CHAPTER – IV

MATERIALS AND METHODS

All the chemicals and reagents used were obtained in high purity either from S.D. Fine Chemicals Pvt. Ltd; Bombay, India or E. Merck Pvt. Ltd., Bombay, India. TLC was carried out using Si-gel (Merck). Column chromatography was carried out on Si gel (Merck, 70-230 mesh) and neutral alumina (S.D. Fine Chemicals Pvt. Ltd., Bombay). The melting points of all the compounds were recorded using Joshiba melting point apparatus and are uncorrected. IR spectra of the compounds were recorded using the KBr pellet method on a Perkin Elmer Spectrum one. ^{1}H-NMR spectra of the compounds were taken on Bruker Avance III 500 MHz (AV 500) NMR spectrometer using $CDCl_3$ as solvent and ^{13}C NMR spectra were recorded on Brukur instrument with $CDCl_3$ as the solvent at 125 MHz. Mass spectra of the compounds were recorded on a JEOL GCMATE II spectrophotometer.

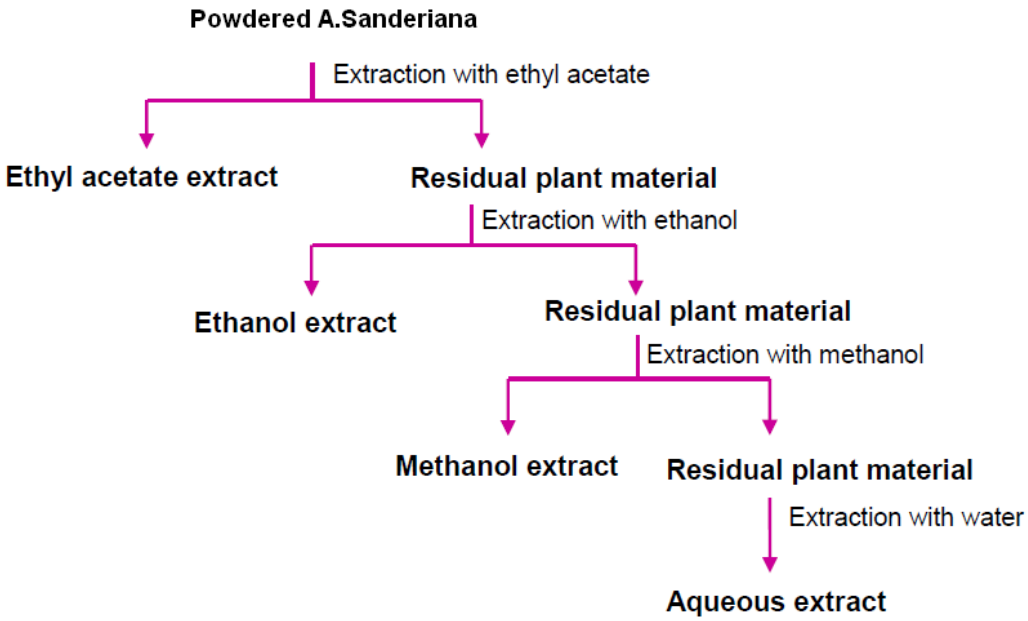
4.1. Selection of extraction solvent for *A. sanderiana* by different methods

The suitability of the extraction method for extraction of dried and fresh *A. Sanderiana* was analysed using three different methods viz., reflux and sonic assisted techniques viz., ultrasonic bath and ultrasonic homogeniser. The extraction of the dried and fresh plant (20 g and 300 g respectively) was carried out for a uniform period of 3 h and 1 h respectively. Scheme 1 represents the procedure adopted for the extraction of dried and fresh *A. Sanderiana*.

The fresh plant was extracted with ethanol by different methods to find the suitability of extraction with ethanol as solvent viz., reflux, ultrasonic bath, ultrasonic homogeniser, hot continuous extraction (soxhlet extraction), microwave

digestion, maceration, infusion, percolation and decoction[1]. The extraction was carried out based on the visual observation till the solvent became colourless indicating the completion of extraction.

Scheme 1. Extraction of dried *A.Sanderiana* by different methods



4.1.1. Preparation of crude extracts

Healthy and disease free *Alocasia sanderiana* plant leaves, stem and root tubers were collected from Valparai, Coimbatore district, Tamil Nadu, India[2] and also Krishnendra Nursery, Jayanagar , Bangalore[3] during January 2012. The plant identified by **Dr. S. Jeeva M.Sc., Ph.D**., Assistant Professor, Research Centre in Botany, Scott Christian College (Autonomous), Nagercoil - 629 003, Kanyakumari, Tamil Nadu, India. The plant leaves, stem and root tubers were washed with tap water and then rinsed with distilled water. Washed plant material was air dried, cut into small pieces and pulverized in a mechanical blender. Powdered plant material in the thimble were introduced into soxhlet apparatus and extracted with 5 × 1000 mL

of ethanol. Evaporation of the solvent yields the crude extracts. The crude extracts of ethanol were used for the phytochemical screening, measurement of antibacterial, antioxidant, anti-inflammatory, anti-diabetic activities and separation, identification and isolation of phytochemical components from each parts of plant.

4.2 Phytochemical screening

The phytochemical screening of the sample was carried out as described by Nweze et al[4]., and Senthilkumar and Reetha[5] . The samples were screened for carbohydrates, alkaloids, flavonoids, steroids, phenols and tannins, saponins, glycosides, proteins and terpenes. Results are tabulated in table 1.

4.2.1. Detection of phenolic compounds and tannins

1. Ferric Chloride Test

The extract (50 mg) was dissolved in 5 mL of distilled water. To this, few drops of neutral 5% ferric chloride solution was added. A dark green colour indicated presence of phenolic compounds.

2. Lead Acetate Test

The extract (50 mg) was dissolved in distilled water and to this 3 mL of 10% lead acetate solution was added. A white precipitate indicates presence of phenolic compounds.

4.2.2. Detection of flavonoids -Alkaline Reagent Test

An aqueous solution of the extract was heated with 10% NH_4OH solution. Yellow fluorescence indicated presence of flavonoids.

4.2.3. Detection of carbohydrates

The extract (200 mg) was dissolved in 10 mL water and filtered. The filtrate was subjected to the following tests:

1. Molisch's Test

To 2 mL of filtrate, two drops of alcoholic solution of α-napthol was added, the mixture was shaken well and 1 mL of con H_2SO_4 was added slowly along the sides of the test tube and allowed to stand. A violet ring indicated presence of carbohydrates.

2. Fehling's Test

1 mL of filtrate was boiled on a water bath. To this, 1 mL of Fehling solutions A and B were added. A red precipitate indicated presence of sugar.

4.2.4. Detection of glycosides

To 2 mL of plant extract, 1 mL of glacial acetic acid and 5% ferric chloride was added. To these 3 drops of concentrated sulphuric acid was added. Presence of greenish blue colour indicates presence of glycosides.

4.2.5. Detection of saponins

The extract (50 mg) was diluted with distilled water and made up to 20 mL. The suspension was shaken in a graduated cylinder for 15 min. A layer of foam indicated presence of saponins.

4.2.6. Detection of steroids

1. Liebermann-Burchard's test

The dry ethanolic extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulphuric acid was added along the sides of the test tube. Formation of green colour indicates presence of steroids.

2. Salkowski reaction

The dry ethanolic extract was shaken with chloroform. To the chloroform layer, concentrated sulphuric acid was added slowly along the sides of test tube. Formation of red colour indicates presence of steroids.

4.2.7. Detection of proteins and amino acids

The extract (100 mg) was dissolved in 10 ml of distilled water and filtered through Whatmann no: 1 filter paper and the filtrate were subjected to tests for proteins and amino acids.

1. Millon's Test

To 2 ml filtrate, few drops of Millon's reagent were added. A white precipitate indicated the presence of proteins.

Millon's reagent:

Mercury (1 g) was dissolved in 9 mL of fuming HNO_3 when the reaction was completed equal volume of distilled water was added.

2. Biuret Test

An aliquot of 2 mL of filtrate was heated with 1 drop of 2 % $CuSO_4$ solution. To this 1 mL of ethanol (95%) was added, followed by excess of KOH pellets. Pink colour in the ethanolic layers indicated presence of proteins.

3. Ninhydrin Test

2 drops of ninhydrin solution (10 mg of ninhydrin in 200 mL of acetone) was added to 2 mL of aqueous filtrate. A characteristics purple colour indicated the presence of amino acids.

4.2.8. Detection of alkaloids

To 2 mL of plant extract, 2 mL of concentrated hydrochloric acid was added. Then 3 drops of Mayer's reagent were added. Presence of green colour or white precipitate indicates presence of alkaloids.

4.2.9. Detection of terpenes- Ischugajiu test

Small portion of each of the decolorized extract residue (0.5 g) was taken in different tubes. The solution of the ethanolic extract was treated with an excess of acetyl chloride and a pinch of zinc chloride. The contents were kept aside for reaction to subside and warmed on water bath. Formation of eosin red colour indicates presence of triterpenoids.

4.3. Antimicrobial activity: Agar-well diffusion method

Agar well diffusion method is used for the rapid determination of the drug or a particular substance on a specific bacterium. This method consists of impregnating small circular disc as positive control and DMSO as negative control with given amount of a chosen concentration of substance. The discs are placed on plates of culture medium that has been seeded with a test bacterial inoculum. After incubation, the diameter of the clear zone of inhibition surrounding the deposit of substance is taken as a measure of the inhibitory power of the particular substance against the particular test organism.

4.3.1. Microorganisms for antibacterial activity

The test microorganisms used for antibacterial analysis[6] were clinical isolates of *Staphylococcus aureus* MTCC3381, *Bacillus cereus* MTCC430, *Pseudomonas aeruginosa* MTCC424, *Klebsiella pneumonia* MTCC432, *Escherichia coli* MTCC739. The bacterial strains were maintained on Nutrient Agar (NA) at the laboratory division of Manian Laboratories Pvt. Ltd, Coimbatore.

Preparation of inocula

The test organisms were subcultured by streaking them on nutrient agar, followed by incubation for 24 h at 37°C. Several colonies of each bacterial species were transferred to sterile nutrient broth. The suspensions were mixed for 15 sec and incubated for 24 h at 37°C on an orbital incubator shaker. Working concentration of the microbial suspension was prepared in 3 mL of sterile saline to turbidity equivalent to 0.5 McFarland scale (i.e., adjusting the optical density to 0.1 at 600 nm), yielding a cell density of 1-2 × 10^5 CFU/mL.

Procedure for antibacterial activity

Nutrient Agar (NA) plates were seeded with 8 h broth culture of different bacteria. In each of these plates, wells were cut out using sterile cork borer. Using sterilized dropping pipettes, different concentrations (1000, 2000 and 3000 µg/well) of plant extract was carefully added into the wells and allowed to diffuse at room temperature for 2 h. The plates were then incubated at 37°C for 18-24 h. Gentamicin (10µg) was used as positive control, and DMSO as negative control. The antimicrobial activity was evaluated by measuring the diameter of inhibition zone.

4.3.2. Microorganisms for antifungal activity

The test microorganisms used for antifungal analysis[7] were clinical isolates of *Candida albicans* MTCC227, *Fusarium solani* MTCC 2935, *Aspergillus fumigates* MTCC 343, *Rhizopus oryzae* MTCC262, Aspergillus *terreus* MTCC1281. The fungi strains were maintained on Sabouraud Dextrose Agar (SDA) at the laboratory division of Manian Laboratories Pvt. Ltd, Coimbatore.

Preparation of inocula

The fungal pathogens were cultured in Sabouraud dextrose agar for 72 h at 27°C and the spores/cells were harvested in sterile saline using a sterile squirrel brush. Working concentration of spore suspension was prepared with sterile saline to turbidity equalent to 0.5 McFarland scale (i.e., adjusting the optical density to 0.1 at 530 nm), yielding a cell density of 1-5 × 10^6 CFU/mL.

Procedure for antifungal activity

Sabouraud dextrose agar plates were seeded with one hundred microliter of spore/cell suspension of different test organisms. In each of these plates, wells were cut out using sterile cork borer. Using sterilized dropping pipettes, different concentrations (1000, 2000 and 3000 µg/well) of plant extract were carefully added into the wells and allowed to diffuse at room temperature for 2 h. The plates were then incubated at 27°C for 48 h. Ketoconazole (10µg) was used as positive control and DMSO as negative control. The antimicrobial activity was evaluated by measuring the diameter of inhibition zone.

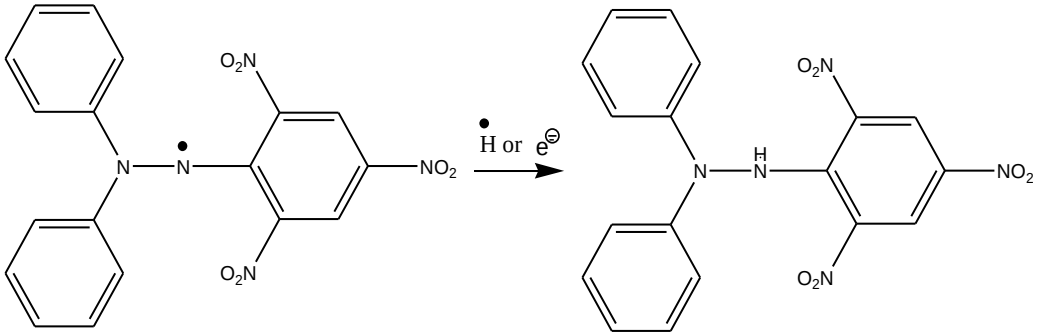
4.4. Antioxidant activity

The antioxidant activity of leaf, stem and root ethanol extracts of *A. Sanderiana* was determined by *in vitro* methods. DPPH free radical scavenging assay and ABTS radical scavenging assay methods were employed to assess the antioxidant potential. All the assays were carried out in triplicate.

4.4.1. Determination of DPPH radical scavenging capacity

Principle

In a solution, DPPH is a stable free radical and appears violet in colour. When DPPH is reduced by the proton donor it turns to colourless or yellow colour. The maximum absorption band is centred at about 517 nm.



2,2'-Diphenylpicryldydrazide radical

(Violet colour)

2,2'-Diphenylpicryldydrazide

(Yellow colour)

Procedure

The antioxidant activity of the sample was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to the method of Blois[8]. The sample extracts at various concentrations (400 - 2000µg) were taken and the volume was adjusted to 100 µl with methanol. 5 mL of 0.1 mM methanolic solution of DPPH• was added and allowed to stand for 20 min at 27°C.

The absorbance of the sample was measured at 517 nm. Percentage radical scavenging activity of the sample was calculated as follows:

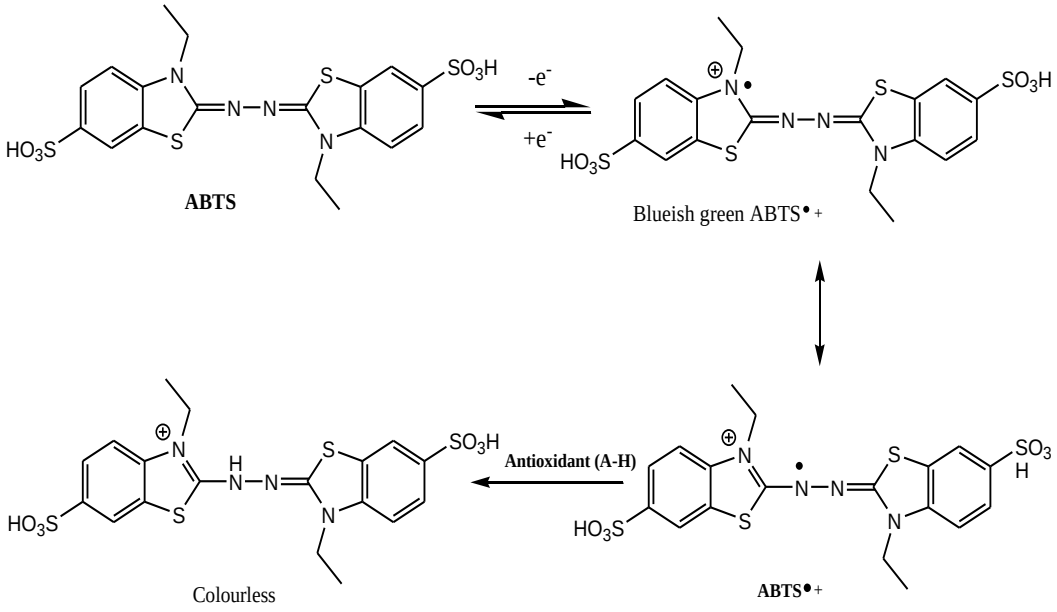
% DPPH radical scavenging activity = (Control OD - Sample OD / Control OD) × 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

4.4.2. Free radical scavenging activity on ABTS●+

Principle

The ABTS reacts with ammonium persulphate under dark condition to form ABTS free radical. The persulphate ions are involved in a nucleophilic attack on ABTS to generate greenish blue ABTS radical (ABTS), which is spectrophotomerically measured at 734 nm. The presence of antioxidant compounds, which donate hydrogen to neutralize the ABTS radicals, results decrease in colour intensity.



Chemicals required

Ammonium persulphate

Potassium persulfate

Ethanol

Procedure

The antioxidant activity of the samples was measured by ABTS radical cation decolorization assay according to the method of Re et al. [9]. ABTS●+ was produced by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium persulfate in the dark for 12–16 h at room temperature. Prior to assay, this solution was diluted in ethanol (about 1:89 v/v) and equilibrated at 30^{o}C to give an absorbance at 734 nm of 0.700 ± 0.02. The stock solution of the sample extracts were diluted such that after introduction of 10 µl aliquots into the assay, they are produced between 20% and 80% inhibition of the blank absorbance. After the addition of 1 ml of diluted ABTS solution to 10 µl of sample (100-500 µg/ml), absorbance was measured at 734 nm at exactly 30 min after the initial mixing. Samples were analyzed in triplicate. Percentage radical scavenging activity of the sample was calculated as follows:

% ABTS radical scavenging activity = (Control OD - Sample OD / Control OD) × 100

The analysis was performed in triplicate. The sample concentration providing 50% inhibition (IC_{50}) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

4.4.3 Reducing power assay

Principle

The reducing power of ethanolic extract of *alocasia* was determined by the slight modification of the method of Oyaizu[9]. Substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric/ferrous complex that has an absorption maximum at 700 nm. The intensity of colour is directly proportional to the reducing power of the test samples.

$$Fe^{3+} + \text{Antioxidant} \rightleftharpoons Fe^{2+} + \text{Oxidized antioxidant}$$

$$Fe^{2+} + Fe(CN)_6^{3-} \rightleftharpoons Fe[Fe(CN)_6]^-$$

Prussion blue colour

Chemicals required

Potassium ferricyanide (1% w/v),

Phosphate buffer (0.2 M, pH 6.6),

Trichloro acetic acid (10%),

Ferric chloride (0.1%)

Ascorbic acid (1%).

Phosphate buffer preparation

Dibasic sodium phosphate (37.50 mL of 0.2 M) is mixed with 62.5 mL monobasic sodium phosphate and diluted to 100 mL with water.

Protocol for reducing power

Various concentrations of the plant extracts in corresponding solvents were mixed with phosphate buffer (2.5 mL) and potassium ferricyanide (2.5 mL). This

mixture was kept at 50ºC in water bath for 20 minutes. After cooling, 2.5 mL of 10% trichloro acetic acid was added and centrifuged at 3000 rpm for 10 min whenever necessary. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and a freshly prepared ferric chloride solution (0.5 mL). The absorbance was measured at 700 nm. Control was prepared in similar manner excluding samples. Ascorbic acid at various concentrations was used as standard. Increased absorbance of the reaction mixture indicates increase in reducing power. Reducing power was measured by varying the concentration of the extract and the contact time.

4.5. Anti-inflammatory activity

The anti-inflammatory activity ethanolic extracts of leaf, stem and root tubers of *A. Sanderiana* was determined by *in vitro* methods. Proteinase inhibiting activity and protein denaturation inhibiting activity methods were employed to assess the anti-inflammatory potential. All the assays were carried out in triplicate.

4.5.1 *In vitro* Proteinase inhibiting activity

The test was performed according to the modified method of Oyedepo et. al[10] and Sakat et al[11]. The reaction mixture (2 mL) contained 0.06 mg trypsin, 1 mL 20 mM Tris HCl buffer (pH 7.4) and 1mL test sample of different concentrations (100 - 500µg/mL). The mixture was incubated at 37^{o}C for 5 min and then 1 mL of 0.8% (w/v) casein was added. The mixture was incubated for an additional 20 min. Then, 2 ml of 70% perchloric acid was added to arrest the reaction. Cloudy suspension was centrifuged and the absorbance of the supernatant was read at 210

nm against buffer as blank. The experiment was performed in triplicate. The percentage inhibition of proteinase inhibitory activity was calculated using the following formula.

$$\% \text{ inhibition} = \frac{(\text{Abs control} - \text{Abs sample}) \times 100}{\text{Abs control}}$$

The IC_{50} value is defined as the concentration of the sample extract to inhibit 50% of protein inhibition under assay condition.

4.5.2 *In vitro* protein denaturation inhibiting activity

The reaction[12] mixture (0.5 mL) consisted of 0.45 mL bovine serum albumin (5% aqueous solution) and 0.05 mL of the sample extracts (100-500 μg/mL). pH was adjusted to 6.3 using a small amount of 1N HCl. The samples were incubated at 37°C for 20 min and then heated at 57°C for 3min. After cooling the samples, 2.5 mL phosphate buffer saline (pH 6.3) was added to each tube. Turbidity was measured spectrophotometrically at 660 nm. For control tests 0.05 mL of distilled water was used instead of extracts while product control tests lacked bovine serum albumin. The percentage inhibition of protein denaturation was calculated as follows.

$$\% \text{ inhibition} = \frac{100 - (\text{O.D of Test} - \text{O.D of Product Control}) \times 100}{\text{O.D of Control}}$$

The IC_{50} value is defined as the concentration of the sample extract to inhibit 50% of protein denaturation under assay condition.

4.6 Anti-diabetic activity

The anti-diabetic activity of leaf, stem and root ethanol extracts of *A. Sanderiana* was determined by *in vitro* methods. α-amylase inhibiting activity and α-glucosidase inhibiting activity methods were employed to assess the anti-diabetic potential. All the assays were carried out in triplicate.

4.6.1 *In vitro* α-amylase inhibiting activity

The α-amylase (0.5 mg/mL) was premixed with extract[13] at various concentrations (100-500 μg/mL) and starch as a substrate was added as a 0.5% starch solution to start the reaction. The reaction was carried out at 37°C for 5 min and terminated by addition of 2 mL of DNS (3,5-dinitrosalicylic acid) reagent. The reaction mixture was heated for 15 min at 100°C and diluted with 10 mL of distilled water in an ice bath. α-amylase activity was determined by measuring spectrum at 540 nm. The % α-amylase inhibitory activity is calculated by the following formula

$$\text{Percentage of inhibition} = \frac{(\text{Abs control} - \text{Abs sample}) \times 100}{\text{Abs control}}$$

The IC_{50} value is defined as the concentration of the sample extract to inhibit 50% of α-amylase activity under assay condition.

4.6.2 *In vitro* α-glucosidase inhibition assay

The α-glucosidase inhibitory effect of plant extracts was determined according to the standard method[14]. For α-glucosidase inhibition, yeast α-glucosidase was dissolved in 100 mM phosphate buffer, pH 7.0, containing bovine serum albumin 2 g/l and sodium azide 0.2 g/l which was used as enzyme source. P-

Nitrophenyl-α-d-glucopyranoside was used as substrate. Extract was weighed and serial dilutions of 100 µg/mL, 200 µg/mL, 300 µg/mL, 400 µg/mL, 500 µg/mL were made up with equal volumes of dimethylsulfoxide and distilled water. 10 micro liters of extract dilutions was incubated for 5 min with 50 µL enzyme source. After incubation, 50 µL of substrate was added and further incubated for 5 min at room temperature. The pre substrate and post substrate absorbance was measured at 405 nm on a microplate reader. The increase in absorbance on substrate addition was obtained. Each test was performed three times and the mean absorption was used to calculate percentage α-glucosidase inhibition. Acarbose was used as positive control with various concentrations. The concentration of acarbose and plant extract required to inhibit 50% of α-amylase activity under the conditions is defined as the IC_{50} value. Percentage α-glucosidase inhibition was calculated according to the following formula;

$$\text{Percentage of inhibition} = \frac{(\text{Abs control} - \text{Abs sample}) \times 100}{\text{Abs control}}$$

4.7. Physicochemical analysis

Physicochemical studies[15,16] like total ash, acid insoluble ash, water insoluble ash, water soluble extractive value and loss on drying at 105°C were carried out as per the WHO guidelines for individual leaf, stem and root tubers ethanolic extracts of *alocasia sanderiana*.

4.7.1. Determination of total ash value

An empty silica crucible along with lid, previously ignited for 1 h was weighed. 1 gm of sample was transferred into the crucible and the crucible was covered with lid and weighed again. The crucible was ignited for 3 h around 450°C

temperature. It was ignited successively for 1 h periods where ignition to constant weight is indicated. Upon the completion of each ignition, the crucible was covered and allowed to cool in a desicator to room temperature before weighing.

$$\text{Ash value} = (\text{Residue Weight} / \text{Sample Weight}) \times 100$$

4.7.2. Determination of acid insoluble ash content

Ash obtained from the total ash was boiled with 25 mL of 2 N HCl for a few minutes and filtered through an ashless filter paper. The filter paper was transferred into a tarred silica crucible and incinerated at 450°C in a muffle furnace until free from carbon. The crucible was cooled and weighed. Percentage of acid insoluble ash was calculated.

4.7.3. Determination of water insoluble ash

Ash obtained from the total ash was boiled with 25 mL of distilled water for a few minutes and filtered through an ashless filter paper. The filter paper was transferred into a tarred silica crucible. Incinerated at 450°C in a muffle furnace until free from carbon. The crucible was cooled and weighed. Percentage of water-soluble ash was calculated.

4.7.4. Determination of water soluble extractive value

About 1gm of air-dried extract was taken with 100 mL of chloroform water in a closed flask for 24 h shaking frequently during the first 6 h and then allowed to stand for 18h. Thereafter, it was filtered rapidly taking precautions against loss of the water. The 25 mL filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C weighed. The percentage of the water soluble extractive value was calculated.

4.7.5. Determination of moisture content

1gm of each sample was placed in pre-weighed flat porcelain dish, dried in the oven at 100^{o}C ±5^{o}C till the constant weight was obtained. The loss of weight was calculated.

Moisture Content = (Loss of sample weight / Sample Weight) × 100

Physicochemical studies like total ash, acid insoluble ash, water insoluble ash, water soluble extractive value and loss on drying at 105°C were carried out as per the WHO guidelines for individual leaf, stem and root tubers extract of alocasia sanderiana values are recorded in the Table 11.

4.8. Isolation and identification of compounds

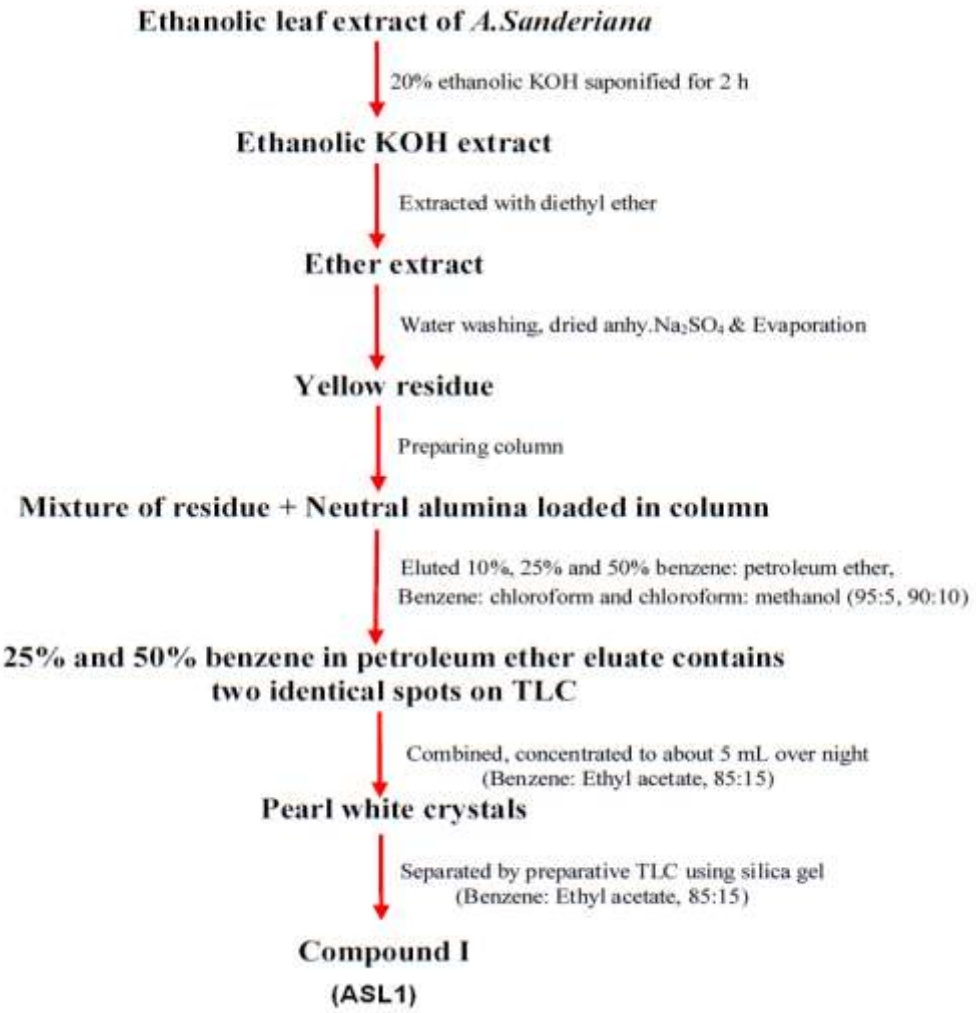
Ethanolic leaf extract (10 g in 300 mL) of *A. sanderiana* was saponified with 20 % ethanolic KOH for 2 h. During saponification, the loss in volume is being compensated by adding distilled water from time to time. The unsaponifiable portion was then extracted with diethyl ether (3 × 300 mL). The pooled extract was then washed with distilled water and dried over anhydrous sodium sulphate. All the ether layers were combined and evaporated leading to the formation of a yellow residue.

The yellow residue was dissolved in 10 ml of chloroform and adsorbed by neutral alumina (20 g). After evaporation of the solvent, it was loaded onto a neutral alumina column (150 g) built in petroleum ether (60-80°C). The column was eluted with petroleum ether (60-80°C), 10%, 25% and 50% benzene: petroleum ether (60-80°C), then with benzene, followed by graded mixtures of benzene: chloroform (95:5) and chloroform: methanol (90:10). The elutions were monitored on TLC (Silica

Gel G, visualization; vanillin-sulphuric acid, heated at 110°C). The 25% and 50% benzene in petroleum ether elute gave a spot on TLC (benzene: ethyl acetate, 85:15).

These spots are concentrated to about 5 mL (benzene: ethyl acetate, 85:15) and left overnight, resulting in the formation of pearl white crystals which were tested to Liebermann Burchard's test for steroids. These were separated by preparative TLC using silver nitrate impregnated silica gel (benzene: ethyl acetate, 85:15) into compounds, recrystallised from benzene and designated as compound I (ASL1).

Scheme 2. Isolation of Compounds from the ethanolic Leaf extract of *A. Sanderiana*



The concentrates of the other elnates gave only brown coloured resin, which was not processed further. These compounds were further purified by crystallization. The compound was then characterized by melting point, solubility and different spectral studies like IR, NMR (^{1}H and ^{13}C) and Mass spectrometry.

4.9. Characterization of new Compounds

Preliminary colour tests were carried out for the isolated new compound ASL1. Melting points were determined in the digital melting point apparatus. The isolated new compound was characterized by various spectral techniques namely IR, 1D NMR, 2D NMR and Mass spectral techniques. The instruments/equipments used for different analysis are given below.

S.No	Instrument	Company
1	Melting point apparatus	Joshiba
2	FT-IR (4000-400) spectrophotometer	The Perkin Elmer Spectrum one
3	CHNS Analyzer	Elementar Vario EL III
4	NMR 500MHz spectrophotometer	Bruker Avance III 500 MHz (AV 500)
5	MS-(EI)	JEOL GCMATE II
6	HPTLC	Camag
7	Rotary vacuum evaporator	Equitron
8	Sonicator	PCi

4.9.1. Melting point apparatus

Melting point of the compound was determined using the digital melting point apparatus (Make: Joshiba). Benzoic acid was used as the standard and the values are uncorrected.

4.9.2. FT-IR spectrophotometer

The IR Spectrum of the isolated compound was recorded Perkin Elmer Spectrum1 FT-IR instrument using KBr pellet. Entire region of 450-4000 cm^{-1} is covered by this instrument. The spectrometer works under purged conditions. Solid samples are dispersed in KBr pellets. This instrument has a typical resolution of 1.0 cm^{-1}. Signal averaging, signal enhancement, base line correction and other spectral manipulations are possible. Infrared spectrum is useful in identifying the functional groups like -OH, -CN, -CO, -CH, $-NH_2$, etc.

4.9.3. CHNS Analyzer

The percentage of elements C, H, N and S of isolated new compound can be individually analyzed over a wide range of sample matrices and concentrations with this instrument Make/**Model**: Elementar Vario EL III.

4.9.4. NMR (500 MHz) Spectrophotometer

Isolated compound was characterized using ^{1}H NMR, ^{13}C NMR and HSQC techniques. NMR spectrum was recorded in $CDCl_3$ solvent using TMS as a standard in Bruker Avance III 500 MHz (AV 500) spectrometer.

4.9.5. Mass spectrum (EI)

The molecular mass and structure of the isolated compound, has been confirmed by recording mass spectrum in JEOL GCMATE II GC-MS, double focusing instrument.

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