and 4.22 ppm, respectively. The ^{13}C-NMR spectrum showed one carbonyl group at δ_C 178.9 ppm. The broad band and DEPT spectra, showed the existence of nine carbon signals including a quaternary carbon, four methine carbons, three methylene carbons and a methyl group. DEPT 90 spectrum showed the existence of four methine (CH) group while DEPT 135 spectrum showed the existence of a methyl group (CH_3) whose peak exist upfield on the positive axis of the spectrum, four methine (CH) whose peaks are found down field on the same positive axis with the methyl group and three methylene (CH_2) signals are located on the negative axis of the DEPT 135 spectrum which was the opposite of the positioning of CH and CH_3. The long range HMBC correlation of H-3 at δ_H 4.21 appeared with the C-4 at δc 29.8, C-5 at δ_C 34.3 ppm while H-4 (δ_H 1.84 ppm) showed correlation with the C-5 and C-9 at δ_C 34.3, 47.9; H-8 at δ_H 2.28 ppm revealed correlation with C-7 and C-9 at δ_C 74.4 and 47.9 ppm, respectively; H-9 showed correlation with C-5, C-8, C-10, C-1 at δ_C 34.4, 44.9, 14.3 and 178.9 ppm, respectively; H-10 at δ_H 1.47 resonated with C-5, C-7, C-8, C-9 at δ_C 34.3, 74.4, 44.9 and 47.9, respectively). The EIMS data (low and high resolution) of compound A suggested the molecular mass of 170.0gmol^{-1} with molecular formula $C_9H_{14}O_3$. Thus, Compound A was elucidated as (4As, 6S, 7R, 7aS) -6-hydroxy-7-methyl-4,4a,5,6,7,7a-hexahydro-3H-cyclopenta[c] pyran-1-one (boonein) whose spectral data were comparable with those previously reported for boonein by Marini-bettolo (1983). All the proton and carbon signals

were apportioned, based on the $^{1}H^{1}$HCOSY, DEPT analysis, HMQC and HMBC (Figures 4.14-1-4.14.9). The chemical structure of compound A is shown in figure 5.13.1

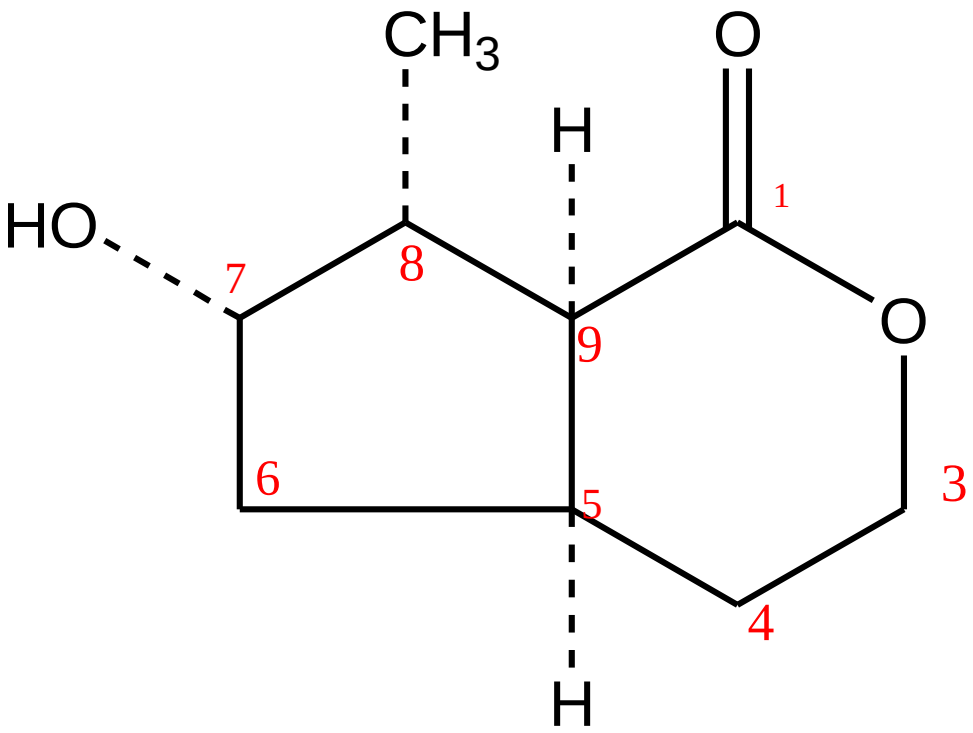


Figure 5.13.1**:** Chemical Structure of Compound A **(Boonein)**

IUPAC Name: (4As, 6S, 7R, 7aS) -6-hydroxy-7-methyl-4,4a,5,6,7,7a-hexahydro-3H-cyclopenta[c] pyran-1-one (boonein)

Chemical formula: $C_9H_{14}O_3$

5.13.2 Structural characterisation and Identification of Compound B (β-amyrin)

Compound B was obtained as a white powder from DCM: Hex (80:20) fraction of column chromatography which did not fluoresce under UV light at both the short and long wavelength (254 and 365 nm) but when sprayed with chromogenic reagents (ceric sulphate and 10% sulphuric acid) gave purple colour. Five out of the six double bond equivalents were adjusted in a pentacyclic carbon framework; one manifested as a CC double bond. The ^{1}HNMR spectrum revealed the existence of eight methyl singlets at C23-C30 with δc (15.7, 28.5, 16.5, 17.0, 26.1, 28.6, 33.4, 30.0) ppm: including an olefinic proton at δ_H 5.16 ppm and an oxygenated proton at δ_H 3.12 ppm, further suggesting oleanane type triterpenoid nucleus. All the proton and carbon signals were positioned, based on the ^{1}H^{1}HCOSY, DEPT analysis, HMQC and HMBC (Figures 4.14.10-4.14.15). The EIMS data (low and high resolution) gave the molecular mass as 426.8 gmol^{-1}, which corresponds with the molecular formula $C_{30}H_{50}O$. Thus, Compound B was elucidated as 3b-hydroxylolean-12-ene whose spectral data were comparable with those previously reported and previously identified β-amyrin by Okoye *et al*. (2014). The chemical structure of compound B is shown in figure 5.13.2

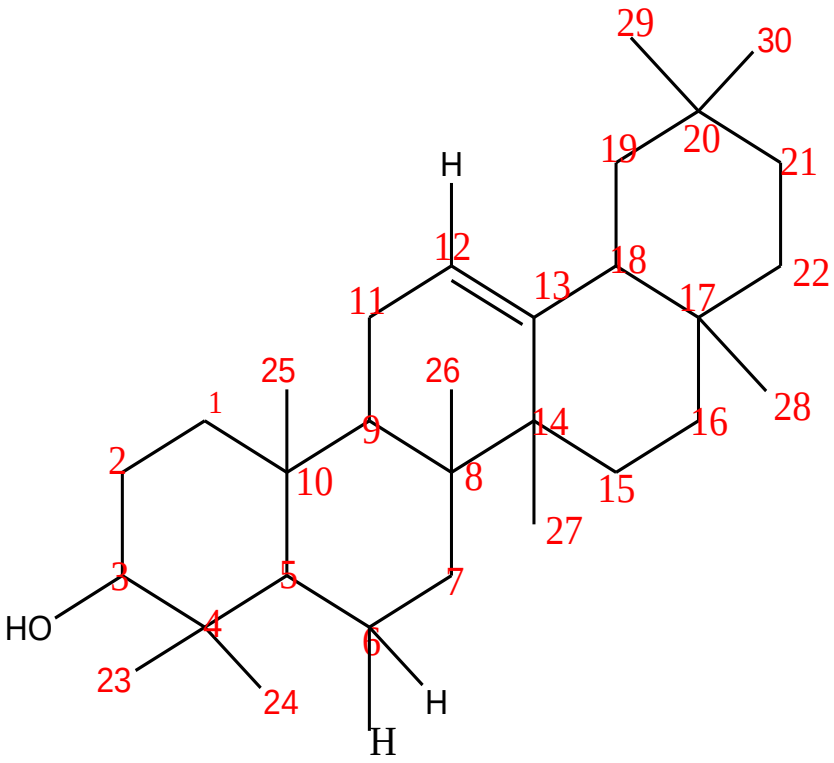


Figure 5.13.2**:** Chemical Structure of Compound B **(β-amyrin)**

IUPAC Name: 3b-hydroxylolean-12-ene (β-amyrin)

Chemical formula: $C_{30}H_{50}O$

5.14 Conclusion

Alstonia boonei and *Alstonia congensis* are often confused due to their similarities in numerous areas of morphological characteristics and medicinal uses. The foliar epidermal and anatomical characters presented in this study for both *A. boonei* and *A. congensis* are useful for identifying them individually as well as differentiating one species from the other. The generic variation observed in the qualitative and quantitative microscopical studies as described in this Thesis such as the epidermal cell size, vein-termination, vein-islet number and stomatal number of *A. boonei* and *A. congensis* are always constant and could therefore be used for correct identification of the two species, individually and independently of each other. Using micro-morphological differences, the significance of anatomical characters is generally accepted for taxonomic differentiation. The assemblage of the results in this study would represent some of the data required in the compilation of the pharmacopoeial monograph for each of the two *Alstonia* species with subsequent incorporation into the next editions of the 2013 West African Herbal Pharmacopoeia and the 2008 Nigerian Herbal Pharmacopoeia.

DNA barcoding was utilised in delineating accessions of *A. boonei* and *A. congensis* successfully. Hence, the study has demonstrated that the microscopic, other pharmacognostic and molecular techniques could be used to complement taxonomical identification of closely related *Alstonia* species.

Physicochemical analysis, which form part of the parameters used in the standardization and monographs of medicinal plants was also employed in this study to add onto other properties for comprehensive standardisation of *A. boonei* and *A. congensis*. For example, the amounts of earthy materials and minerals indicated by the total ash values in the leaf has showed that it must not be more than 8.5% in *A. boonei* and 6.8% in *A. congensis*. The moisture contents obtained were within the recommended limits for *A. boonei* and *A. congensis* as recorded in herbal pharmacopoeias. The protein, carbohydrate and crude fibre values obtained in the proximate analysis showed that the two plants possessed nutritive value that influences the medicinal potentials of the plants.

Also, the elemental analysis in the present study revealed the presence of macro and micro elements which could be of therapeutic importance. Calcium content was found at the

highest concentration in both the leaves and stem-barks of *A. boonei* and *A. congensis* which could contribute to its therapeutic profile as antiarthritic drug since calcium helps in bone formation and strengthening. The phytochemical screening showed the presence of different classes of phytochemicals in *A. boonei* and *A. congensis,* which are responsible for the variety of pharmacological activities, supporting the well-known belief that plants are natural chemical laboratories for the synthesis of variety of compounds.

This study demonstrated that aqueous crude extracts and DCM fractions of both *A. boonei* and *A. congensis* exhibited dose-dependent antispasmodic activities causing peristaltic reduction of rat ileum, the ethyl acetate fractions exhibited both antispasmodic and spasmodic activities, while the aqueous fraction of *A. congensis* exhibited spasmodic activity alone. The DCM fractions exhibited the highest antispasmodic activities which showed that the constituents responsible for the observed antispasmodic activities were non-polar compounds.

In the experiments using castor-oil to induce diarrhoea, the crude extracts and the DCM fractions of the two *Alstonia* species were able to lessen the total number of defaecation and wet faeces as well as increase the onsets of diarrhoea. *A. boonei* stem-bark extract exhibited higher antidiarrhoeal activities than *A. congensis*. The AP1, AP2 and AP3 herbal preparation mixtures of *A. boonei* stem-bark and *Picralima nitida* seeds showed antidiarrhoeal activities but the highest activity was found in AP3, which contained twice the content of *A. boonei* stem-bark.

Two chemical constituents of *A. boonei* stem-bark were isolated and characterised as boonein and β-amyrin from the DCM fraction. They were elucidated with spectroscopic techniques, 1-D NMR (^{1}H NMR, ^{13}C NMR, DEPT), 2-D NMR (COSY, HSQC, HMBC, NOESY) and EI-MS. These compounds, with some other constituents of the plant may have been responsible for the therapeutic effects (including the antidiarrhoeal activities), observed ethno-medically and as validated by the present study. Both isolated compounds A and B relaxed the spontaneous and high potassium induced contractions of isolated rat ileum while their antispasmodic activities are being reported for the first time in this study. **Boonein** possessed a stronger antispasmodic action than **β-amyrin,** hence, may represent one of the major antidiarrhoeal agents in *A. boonei*.

5.15 Recommendations

The following recommendations are vital in the light of the findings of this research::

1. Other DNA barcoding primers should be employed to complement the ITS primers used in the delineation of *A. boonei and A. congensis.*
2. *A. boonei* stem-bark decoction gave the formulated *DIASTIL SYRUP* in this Thesis which can enter into the primary health care (PHC) level utilisation for the management of diarrhoea and other hypermotility-related gastrointestinal disorders, e.g. stomach or duodenal ulcers
3. Isolation and Identification of other constituents from *A. boonei* and *A. congensis* stem-barks may yield more potent antidiarrhoeal agents
4. Synthesis of boonein should lead to conventional antidiarrhoeal dosage forms, e.g. tablet solid dosage form.

5.16 Contributions to knowledge

1. Establishment of pharmacognostic characters, which could be used in identifying the two similar species; *A. boonei* and *A. congensis* which led to the compilation of their monographs in this Thesis;
2. The monographs may be used for updating the Nigerian Herbal Pharmacopoeia (NHP, 2008) and the West African Herbal Pharmacopoeia (WAHP, 2020) ;
3. Delineation of *A. boonei* and *A. congensis* at molecular level with DNA barcode using ITS 1 and ITS 4 primers, is being reported for the first time;
4. Scientific validation of ethno-medical claims on *A. boonei* and *A. congensis* in the treatment of diarrhoea, thus, supporting the hypothesis of this study;
5. Antispasmodic activities of boonein and β-amyrin, isolated from the DCM fraction of *A. boonei* are being reported for the first time;
6. A new antidiarrhoeal herbal medicine (DIASTIL SYRUP) containing *A. boonei* stem-bark was formulated and produced as a packaged stable and patentable remedy.

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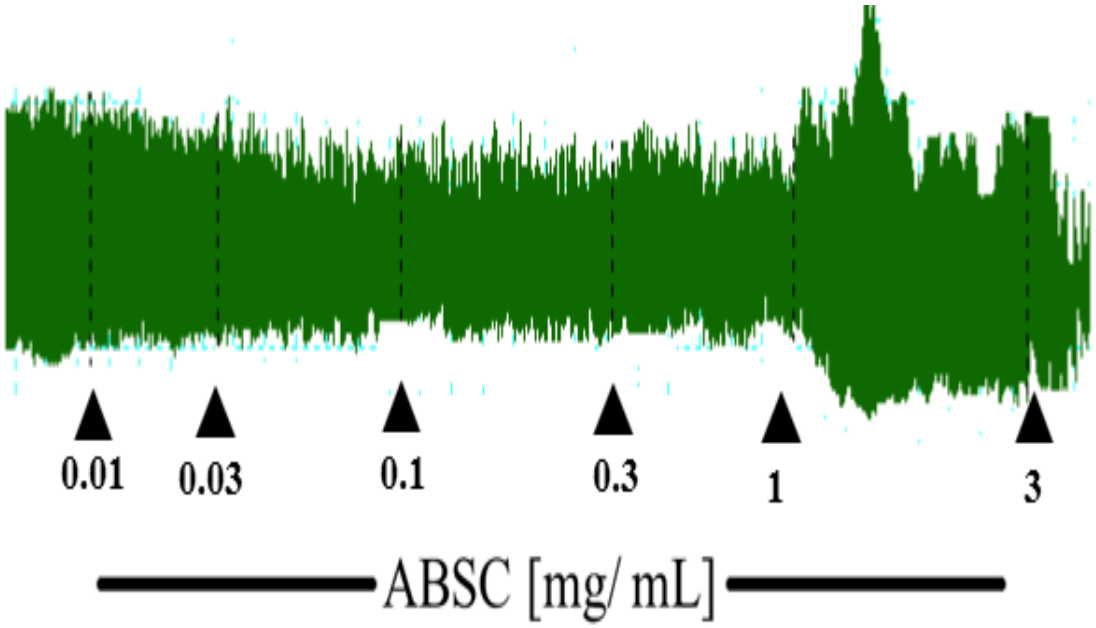
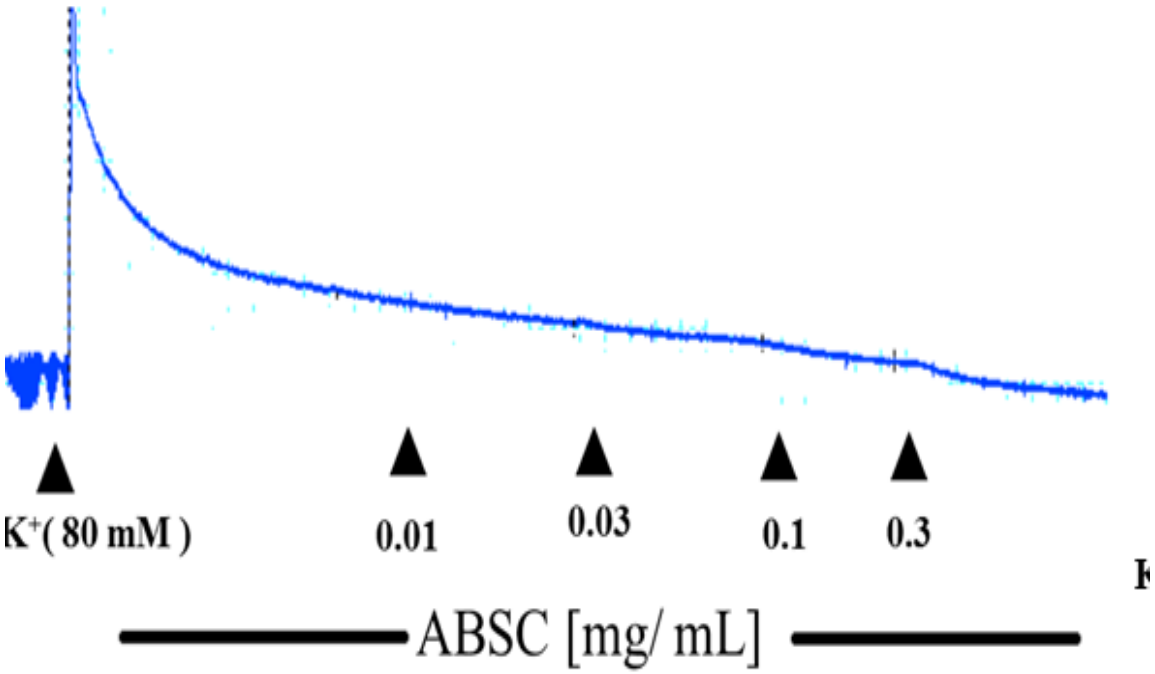
APPENDICES

Appendix I



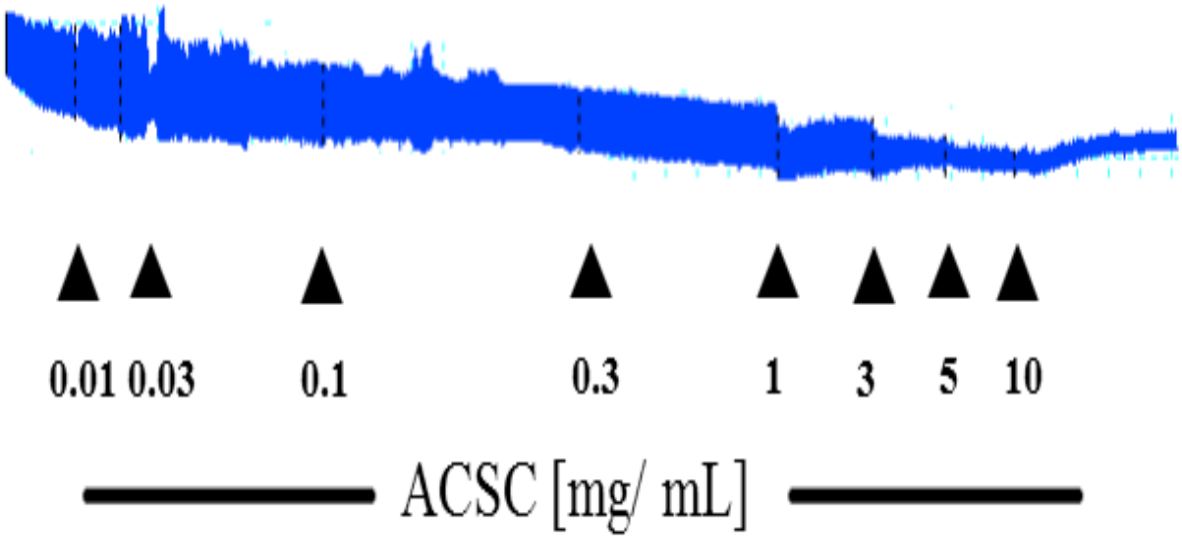
Antispasmodic effect of crude extract of *Alstonia boonei* stem-bark ($mgmL^{-1}$) on spontaneous contraction of isolated rat ileum tissue

Appendix II



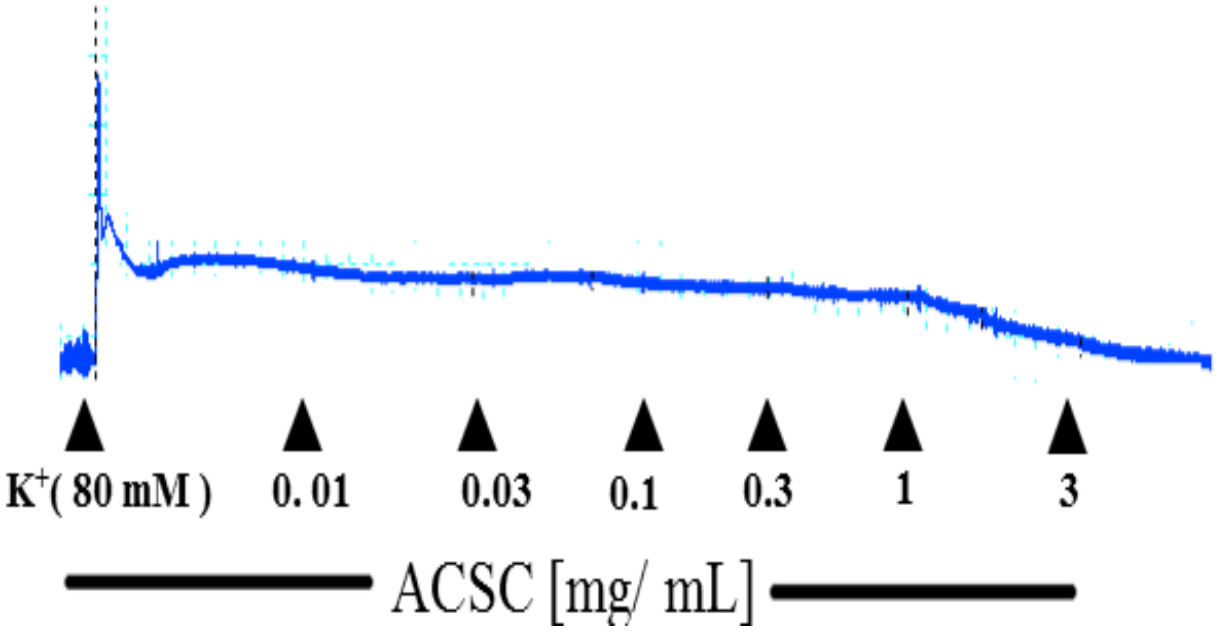
Antispasmodic effect of crude extract *Alstonia boonei* stem-bark ($mgmL^{-1}$) on induced of isolated rat ileum tissue

Appendix III



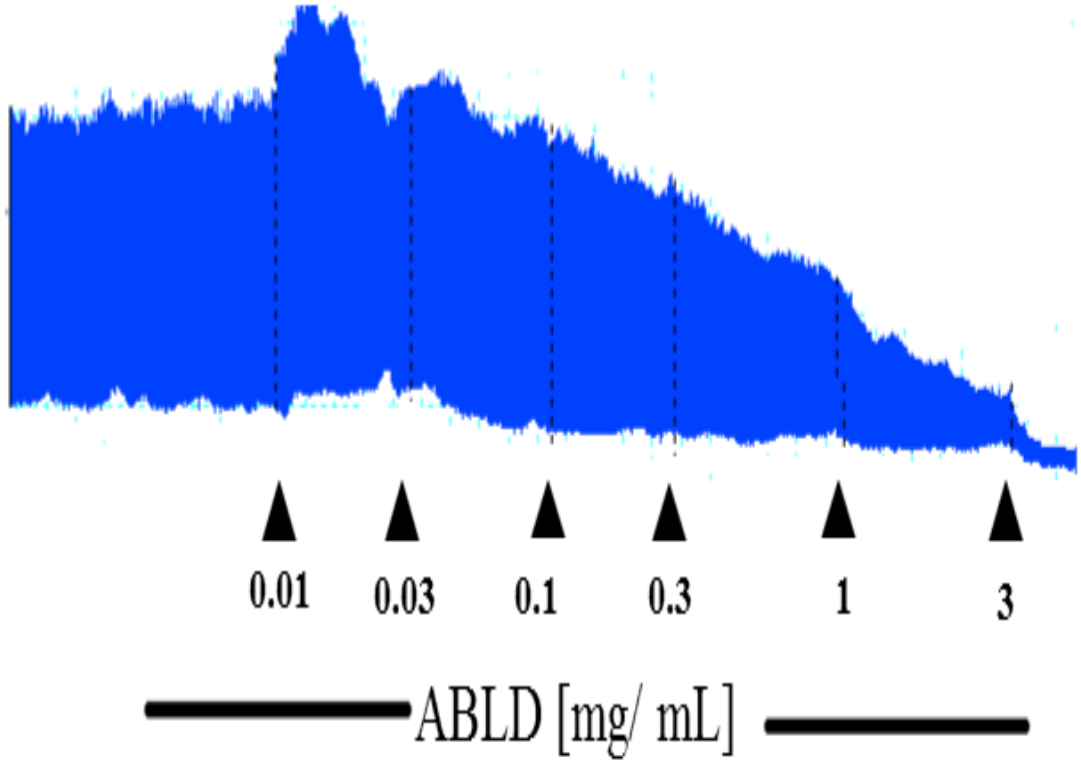
Antispasmodic effect of crude extract of *Alstonia congensis* stem-bark ($mgmL^{-1}$) on spontaneous contraction of isolated rat ileum tissue

AppendixIV



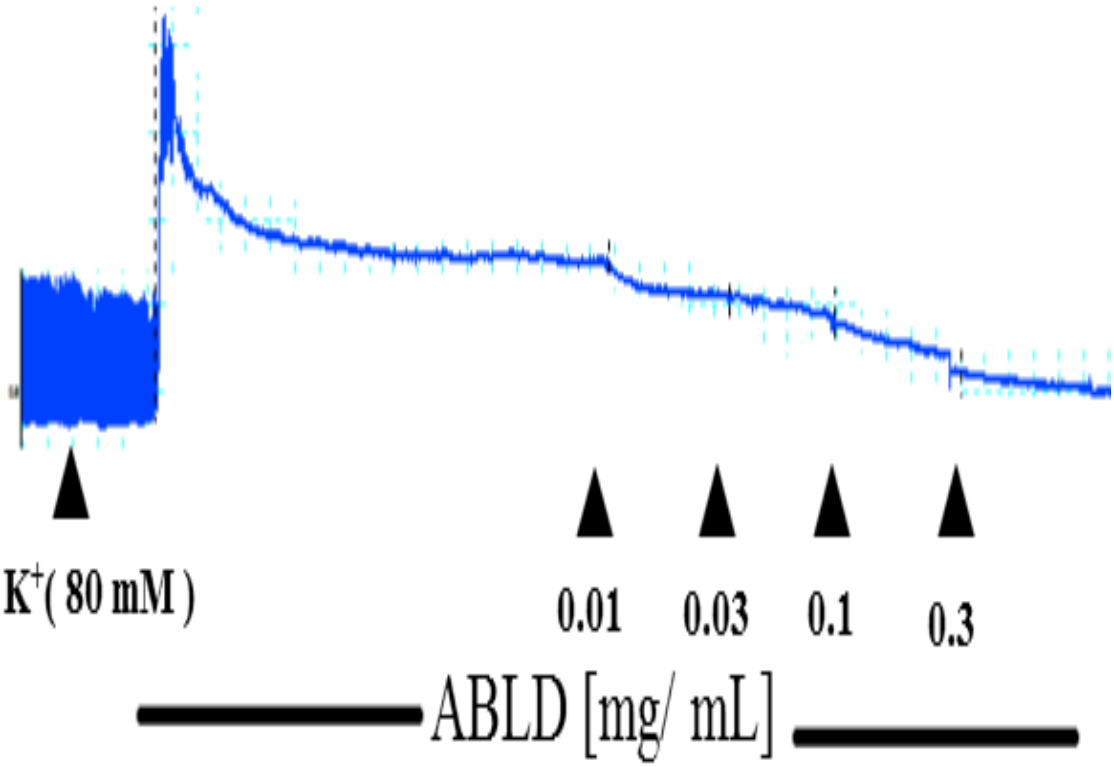
Antispasmodic effect of crude extract *Alstonia congensis* stem-bark ($mgmL^{-1}$) on high [K^+] induced of isolated rat ileum tissue

Appendix V



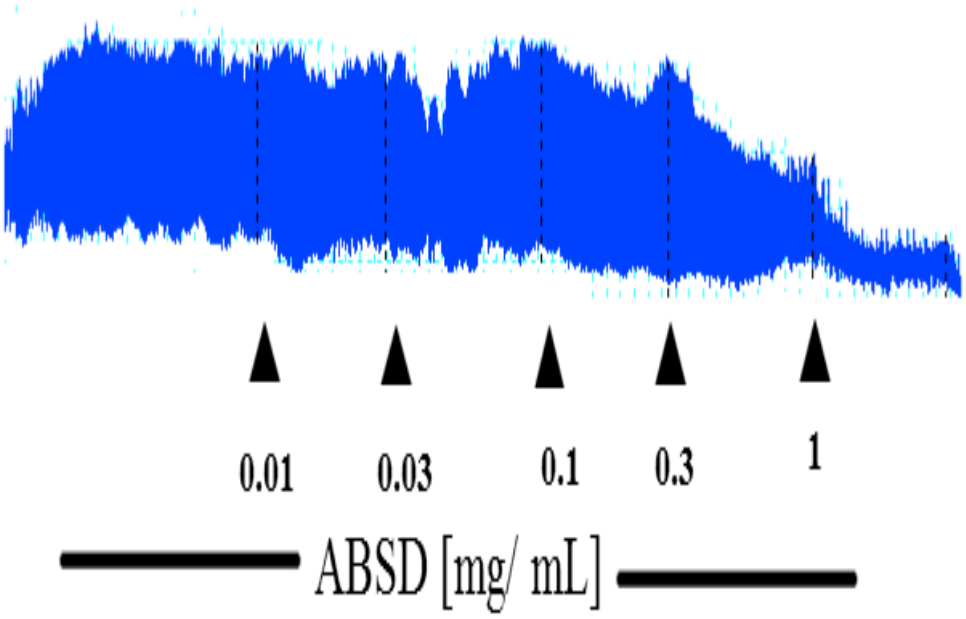
Antispasmodic effect of DCM fraction of *Alstonia boonei* leaf ($mgmL^{-1}$) on spontaneous contraction of isolated rat ileum tissue

Appendix VI



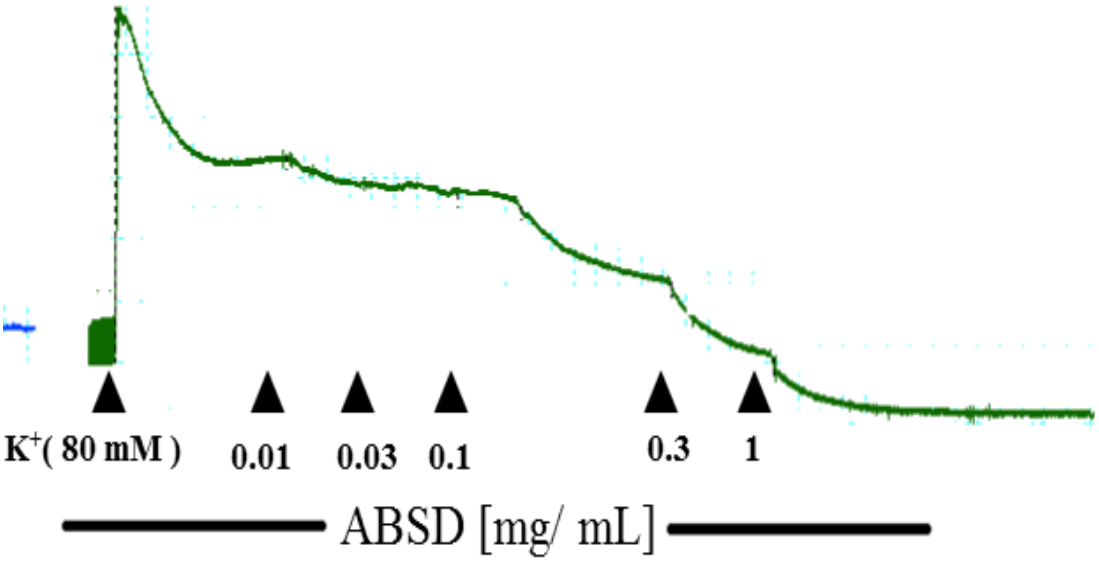
Antispasmodic effect of DCM fraction of *Alstonia boonei* leaf ($mgmL^{-1}$) on high [K^+] induced of isolated rat ileum tissue

Appendix VII



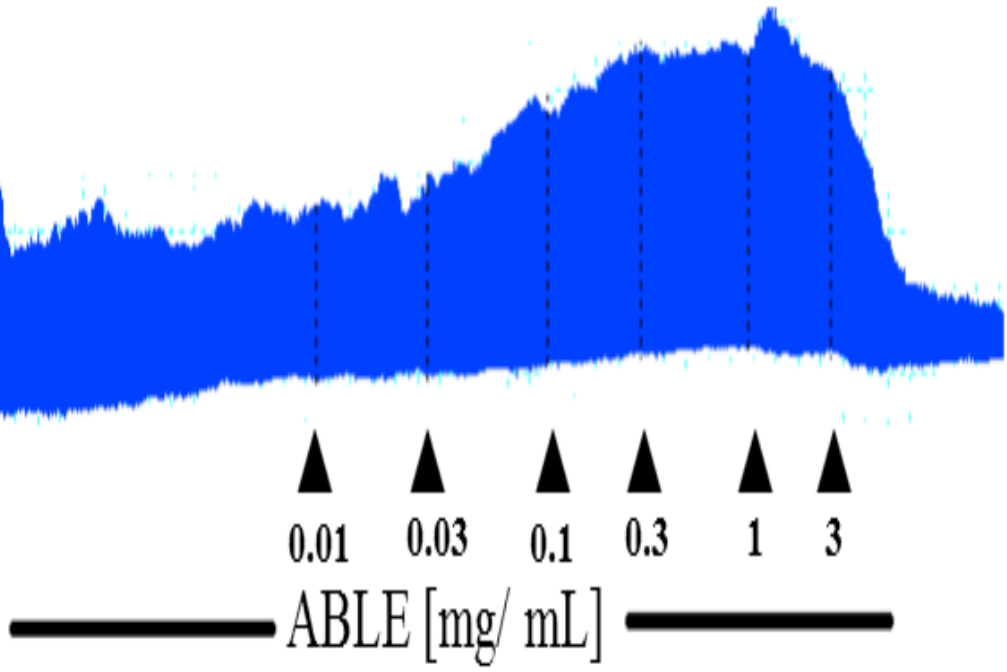
Antispasmodic effect of DCM fraction of *Alstonia boonei* stem-bark ($mgmL^{-1}$) on spontaneous contraction of isolated rat ileum tissue

Appendix VIII



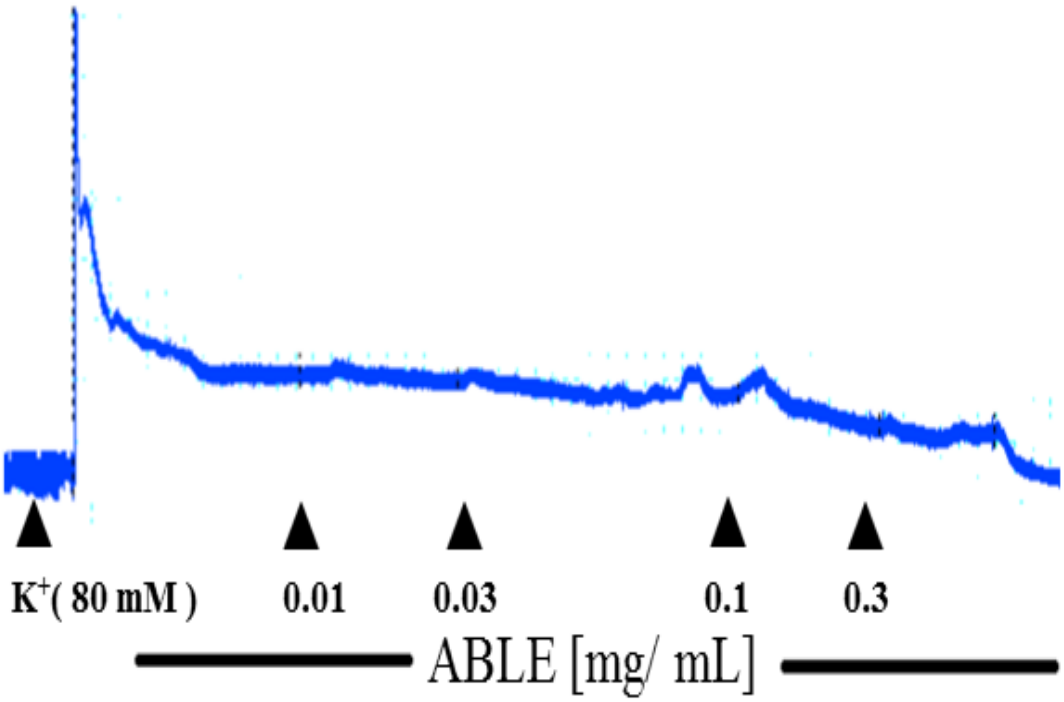
Antispasmodic effect of DCM fraction of *Alstonia boonei* stem-bark ($mgmL^{-1}$) on high potassium induced of isolated rat ileum tissue

Appendix IX



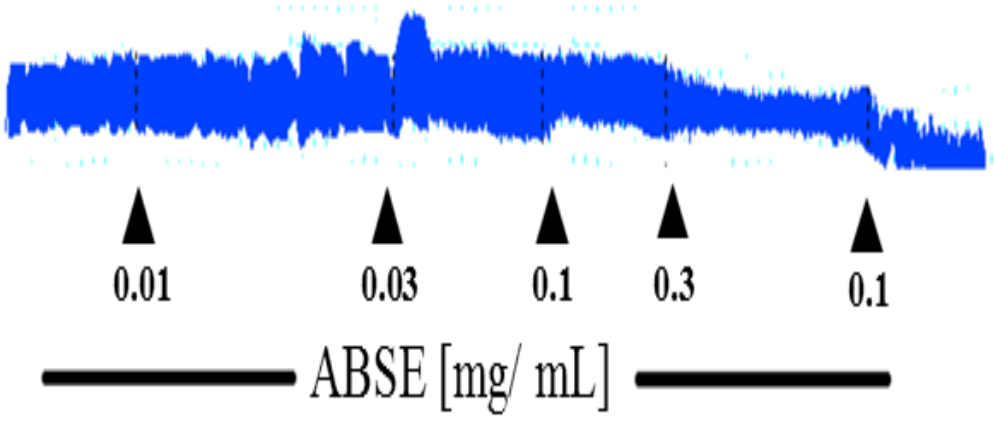
Antispasmodic effect of Ethyl acetate fraction of *Alstonia boonei* leaf ($mgmL^{-1}$) on spontaneous contraction of isolated rat ileum tissue

Appendix X



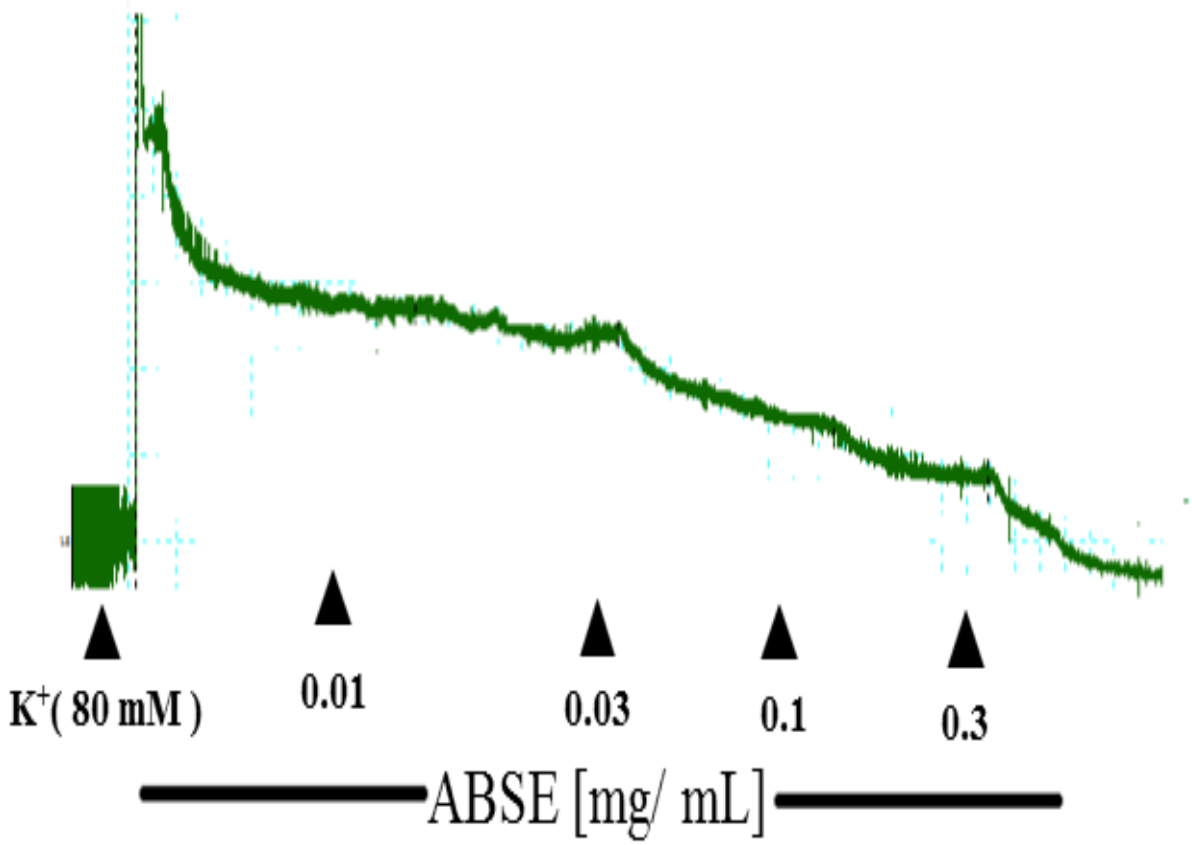
Antispasmodic effect of ethyl acetate fraction of *Alstonia boonei* leaf ($mgmL^{-1}$) on high potassium induced contraction of isolated rat ileum tissue

Appendix XI



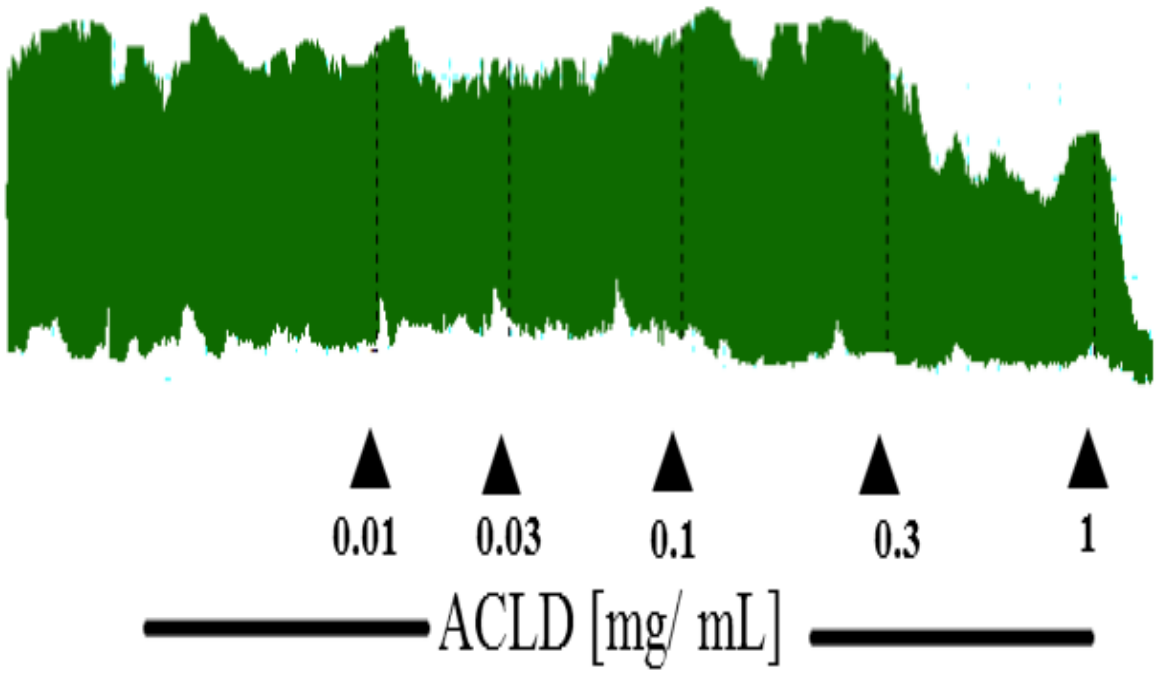
Antispasmodic effect of Ethyl acetate fraction of *Alstonia boonei* stem-bark ($mgmL^{-1}$) on spontaneous contraction of isolated rat ileum tissue

Appendix XII



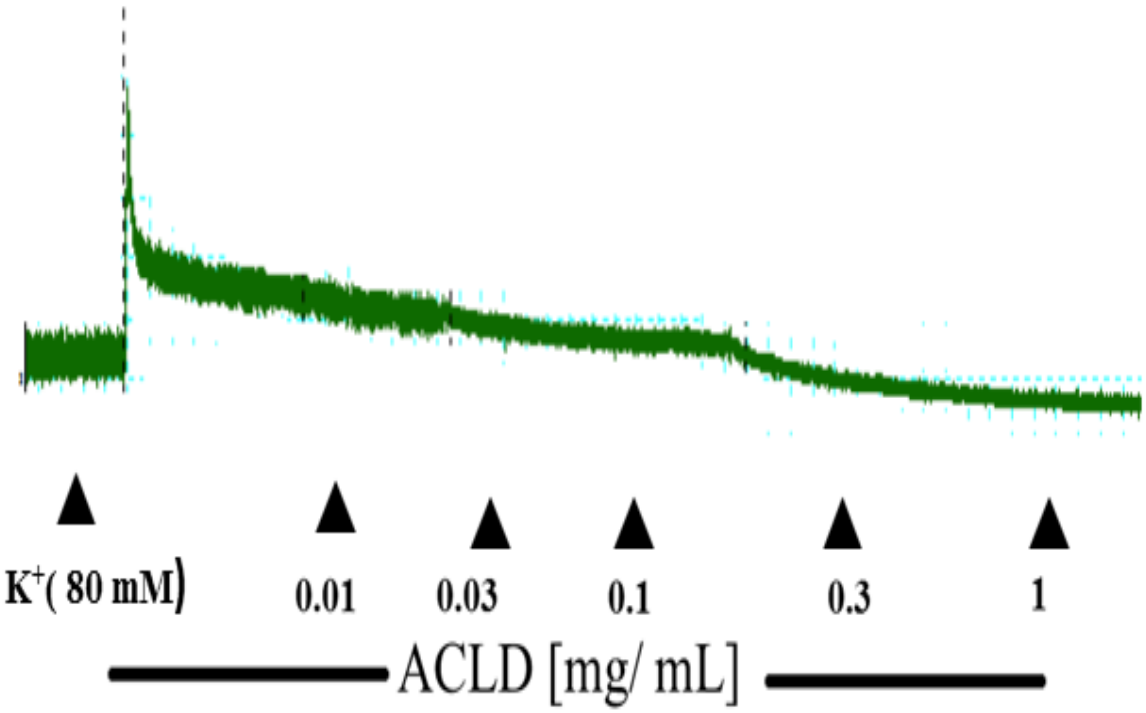
Antispasmodic effect of Ethyl acetate fraction of *Alstonia boonei* stem-bark ($mgmL^{-1}$) on high potassium induced contraction of isolated rat ileum tissue

Appendix XIII



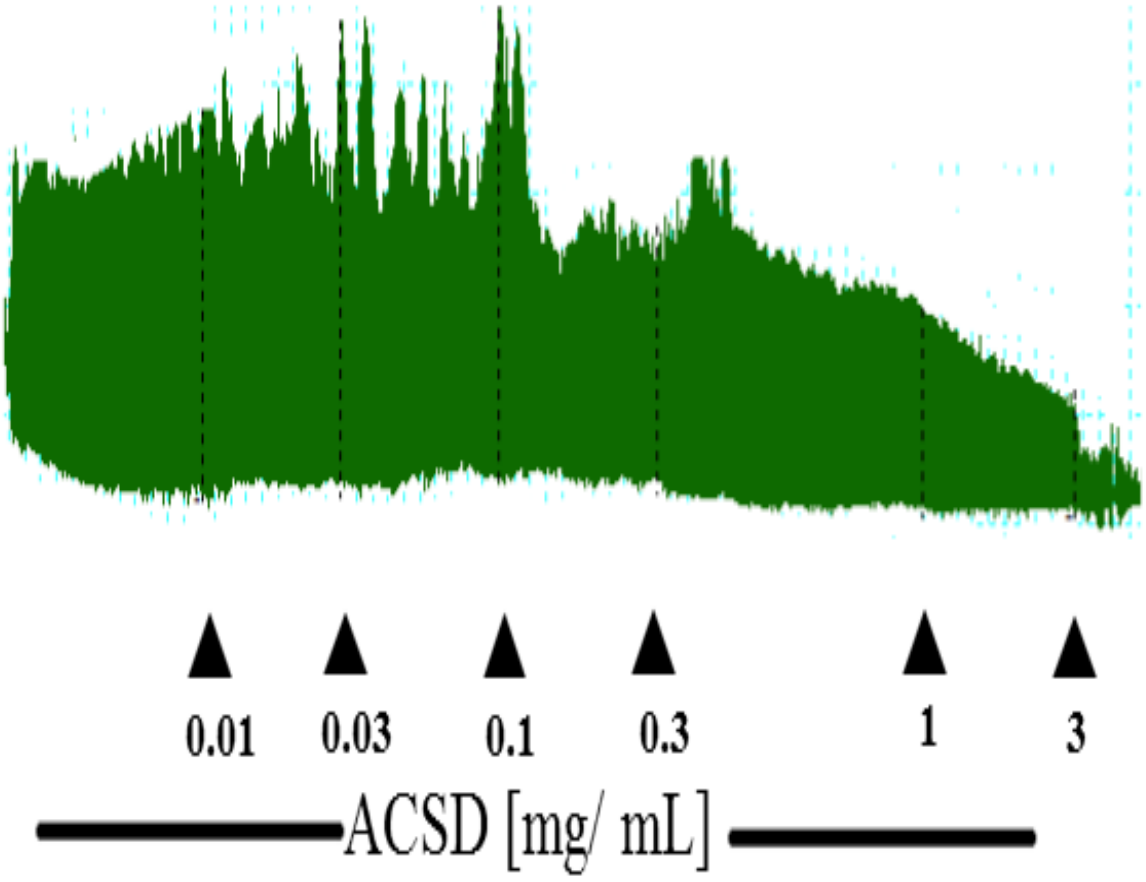
Antispasmodic effect of DCM fraction of *Alstonia congensis* leaf ($mgmL^{-1}$) on spontaneous contraction of isolated rat ileum tissue

Appendix XIV



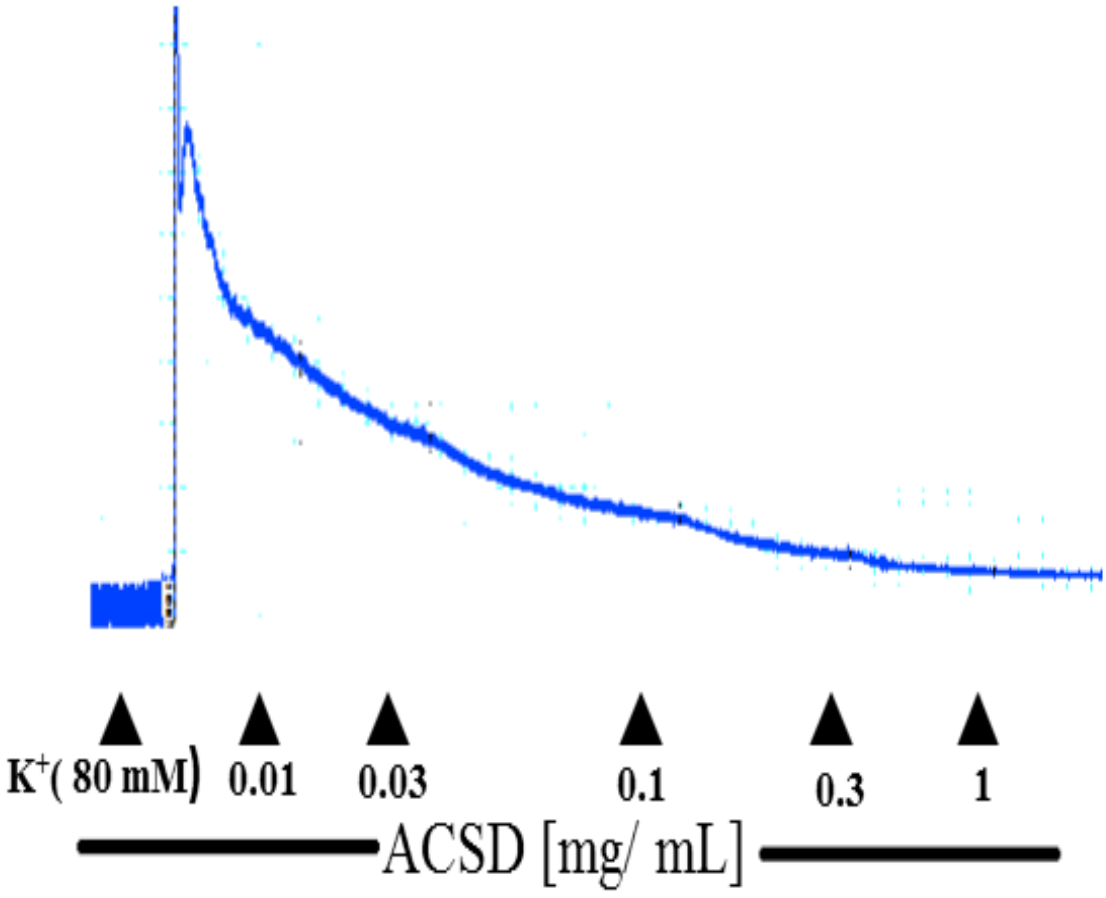
Antispasmodic effect of DCM fraction of *Alstonia congensis* stem-bark ($mgmL^{-1}$) on high potassium induced contraction of isolated rat ileum tissue

Appendix XV



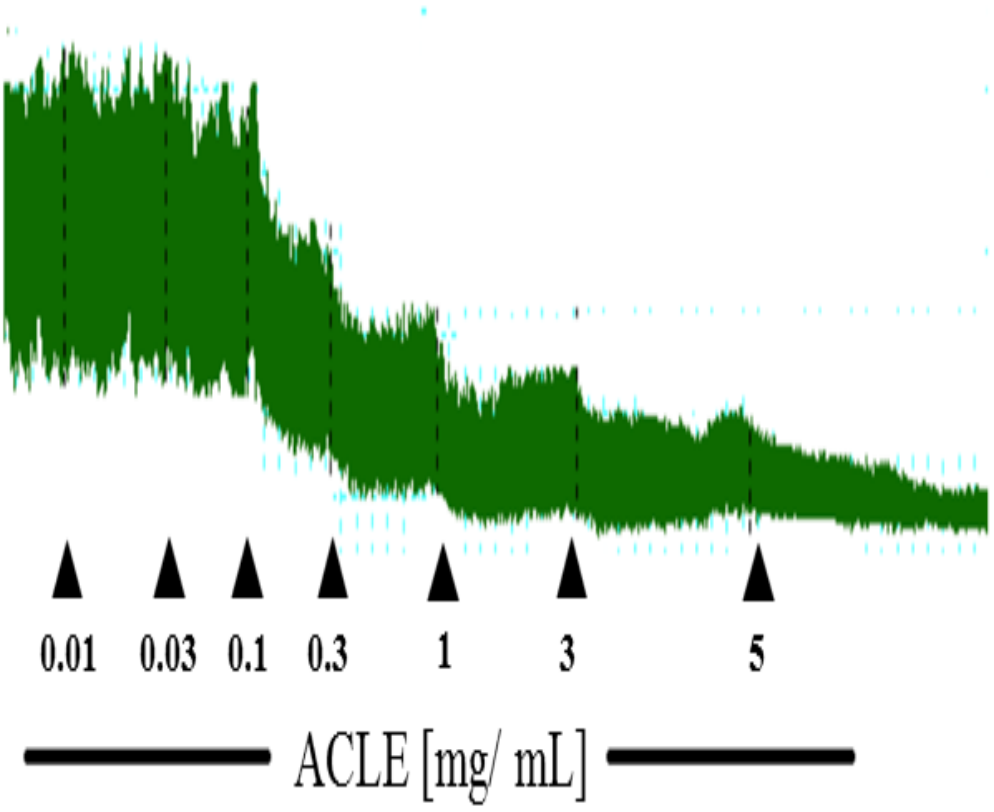
Antispasmodic effect of DCM fraction of *Alstonia congensis* stem-bark ($mgmL^{-1}$) on spontaneous contraction of isolated rat ileum tissue

Appendix XVI



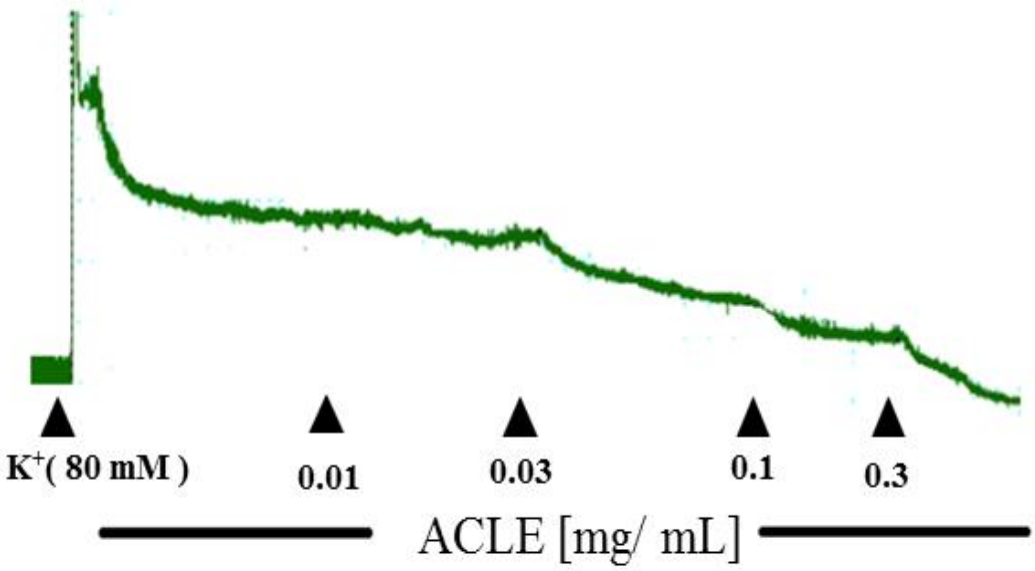
Antispasmodic effect of DCM fraction of *Alstonia congensis* stem-bark ($mgmL^{-1}$) on high potassium induced contraction of isolated rat ileum tissue

Appendix XVII



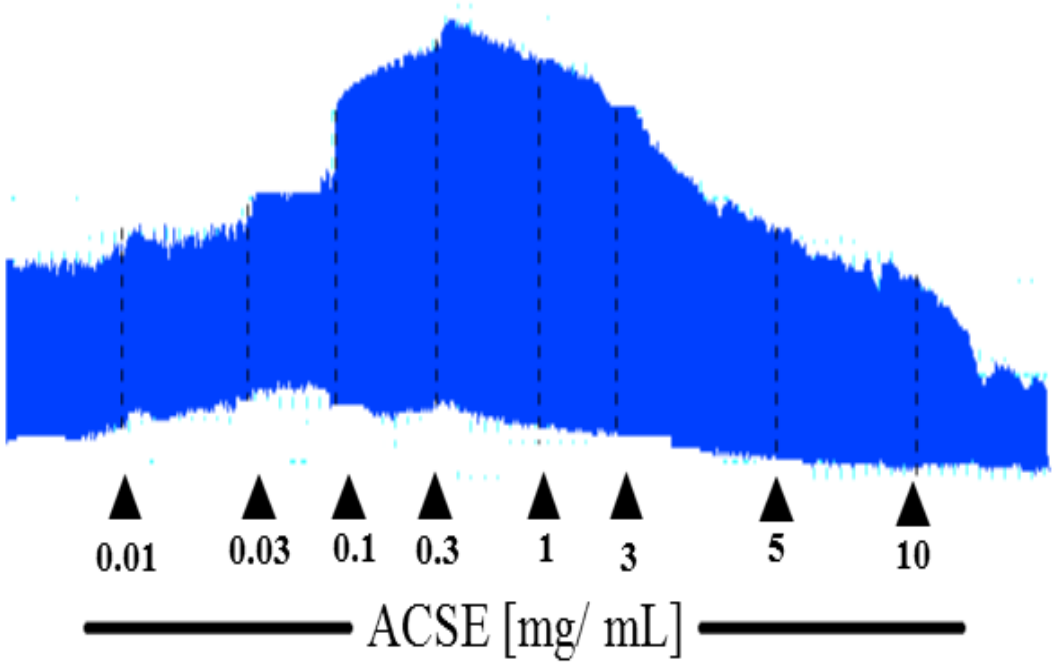
Antispasmodic effect of *Alstonia congensis* ethyl acetate leaf fraction ($mgmL^{-1}$) on spontaneous contraction of isolated rat ileum tissue

Appendix XVIII



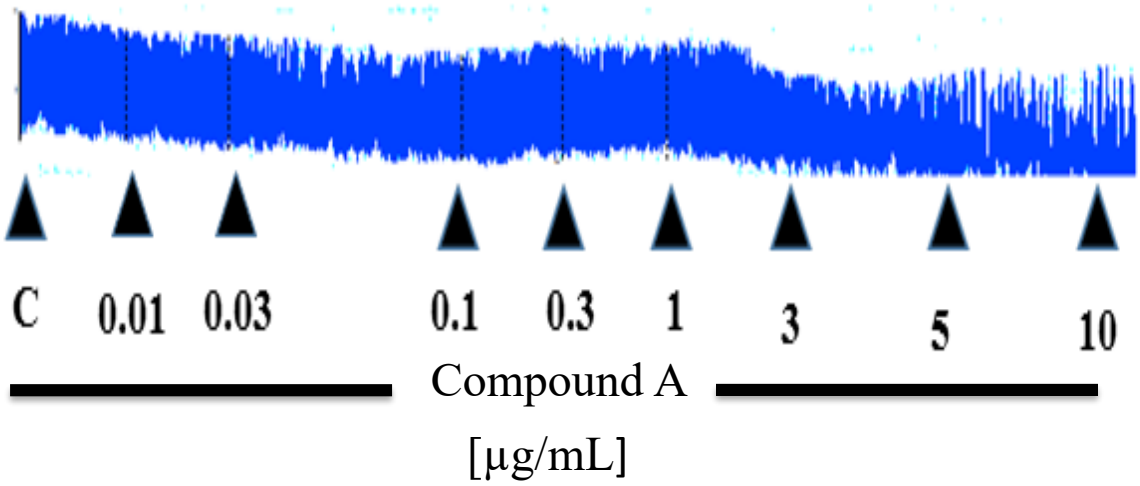
Antispasmodic effect of *Alstonia congensis* ethyl acetate leaf fraction ($mgmL^{-1}$) on high potassium induced contraction of isolated rat ileum tissue

Appendix XIX



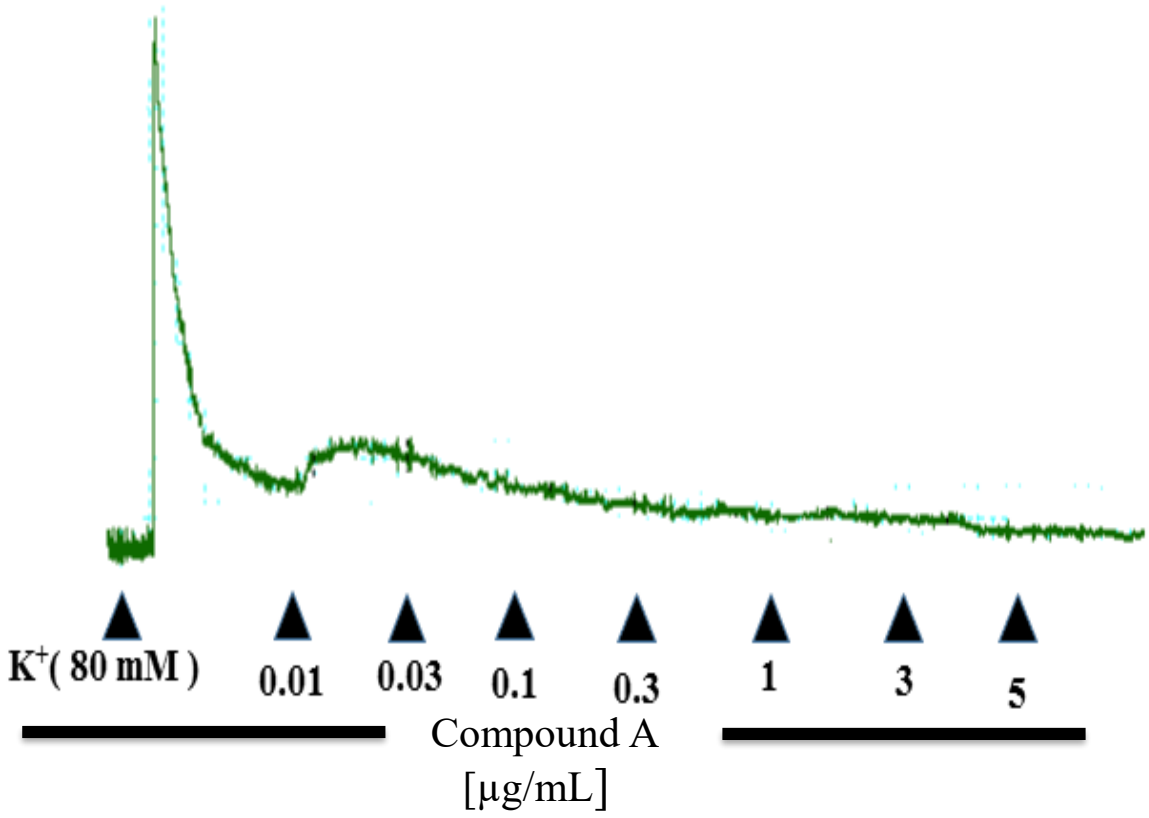
Antispasmodic effect of *Alstonia congensis* ethyl acetate stem-bark fraction ($mgmL^{-1}$) on spontaneous contraction of isolated rat ileum tissue

Appendix XX



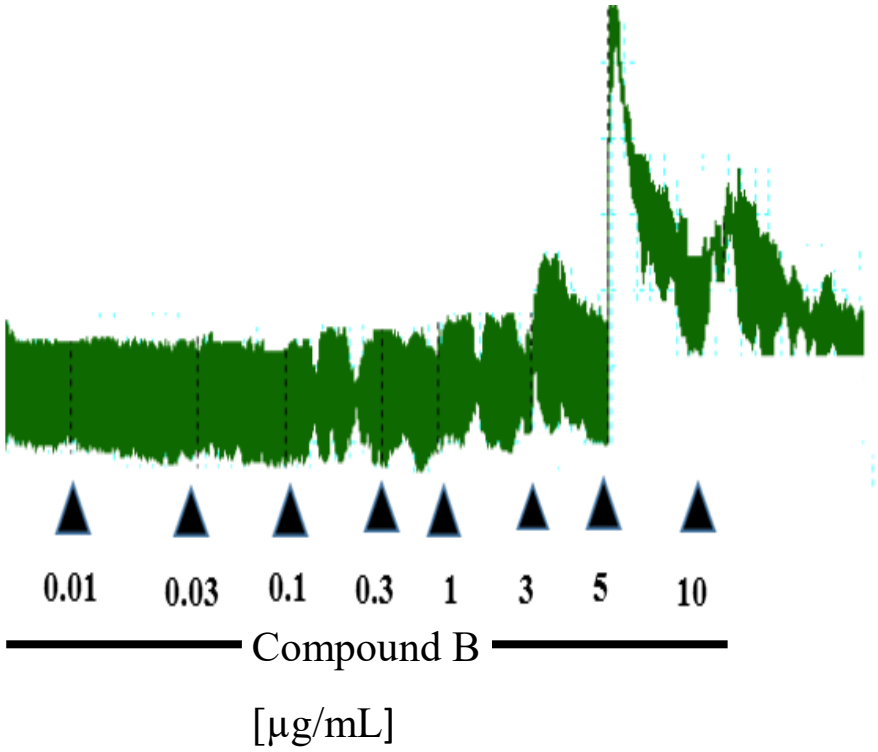
Antispasmodic effect of Compound A isolated from the DCM fraction *Alstonia boonei* stem-bark aqueous extract (µg/mL) on spontaneous contraction of isolated rat ileum tissue

Appendix XXI



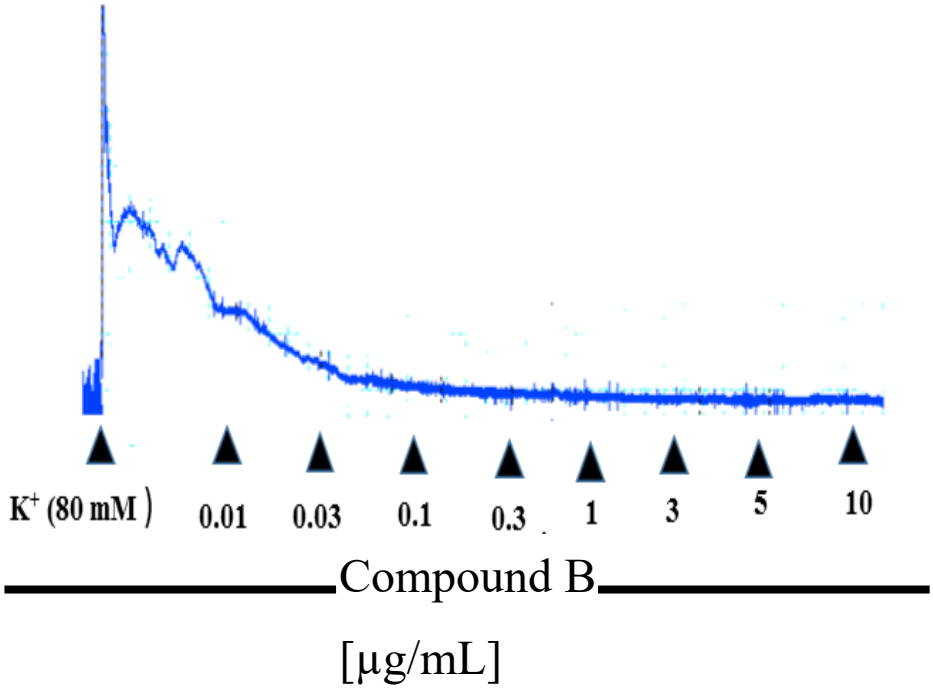
Antispasmodic effect of Compound A isolated from the DCM fraction *Alstonia boonei* stem-bark aqueous extract (µg/mL) on high potassium induced contraction of isolated rat ileum tissue

Appendix XXII



Antispasmodic effect of Compound B isolated from the DCM fraction *Alstonia boonei* stem-bark aqueous extract (µg/mL) on spontaneous contraction of isolated rat ileum tissue

Appendix XXIII



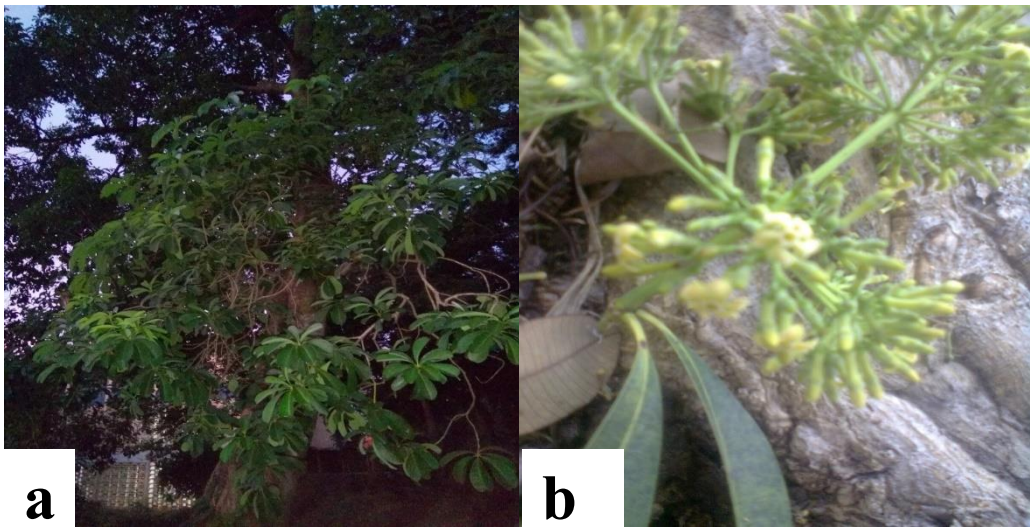
Antispasmodic effect of Compound B isolated from the DCM fraction *Alstonia boonei* stem-bark aqueous extract (µg/mL) on high potassium induced of isolated rat ileum tissue

Appendix XXIV

MONOGRAPH FOR A. BOONEI

***Alstonia boonei* De Wild.**

FAMILY APOCYNACEAE



a- *Alstonia boonei* tree and leaves; b- *Alstonia boonei* inflorescence; c- dry outer surface of *Alstonia boonei* stem-bark; d- dry inner surface of *Alstonia boonei* stem-bark

Common name: Stool wood, fever wood

Nigerian names: awun, ahun (Yoruba), egbu, egbu ora (Ibo), Ukhu (Bini), Okugbe (Itsekiri), Ukpukuhu (Urhobo) (Okoye *et al*., 2014).

Geographical distribution

A. boonei is a light demanding species of Alstonia widely distributed in the tropics of Africa including Senegal, Gambia, Ivory Coast, Ghana, Nigeria, Cameroon. It is commonly found in rocky sites or dry land and sometimes in marshy area (Okoye *et al*., 2014).

Brief description of the plant

It is a large evergreen tree of about 40 m high and 1.2- 1.4 m in diameter, known for its plenteous milky sap production. Bark is scaly, grey to greyish green in colour, inner bark is off white to creamy to yellowish colour. Leaves are simple and entire, arrange in whorls of 4–8, subsessile to petiolate, glabrous, no stipules, petiole 0.5-1cm long and stout; blade is oblanceolate to obovate, apex is acute to emarginate, base cuneate, margin is entire with coriaceous texture and dark green above and light green underneath, midrib is more conspicuous below. Flowers are regular and hermaphrodite, creamy in colour. The fruits are made of two linear follicles, greenish in colour with hairy flat seed (Burkhill, 1985; Orwal *et al*., 2009).

Herbarium specimen

The herbarium sample has been deposited at the Faculty of Pharmacy Herbarium, Department of Pharmacognosy, Obafemi Awolowo University, Nigeria with FPI number 2169

Ethnomedicinal uses

The stem-bark of *A. boonei* and a bunch of *Piper guineensis* fruit are infused in local gin. A teaspoon of the infusion is administered daily for treatment of impotence. Infusion of bark is used to treat malaria, diarrhoea (Igberaese &Ogbole, 2018), analgelsic, asthma, antidote and used in treaing painful micturition and rheumatic conditions, round worm and other intestinal parasites expeller, menstrual pain, hypoglycaemic, hyperyension (Ojewole, 1984; Asuzu and Anaga, 1991, Abbiw, 1990, Iwu, 1993)

Morphological part used as drug

Stem-bark

Other part used

leaf

Definition of the drug

consists of fresh or dried stem-bark of *Alstonai boonei* De Wild. family Apocynaceae

Description of the drug

Macroscopy

Bark is scaly, grey to greyish green in colour on the outer surface and off white to cream to yellowish colour on the inner surface

Microscopy

The epidermises of the leaf are polygonal in shape; the leaf is hypostomatic. The upper epidermis contains coastal cells and starch grains, stomata is only found on the lower epidermis, epidermis contains papillae, stomata are anomocytic, paracytic and or cyclotic. Mid-rib contains secretory cell, xylem vessels which are isolated, schlerenchymatic sheaths covering the phloem on the abaxial side are not coonected. Stem-bark transverse section of the bark contains cork cell which is polygonal and arranged in radial rows, cortex consists of ground mass of parenchymatous cells with lignified brachysclereids, cortex also contain prismatic calcium oxalate crystals, phloem and starch grains

Powdered drug

Cartoon colour with slight characteristic odour, containing fragment of wood from the stem, cork, cortex, calcium oxalate crystals, stone cells, fibres, phloem

Chemical and bioactivity profile

The stem-bark contains indole alkaloid including echitamine, echitamidine, iridoid like boonein as well as tanins, flavonoid, terpenoids like alpha and beta amyrin,. It possesses antispasmodic, antimalarial,analgesic and antidiarrrhoeal activity

Test for identity and purity

Moisture content (African Pharmacopoeia Methods, 1986): Not more than 8.5 %

Total Ash: Not more than 5.9 %

Acid-insoluble ash: Not more than 0.7 %

Alcohol soluble extractive: Not less than 4.5 %

Water soluble extractive: Not less than 5.0 %

Palisade ratio: 5.2

Vein-islet number: 9.0

Vein- termination number: 6.2

Chemical tests and assay

Alstonia boonei can be assayed by the qualitative and quantitative estimation of the total alkaloids and iridoids constituents using appropriate chromatographic techniques such as TLC and HPLC

Indications and dosage

An adult patient would be expected to take between 10-20 mL once or twice daily while children above 5 years would take between 5- 10 mL once or twice daily of the *Alstonia boonei* stem-bark decoction

Contraindications/ adverse effects/ precautions

Contraindicated in pregnancy, constipation, liver or kidney diseases

Storage conditions

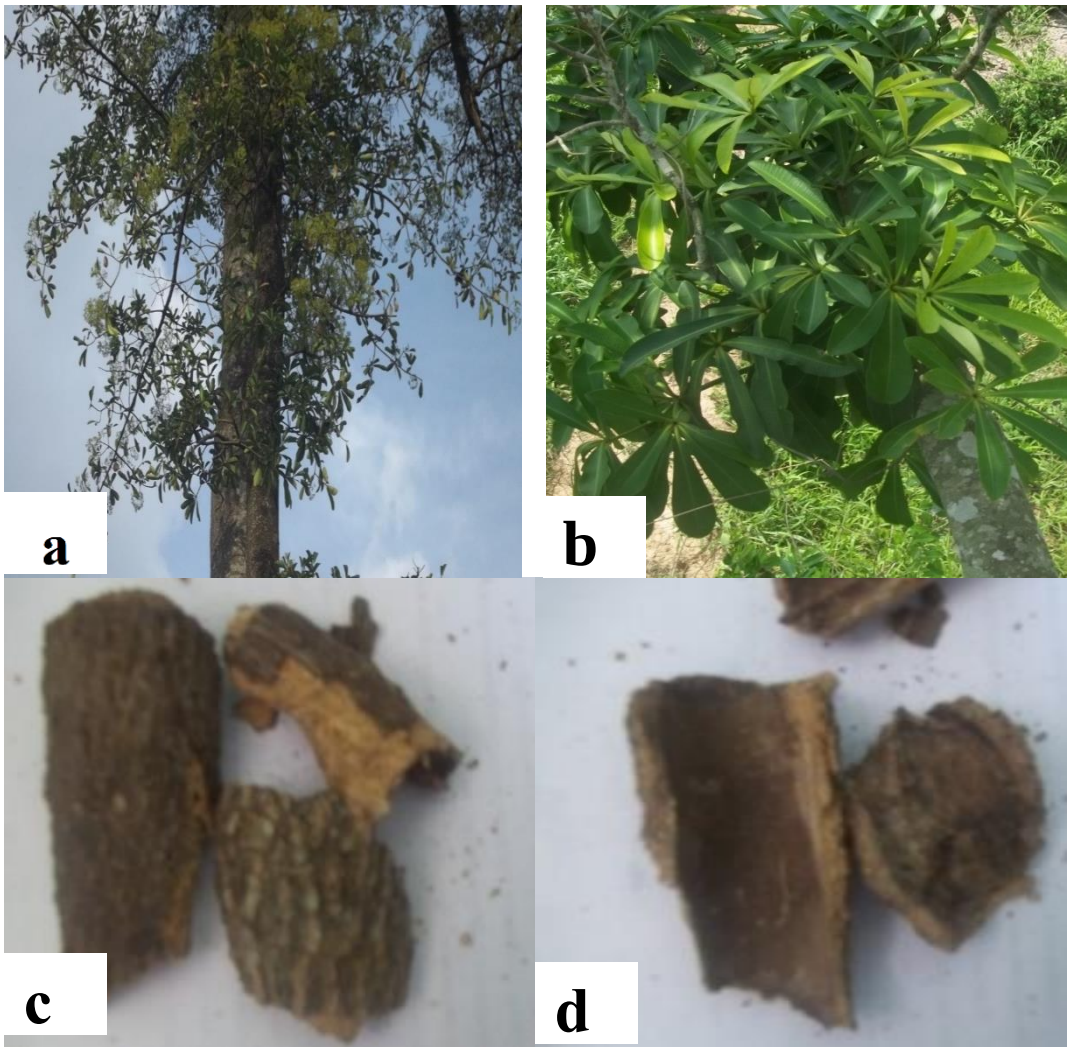
In an air tight, dry container, protected from light and in cool dry place

Appendix XXV

MONOGRAPH FOR *A. CONGENSIS*

***Alstonia congensis* Engl.**

FAMILY APOCYNACEAE



a- *Alstonia congesis* tree, leaves and infloresence; b- *Alstonia congesis* leaves; c- dry outer surface of *Alstonia congesis* stem-bark; d- dry inner surface of *Alstonia congesis* stem-bark

Common name: Stool wood, fever wood

Nigerian names: awun, ahun (Yoruba), egbu, egbu ora (Ibo), Ukhu (Bini), Okugbe (Itsekiri), Ukpukuhu (Urhobo) (Okoye *et al*., 2014).

Geographical distribution

Alstonia congensis is fast going into extinction, its found in tropical rainforest, occurring from the south-western Nigeria, Central African Republic, Democratic Republic of Congo and northern Angola, Cameroon and Liberia. Specimens for scientific identification were first collected from Congo. Hence, the specific epithet was coined from the Country (Orwa *et al*., 2009). The species usually occurs in swampy area with *Elaeis guineensis, Dioscorea preusii, Cnestis hirsutus*, *Funtumia africana*, Raphia, although it is sometimes found on drier soil.

Brief description of the plant

Alstonia congensis is an evergreen tree of about 30 m high, with copious latex. Its stem bark is greyish and rough, with large lenticels. The fresh inner bark is creamy, but brownish when dried. Its leaves are arranged in whorls of 6-8, simple, entire, stipules absent, petiole 0.5 to 1 cm, flattened and grooved with narrowly triangular colleters at base. The blade is obovate with cuneate base; the leaf may assume rounded, acuminate or retuse shapes at the apex (Orwal *et al*., 2009).

Herbarium specimen

The herbarium sample has been deposited at the Faculty of Pharmacy Herbarium, Department of Pharmacognosy, Obafemi Awolowo University, Nigeria with FPI number 2170

Ethnomedical uses

Decoction of *A. congensis* leaf is used to treat diarrhoea both in adult and children in DR Congo (Nsaka *et al*., 2012). Neuwinger (2000) posits that *A. congensis* stem bark decoction is used to treat malaria, gonorrhea, rheumatic pain and dysmenorrhea. It has galactagogue and antihelmintic effects. The latex is used to treat leucorrhoea, ulcers, scabies, yaws and headache, and its lightly roasted leaves may be smoked in a pipe, as a remedy for cough (Burkhill, 1985).

Morphological part used as drug

Stem-bark

Other part used

leaf

Definition of the drug

consists of fresh or dried stem-bark of *Alstonia congensis* Engl. family Apocynaceae

Description of the drug

Macroscopy

Bark is scaly, grey to greyish green in colour on the outer surface and off white to cream to yellowish colour on the inner surface

Microscopy

The epidermises of the leaf are polygonal in shape; the leaf is hypostomatic. The upper epidermis contains coastal cells and starch grains, stomata is only found on the lower epidermis, epidermis contains papillae, stomata are anomocytic, paracytic and or cyclotic. Mid-rib contains xylem which are interconnected, schlerenchymatic sheaths which are joined together and bicollateral vascular bundle. Stem-bark transverse section of the bark contains cork cell which is polygonal and arranged in radial rows, cortex consists of ground mass of parenchymatous cells with lignified brachysclereids, cortex also contain prismatic calcium oxalate crystals, phloem and starch grains

Powdered drug

Cartoon colour with slight characteristic odour, containing fragment of wood from the stem, cork, cortex, calcium oxalate crystals, stone cells, fibres, phloem

Chemical and bioactivity profile

The stem-bark contains indole alkaloid including echitamine, echitamidine as well as tanins, flavonoid, terpenoids like alpha and beta amyrin,. It possesses antispasmodic, antimalarial,analgesic and antidiarrrhoeal activity

Test for identity and purity

Moisture content (African Pharmacopoeia Methods, 1986): Not more than 7.2 %

Total Ash: Not more than 4.8 %

Acid-insoluble ash: Not more than 0.6 %

Alcohol soluble extractive: Not less than 4.0 %

Water soluble extractive: Not less than 4.3 %

Palisade ratio: 3.7

Vein-islet number: 6.2

Vein- termination number: 6.2

Chemical tests and assay

Alstonia congensis can be assayed by the qualitative and quantitative estimation of the total alkaloids and other constituents using appropriate chromatographic techniques such as TLC and HPLC

Indications and dosage

An adult patient would be expected to take between 10- 20 mL once or twice daily while children above 5 years would take between 5- 10 mL once or twice daily of *A. congensis* stem-bark decoction

Contraindications/ adverse effects/ precautions

Contraindicated in pregnancy, constipation, liver or kidney diseases

Storage conditions

In an air tight, dry container, protected from light and in cool dry place