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ISOLATION OF STEROLS FROM *OCIMUM SANCTUM* L. LEAVES AND IT'S INHIBITORY ACTION ON GLUCOAMYLASE IN VITRO

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ABSTRACT

Diabetes Mellitus is the major chronic disease caused by an improper balance of glucose homeostasis. The hydrolysis and intestinal absorption are carried out by a group of hydrolytic enzyme, which includes salivary α-amylase and intestinal α-glucosidases, respectively. Plant based remedies have always been an integral part of traditional medicine throughout the world. *Ocimum Sanctum L.* (Tulsi) extracts are used as Ayurvedic remedies for common cold, headache, stomach disorders, inflammation, heart diseases, various forms of poisoning and malaria The active fraction containing mixture of stigmasterol and β-sitosterol was isolated by silica gel column chromatography and structure was determined using spectroscopic analysis. The inhibitory effect of glucoamylase parameter was determined *in vitro.* The isolated mixture of compounds shows moderate inhibitory effect at 1µg/mL concentration with 33.33% inhibition. The enzyme inhibition could be part of the mechanism by which they are used in the treatment of the type-II diabetes. However, stigmasterol and β- sitosterol may play an important role in glucoamylase inhibition action. Research studies leading to extraction, isolation, identification and biological evaluation of plant constituents have now formed the major field of study.

KEYWORDS: *Ocimum Sanctum* L., stigmasterol, β- sitosterol, glucoamylase.

INTRODUCTION

Diabetes Mellitus is the major chronic disease caused by improper balance of glucose homeostasis. Dietary carbohydrate, such as starch, is the major source of glucose in the blood. The hydrolysis and intestinal absorption are carried out by a group of hydrolytic enzyme, which includes salivary α-amylase and intestinal α-glucosidases, respectively. Inhibitor of intestinal α-glucosidase enzyme retards the rate of carbohydrate digestion, thereby providing an alternative means to reduce postprandial hyperglycemia.[1]

Plant based remedies have always been an integral part of traditional medicine throughout the world. *Ocimum Sanctum L.* (Tulsi) extracts are used in Ayurvedic remedies for common cold, headache, stomach disorders, inflammation, heart diseases, various forms of poisoning and malaria.[2] The phytoconstituents of *Ocimum Sanctum* leaves include carvacoral, bornyl acetate, caryophyllene and eugenol; the last of these which accounts for 71% of the volatile oils. Volatile oils have analgesic, antibacterial and insecticidal activity.[3] The leaves show variety of activities including antibacterial[4,5], hypoglycemic[6], antifertility[7], hepatoprotective[8], immunomodulatory[9], anti-asthematic[10] and anti-inflammatory.[11] Oral administration of alcoholic extract of *Ocimum Sanctum* leaves led to marked lowering of blood sugar level in normal, glucose fed hyperglycemic and streptozotocin induced diabetic rats.[12] The tetracyclic triterpenoid isolated from aerial part of *Ocimum Sanctum* L. has a great anti-diabetic potential.[13]

Natural products from medicinal plants, either as pure compounds or as standardized extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. Due to an increasing demand for chemical diversity in screening programs, seeking therapeutic drugs from natural products, interest in plant chemistry has grown throughout the world. Research studies leading to extraction, isolation, identification and biological study of plant constituents have now formed the major field of the study. In-vitro methods play an important role for the pre-clinical studies for any activity, which supports the in-vivo studies. We observed similar potentiation of glucoamylase inhibition by the active compounds Gallic Acid and Epigallocatechin gallate obtained from the fruit rind of *Terminalia Bellerica*.[14,15]

The present manuscript describes the isolation and characterization of stigmasterol and β- sitosterol from the

ethanolic extract of the leaves of *Ocimum Sanctum* L. and evaluation of glucoamylase inhibition *in vitro*.

MATERIALS AND METHODS

Plant material

The fresh leaves of *Ocimum Sanctum* L. were collected from Keshavshrusti, Bhayander, Mumbai and authentificated from Blatter Herberium, St. Xavier's College, Mumbai. Voucher specimens were submitted in a herbarium of same institute for future reference. The leaves were dried in shade and stored in the dark until use.

Chemicals

Glucoamylase (Sisco Research Laboratories),Glucose (Sisco Research Laboratories), Starch (SDF Mumbai), 3,5-Dinitro Salicylic Acid (Spectro Chem), Silica gel for column chromatography 60-120 mesh (SDF chemicals), Silica gel H for TLC (SDF chemicals), Sodium Acetate (SDF chemicals), Glacial Acetic Acid (SDF chemicals). All chemicals were extra pure and analytical grade while the water was glass distilled.

Preparation of ethanolic extract of *Ocimum Sanctu*m L. leaves extract

The *Ocimum Sanctum* leaves were dried at room temperature and then powdered. Powdered leaves (200g) were placed in conical flask with ethanol (750mL) and allowed to stand at room temperature overnight. The ethanolic extract was filtered and collected. This process of extraction was repeated for a week replacing the solvent after every 24 hours. The combined extract was concentrated under vacuum and ethanol free residue (23.5g) was recovered which was 11.72% of the total mass.

Glucoamylase inhibition assay

0.5 mL of the reaction mixture containing 0.1 mL modulator, 0.3 mL of 100mM acetate buffer (pH 4.5) and 0.1 mL of glucoamylase (1.3µg) were incubated at 37°C for 30 minutes. Then; 0.5 mL of starch solution (5 mg/mL prepared in 100mM acetate buffer pH 4.5) was added and incubated at 37°C for 30 minutes. The reaction was terminated by keeping the test tubes in boiling water bath for 1-2 minutes, cooled under running tap water. 2mL of DNS reagent (3, 5-Dinitro Salicylic Acid) was added and the test tubes were kept in boiling water bath for 15 min. the test tubes were cooled and diluted with 7mL distilled water. The absorbance was recorded at 540 nm using spectrophotometer and liberated glucose was estimated by DNS method.[16] A unit activity is defined as the mg of glucose liberated per mg of protein per minute.

Purification

20g of ethanolic extract was subjected for silica gel (60-120 mesh) column chromatography and eluted with pet ether 100% (1-A), pet ether: $CHCl_3$ 1:1 (1-B), $CHCl_3$ 100% (1-C), $CHCl_3$: Ethyl Acetate 1:1 (1-D), Ethyl Acetate 100% (1-E), Methanol 100% (1-F). Fraction 1-B was further subjected to silica gel column chromatography for further purification. The column was eluted with solvent systems, Pet ether 100% (2-A), Pet ether: $CHCl_3$ 9:1 (2-B), Pet ether: $CHCl_3$ 8:2 (2-C), Methanol 100% (2-D). The fraction 2-C was recolumn chromatographed and eluted with solvent systems, Pet ether 100% (3-A), Pet ether: $CHCl_3$ 95:5 (3-B), Pet ether: $CHCl_3$ 90:10 (3-C), Pet ether: $CHCl_3$ 85:15 (3-D) and $CHCl_3$ 100% (3-E). The fraction 3-D was then recrystallised using the solvent ethanol which resulted in needle shaped white crystals This fraction exhibited a single spot (R_f 0.5) on TLC using a Pet ether: Chloroform (7:3, v/v) solvent system.

Identification tests for steroids[17]

Salkowski reaction

Few crystals were dissolved in chloroform and a few drops of sulphuric acid were added to the solution. Reddish colour was seen in chloroform.

Libermann-Burchard Reaction

Few crystals were dissolved in chloroform and few drops of sulphuric acid were added followed by the addition of 2-3 drops of acetic anhydride. Solution turned violet, blue and finally green. Hence, the presence of steroids was confirmed.

Spectral Characterization

Different spectroscopic methods were used to elucidate the structure of isolated compound. Among the spectroscopic techniques, IR, ^{1}H-NMR, ^{13}C-NMR and GC-MS were carried out. The Infra red spectrum was recorded on FTIR Perkin Elmer, ^{1}H-NMR and ^{13}C-NMR spectra were recorded using $CDCl_3$ as solvent on Bruker Avance II 300MHz and 75MHz NMR spectrometer, respectively and GC-MS spectrum was recorded at high resolution on Perkin Elmer mass spectrometer, in the Department of Chemistry, University of Mumbai, Kalina, Santacruz (E), Mumbai.

Instrumentation and GC-MS Condition

GC-MS analysis was performed on Thermo Fisher Scientific, Germany Trace GC ultra-gas chromatograph coupled to a Triple Quadrupole mass analyzer (Thermo Scientific Polaris-Q Ion trap model, Germany). 1µL of sample was injected at constant injector temperature 250°C in the splitting ratio 1:1. GC separation was performed on DB-5MS capillary column (30m X 0.25mm X 0.25µm) (Agilent Technology, Santa Clara, CA, USA). The column temperature was initially held at 60°C for 1 min and then temperature was raised to 300°C at rate of 10ºC min^{-1} followed by isothermal period of 5min. Ultra-high purity helium gas with an inline oxygen trap was used as carrier gas at a flow rate of 1mL min^{-1}. The total run time was 30 min. The transfer line temperature was held at 220°C. MS source temperature was 200°C. The MSD was acquiring data in the full scan mode (mass range 50-600amu) with the multiplier voltage 2225V and analyzed in the Electron Impact mode, ionization energy was 70eV.

Spectral data
Elemental Analysis (Observed)
C=82.012%, H=11.749%, O=6.239%.

FT-IR spectrum
3429.48cm^{-1}, 2957.98cm^{-1}, 2937.48cm^{-1}, 2862.74cm^{-1}, 1638.36cm^{-1}, 1464.26cm^{-1}, 1381.97cm^{-1}, 1061.82 cm^{-1}.

^{1}H NMR spectrum ($CDCl_3$, 300MHz)
δ5.35ppm (1H, d, H-6), δ5.012ppm (1H, dd, H-22), δ5.154ppm (1H, dd, H-23), δ3.5ppm (1H, m, H-3), δ2.624ppm (1H, s, H-20).

^{13}C NMR spectrum ($CDCl_3$, 70MHz, δ in ppm)
37.25 (C-1), 31.65 (C-2), 71.79 (C-3), 42.21 (C-4), 140.75(C-5), 121.69 (C-6), 31.89 (C-7), 31.87 (C-8), 51.23 (C-9), 36.50 (C-10), 21.07 (C-11), 39.77 (C-12), 42.31 (C-13), 56.76 (C-14), 24.29 (C-15), 29.10 (C-16), 55.95 (C-17), 12.03 (C-18), 19.38 (C-19), 40.47 (C-20), 21.20 (C-21), 138.30 (C-22), 129.27 (C-23), 45.80 (C-24), 39.77 (C-25) 19.03 (C-26), 21.07 (C-27), 25.39 (C-28), 11.97 (C-29).

RESULTS AND DISCUSSION

From the positive tests for steroids, it is assumed to be a compound containing steroidal nucleus. The solid obtained was white and had crystalline needle like structure with melting point 144-146^{o}C. In the IR Spectrum analysis, the observed absorption bands are 3429.48cm^{-1} that is characteristic of -OH stretching. Absorption at 2937.48cm^{-1} and 2867.88cm^{-1} assigned to aliphatic C-H stretching of methylene and methyl groups. Other absorption frequencies include 1638.36cm^{1} as a result of C=C absorption, however, this band is weak. 1464.26cm^{-1} is a bending frequency for cyclic (-CH_2) and 1381.97cm^{-1} for -OH deformation. The absorption frequency at 1061.82cm^{-1} signifies cycloalkane.

In ^{1}H spectrum, a complicated pattern observed between δ0.5ppm and δ3.0ppm is the fingerprint region. This unresolved region arises due to the numerous hydrogen atoms present in the ring system and side chain. As the numbers of Hydrogen atoms are large, the signals due to the individual Hydrogen atoms are indistinguishable. The proton corresponding to the H-3 of a sterol moiety appears as a multiplet at δ3.5ppm. The H-22 and H-23 olefinic protons of the side chain appeared each as one proton double doublet at δ5.012 (J=15.0 and 8.5Hz) and δ5.154ppm (J=15.0 and 8.5Hz). A one proton signal appeared at δ5.35ppm (J=4.8Hz) as a doublet indicating the H-6 endocyclic double bond proton. The singlet appearing at δ2.624ppm indicates the presence -CH_3 group. Hydroxyl hydrogen gives signal at δ6.0ppm and is broad and which disappears during deuterium exchange.

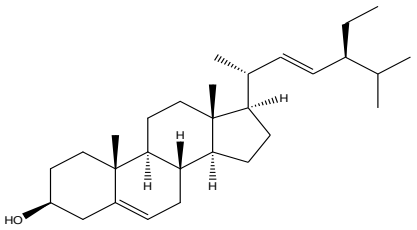
The ^{13}C NMR shows recognizable signals at δ140.8 and 121.7ppm, which are assigned to C-5 and C-6 double bonds respectively as in Δ^5 spirostene. The alkene carbons appeared at δ140.8, 121.7ppm and 138.3, 129.3ppm which are assigned to C-5, C-6 and C-22, C-23 double bonds, respectively.[18] The δ value at 71.8 ppm is due to C-3 β-hydroxyl group.

The GC-MS spectrum of the isolated compound shows to be a mixture of two compounds having molecular ions at m/z 412 and m/z 414 and the characteristic peaks are given at m/z 271 [M-139] and 273 [M-141] due to the loss of $C_{10}H_{19}$ and $C_{10}H_{21}$. The molecular weights and the fragmentation pattern indicate that the compounds present in this sample are stigmasterol and β-sitosterol respectively.

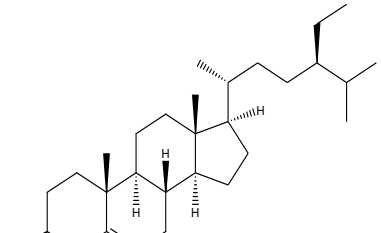
The mass spectrum shows the molecular ion peak at m/z 412, with fragments at m/z (relative intensity) 412(94), 394(36), 379(45), 327 (13), 313 (19), 300 (28), 271(59), 255(74), 159(100), 145(85), 95(45), 69(37). These fragments were compared with the reported in the literature.[19]

The mass spectrum showed the molecular ion at m/z 414 with fragments at m/z (relative intensity) 414 (72), 396 (77), 329 (96), 303 (23), 273 (42), 255 (68), 213 (100), 145 (90), 107 (65), 95 (42), 81 (50), 69 (16), these data was compared with the reported in the literature for this compound.[19]

In ^{13}C NMR spectrum the difference between the two compounds is the presence of C_{22}=C_{23} double bond in stigmasterol and C_{22}-C_{23} single bond in β-sitosterol hence, the lack of practical difference in the R_f despite the use of several solvent systems. Furthermore, literatures have shown that β-sitosterol is difficult to be obtained in pure state.[20-22]



Stigmasterol



β-sitosterol

From the above findings, mixture of stigmasterol and β-sitosterol were isolated from ethanolic extract of the leaves of *Ocimum Sanctum* L. and chemical structures elucidated respectively by means of spectral techniques.

Effect of isolated component on glucoamylase *in vitro*

0.1mg/mL of stock solution of the isolated compound was prepared in 1, 4-Dioxane. The effect of purified compound with various concentrations ranging from 1-10µg/mL on glucoamylase activity *in vitro* was studied. **(Fig. 1).**

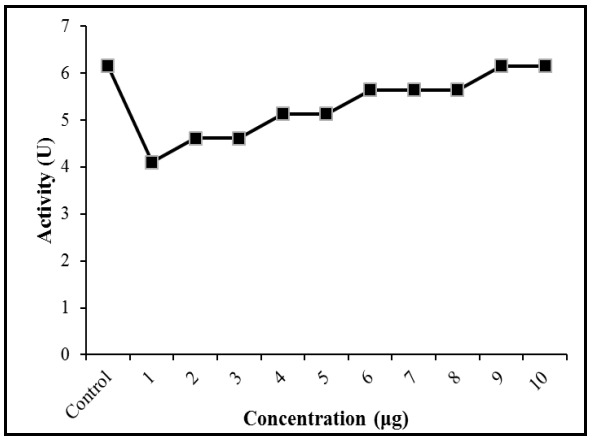


Fig. 1: *In vitro*, Effect of stigmasterol and β-sitosterol mixture on glucoamylase enzyme.

CONCLUSION

Sterols are derivatives of tetracyclic hydrocarbon, cyclopentanoperhydroxy phenantherene ring and are members of triterepenoid isoperoxidases. Sterols are known to be the starting materials for a number of plant steroids which are used in pharmaceutical industry. β-sitosterol is the most abundant phytosterols and occurs either free or as its glucoside, frequently accompanied by its mono-unsaturated analogue, stigmasterol, which also appears either free or as its glucoside.[23] Anti-inflammatory, analgesic and anti-pyretic activity of β-sitosterol have been reported previously.[24] β-sitosterol could also modulate antioxidant enzymes in vitro.[25] A purified mixture of β-sitosterol and stigmasterol isolated from the root extract of *Pluchea indica* and successfully established its anti-snake venom activity in experimental animal.[26]

The mixture of stigmasterol and β-sitosterol isolated from the leaves of *Ocimum Sanctum* was found to be moderate inhibitor of glucoamylase, thus may act as an agent to control blood glucose levels in hyperglycemic condition. From the present study, it was apparent that plant exhibits or have the potential to show, a diverse array of biological activities. However, so far only a fraction of these plants have been investigated. There is an urgent need to develop newer, more efficient and reliable bioassays for large scale rapid evaluation of plant extracts.

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