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A STUDY OF ISOLATION AND CHARACTERIZATION OF BIOACTIVE COMPOUND FROM CROSSANDRA INFUNDIBULIFORMIS FLOWER EXTRACT

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ABSTRACT

Plants are a vast source for novel drug compounds. Medicines derived from plants have made large contributions to human health and wellbeing. Plant compounds with medicinal value have been used over many years as drugs due to their safety towards human health. Secondary metabolites expelled out from the plants possess good defense mechanism against microorganism. Diverse beneficial activities have been derived from these metabolites such as antimicrobial, antioxidant, anti-inflammatory activity, anti-diabetic activity, anticancer and so on. Both flowers and leaves also have aphrodisiac activity and wound healing ability. Based on the preliminary phytochemical screening, we recorded the presence of major phytoconstituents such as flavonoids, alkaloids, glycoside, tannins, saponins, steroids and terpenoids. The phytochemical profiling applying High Performance Thin Layer Chromatography (HPTLC) confirmed the presence of five different flavonoids and glycoside. Fourier Transform Infra-Red (FTIR) spectral analysis of the flower sample indicated the presence of functional groups such as aldehydes, carboxylic acids, esters, ethers, alkyl halide, alkane and amines. Elemental composition such as chlorine, silicon, aluminium present in the floral sample was determined from Energy Dispersive X-ray spectrometer (EDX). Among the various solvents used, methanol was found to contain most of phytochemicals in C. infundibuliformis flowers. Therefore methanolic extract was used for isolation and purification of bioactive compound. For separation and isolation of the bioactive compound, column chromatography technique and for checking the purity of the separated fractions thin layer chromatography were adopted. Pure crystal was obtained from column fractionation. The structure of isolated compound was determined using X-ray crystallography method and characterized as 1, 3-benzoxazol-2(3H)-one.

KEYWORDS: Bioactive Compound, CrossandraInfundibuliformis, Flower Extract, human health, Plant compounds, anti-inflammatory activity, anti-diabetic activity

INTRODUCTION

The flower of Crossandrainfundibuliformis (Acanthaceae), possesses medicinal properties such as wound healing, antioxidant, antimicrobial, anti-inflammatory (Sandeep et al., 2015) and anticancer activity (Mallikarjuna et al., 2012). Though it is evident that the plant possesses certain important medicinal properties, the bioactive compound responsible for these is not explored so far. Hence in this chapter, isolation of the bioactive compound using chromatographic technique and characterization of the isolated compound by X-ray crystallography are discussed.

Chromatography: Working principle and types

Chromatography is an efficient method for the separation of components from crude or mixture of extract works on the principle of gravity. Extract which is to be separated into single compound (or fraction) is dissolved in mobile phase solvent and passed through the solid matrix or the stationary phase. From the column, low polar compound will be eluted first whereas high polar compound will be retained on the stationary phase. As the polarity of (mobile phase) solvent is gradually changed, the high polar compounds desorbs from stationary phase. Chromatography may be categorized into two. Based on the shape of the stationary phase, it is classified as horizontal (eg. Thin layer chromatography) and vertical (eg. column chromatography). Based on the physical nature of the mobile phase, it is classified as gas, liquid and supercritical fluid chromatography.

Column chromatography

Reichstein first introduced and demonstrated this technique. Column chromatography (CC) is a widely used technique to separate and collect individual compounds from crude extract by means of suitable solvents. Silica and alumina are the two popular packing materials (referred as the stationary phase) used in CC. All the compounds present in the sample are segregated as bands based on their diverse distribution coefficient in regard to the stationary phase. Column packing is done either by dry or slurry method. Care should be taken while packing such that no air bubbles or cracks are formed as this will lead to uneven packing of stationary phase. Once the column is ready sample can be loaded. Owing to the diverse solubility of compound mixture present in the extract, a broad spectrum of solvents will be used for mobile phase, usually non polar solvents like hexane, petroleum ether, moderately polar solvents such as chloroform, ethyl acetate and high polar solvents like methanol, ethanol etc. From the column, low polar compound will be eluted first whereas high polar compound will be retained on the stationary phase. As the polarity of (mobile phase) the solvent gradually changes, the high polar compounds desorbs from stationary phase. These elute or fractions are collected in set of test

tubes and each fraction could be tested for purity and individual compounds using Thin Layer Chromatography. Fractions with similar spot can be pooled together and further purified to get pure compound.

Thin layer chromatography

Thin layer chromatography (TLC) is an advantageous and fast technique employed for the identification of number of compounds present in the extract. Adsorbents such as silica gel, alumina, or cellulose are used as stationary phase. TLC utilizes different solvents with varying ratio so as to obtain better separation of spots corresponding to the compounds. Developing solvent (or solvent front) moves from bottom to top and the high polar molecules that has lower Rf (Ratio of flow) values will be retained at the bottom whereas the low polar compounds (has high Rf) runs fast and reach the top.

1 Calculation of R values of TLC spots

The solvent front after passing more than half of the TLC plate, the plates were taken out, marking is made and allowed to dry. The plates are then placed in iodine chamber or UV box (as required) and Rf values were calculated as follows:

Rf = Distance traveled by the elute from the starting point / Distance traveled by the solvent front from the starting point (Masuduzzaman et al., 2008).

Methods of structure determination

Quite a few methods such as NMR, X-ray crystallography, neutron diffraction and electron microscopy are available to determine the three-dimensional structure of small molecules and macromolecules like proteins and nucleic acids. Among these methods, X-ray Crystallography provides detailed atomic level information and 3d images of molecules under study.

Other methods have some limitations, as in case of NMR (precision), Electron microscopy (resolution), Neutron crystallography (limited applicability). X-ray diffraction (XRD) studies is applicable for wide range of structure sizes from very small molecules, phytocompounds, simple salts, complex minerals, synthetically prepared organic and inorganic compounds and biological macromolecules (like nucleic acids and proteins and so on).

Of all the methods for structural determination, X-ray crystallography is the capable method that provides explanatory data on atomic distances, bond length, bond angles, torsion angles, molecular architecture, complete configuration, crystal packing, possible order/disorder stoichiometry and thermal vibration parameters, from the single experiment.

X-ray Crystallography

X-ray crystallography is the chief standard method to solve the crystal structure through X-ray diffraction of single crystal. This method provides accurate determination of crystal structure. Working principle of Xray crystallography is on the basis of interaction of X-rays with the matter in the crystalline state. Magnitude of the crystal‟s interatomic distances is in the same order of X-rays wave-lengths (nanometer), hence their interaction results in diffraction patterns.

a. Background theory pertaining to X-ray diffraction and Bragg's law

The X-ray diffraction theory was first interpreted by W. L. Bragg in 1913. When X-rays incident on a crystal, it get scattered as reflection generally termed as diffraction. Wavelengths (λ) of X-rays are on the scale similar as that of crystal atom, hence produced reflections or diffraction patterns. Data collected from XRD is due to relative intensity (I) for every reflection through set of planes in crystal, called as Miller indices (h, k, l) associated with respective scattering angle (2θ) for each reflection. Details of atoms arranged in crystal space lattice are represented by diffracted beams intensities and positions shown in Figure 4.1. X-ray beam is diffracted only when it imposes on crystal planes, and crystal geometry must satisfy particular conditions expressed by Bragg‟s law in equation 1.

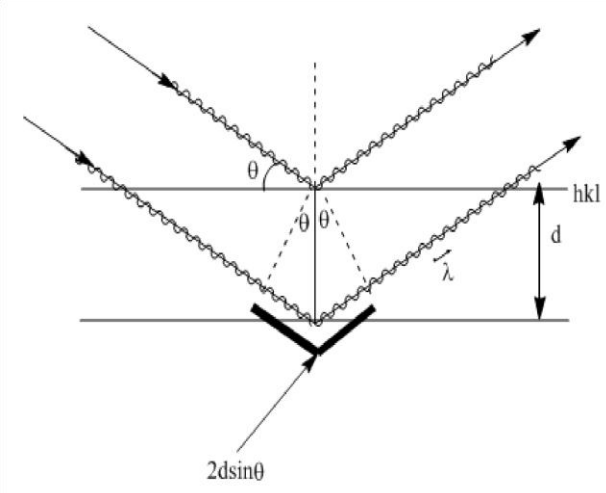

Figure 1 Schematic drawing of Bragg‟s law (Rhodes., 2006)

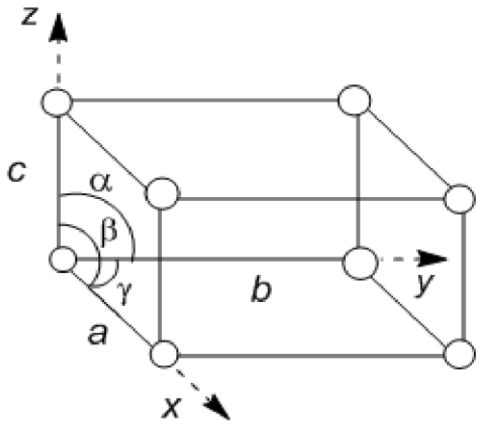

Figure 2 Schematic representation of unit cell where a, b and c are axial lengths, α, β, and γ are the interaxial angles between them, and the coordinates x, y, and z are measured along a, b, and c respectively.

$n\lambda = 2d_{hkl}\sin\theta$ ----------(1)

where, n = an integer, for peak order

λ= Wave length of the radiation (X-ray)

d_{hkl} = interplanar spacing of the hkl planes in the crystal lattice

θ = Bragg angle or diffraction angle

Crystal is an object made up of atoms that are arranged in an orderly pattern with repeating 3d units called „unit cells". Unit cell is described through six parameters as three axial angles (α, β, γ) and lengths (a, b and c) shown in Figure 2 (Rhodes., 2006).

a. X-ray Crystallography workflow

Structure determination of a crystal in X-ray crystallography is achieved by the following stages as shown in Figure 4.3.

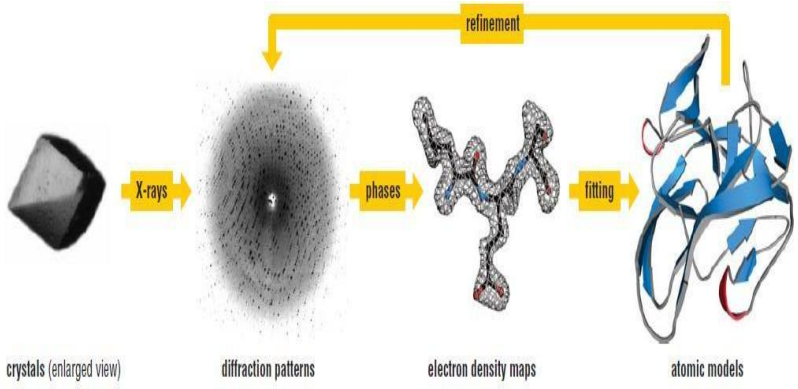

Figure 3 Schematic representation of X-ray Crystallography workflow

c. Stage 1 – Crystal mounting and X-ray Intensities Measurement c1. X- Ray Diffractometer and detector

In X-ray diffraction, the data collection is done using any one of these diffractometers coupled with detectors, namely, SMART CCD area detectors, Bruker APEX CCD and Enraf-Nonius CAD-4. A diffractometer is a device meant for collecting diffracted X-ray intensities via the X-ray photons that fall at the suitably placed detector. It provides crystal position and orientation when the crystal is placed in X-ray beam. Additional accessories like instance camera and high speed computer are attached to it for data analysis.

c2. Crystal growth and selection

The prerequisite for X-ray crystallography is to a obtain pure crystal. This could be achieved by maintaining certain solution conditions for the crystal to grow. Normally crystals grow when the condition of supersaturated solution changes slowly. There are three types of condition for a solution to be saturated. They are unsaturated (no crystals growth occur), Low supersaturated (crystals growth occur but new crystals are not formed) and High supersaturated (condition wherein crystals grow and new ones are formed). To crystallize molecules, any one of the methods such as slow cooling, slow evaporation and vapour diffusion is utilized. In slow cooking method, the temperature of laboratory vessels containing solutions is elevated which results in rapid microscopic crystal formation. In case of slow evaporation, solvent gets evaporated leading to crystals deposition on the walls of container. Good quality crystals could be obtained by this method. Vapour diffusion method involve varying the solvent and results in good quality single crystals with minor quantity of solute (Richard, 2012).

Selection of crystal for X-ray diffraction studies is an important step. It should satisfy the requirements that crystal have uniform interior structure arrangement and in proper shape and size (in other words crystal should not be twisted or complexes with many crystals).

c3. Crystal Mounting

A single crystal under study is affixed to the glass fiber of the goniometer head as a first step. There after goniometer head is positioned exactly to the diffract meter and rotated gradually so as to bombard X-rays onto the crystal.

This step results in the production of diffraction patterns of uniformly spaced spots also known as the reflections. The 2D images which are recorded are converted into 3D model using Fourier transforms, a mathematical method that combines density of electrons with known chemical data of the sample. If crystals are very small, it results in poor resolution.

c4. Data Reduction

Raw data obtained from diffractrometer will have geometrical and physical constrains. In order to elucidate the structure, intensity data must be corrected for Lorentz polarization and absorption effects. It is specified in equation 2.

I hkl = KI hkl / Lp ---------- (2) where p is the Polarization factor, given by equation 3, p =1+cos22θ /2 ---------- (3)

L is the Lorentz factor, depends on the measurement technique used and is given in equation 4.4,

L = 1/sin2θ ---------- (4)

The absorption of X-rays by the crystal is governed by the relation 4.5, I = Ioexp(-μt) ---------- (5)

where μ is the linear absorption coefficient and t is the average distance travelled by the X-ray beam inside the crystal. Absorption correction becomes vital, when the crystal has more absorbing elements for the incident X-ray wavelength.

c5. Determination of Space Group

Symmetry of a unit cell described by Space groups, that comprise of 230 variations. These are determined from systematic absences of hkl reflections. If uncertainty arises in space group, density is taken into account. Intensity statistics are also considered to finalize the space group.

c6. Structure Factor and Electron density map

Structure factor is defined as the combination of phase and amplitude obtained from maximum diffractions, resulted when X-ray reflections scattered from one-unit cell of crystal in any direction. Electron density map is a contour plot that indicates the region of electrons present in the crystal molecule.

CONCLUSION

A variety of chemical compounds are sourced and obtained from microbes, plants and animals abundantly in nature. Man has been in search of such natural products from the ancient times. Since he has been the beneficiary of these materials, his quest for such valuable substances still continues. The structure of isolated compound was characterized as 1, 3-benzoxazol2(3H)-one (also referred as 2-benzoxazolinone or BOA). Next, computational study was carried out with the isolated compound, BOA. Amide and piperazine derivatives of BOA were modeled in silico. The drug-likeness of the derivatives was computed and all the derivatives were found to follow the Lipinski rule of five. These derivatives were used as ligands and docked against melanoma cancer target, BRAF protein. Among the modeled ligands, two of them, namely, N-(4methoxyphenyl)-2-(2-oxo-1,3-benzoxazol-3(2H)-yl) acetamide (compound ID: A28) and 1-S-p-Nitrophenyl 1,1- diS- oxo 4- [2-(2-oxo-1,3-benzoxazolidin-3yl) acetyl]-1-piperazinecarbothioate (compound ID: P23), were found to have favorable docking score and binding energy. The notable binding efficiency of P23 may be due to the basicity of piperazine nitrogen and the presence of methyl and amine nitrogen attachment to the N-phenyl ring in A28.

On the basis of the results obtained from the computational study, chemical synthesis of two BOA derivatives, A28 and P23 (which were found to be efficient ligands against melanoma cancer target) was successfully carried out and purity was checked using TLC. Characterization of the synthesized compounds was done by considering the physic-chemical properties, FTIR and NMR (H and C) spectroscopies. Three compounds, namely the isolated compound (1,3-benzoxazol2(3H)-one) and the synthesized compounds A28 and P23 were evaluated for the anticancer activity. MTT assay was carried out for the evaluation of anticancer efficacy of the three compounds. All the compounds were cytotoxic towards melanoma cancer cell line. P23 expressed significant activity while the other two compounds A28 and BOA had a moderate effect.

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