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Preparation and Evaluation of Conessine Enriched Extract from *Holarrhena antidysenterica* Bark for Enhanced Antibacterial Properties

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Abstract**—** *The current study was designed with a view to develop an efficient method to recover extract of Holarrhena antidysenterica bark with high conessine content decipher enhanced antimicrobial action. H. antidysenterica bark powder was extracted by reflux using methanol as a solvent containing varying percentage of di-ethylamine (2.5%, 5%, and 10% respectively). Extract was further subjected to chromatographic separation to isolate pure conessine. Isolated conessine was characterized by 1H and 13C NMR spectroscopy. Estimation of conessine content in the extracts was performed by high performance thin layer chromatography (HPTLC). The comparative antimicrobial properties of all the extracts were assessed in both gram +ve and gram -ve bacteria by performing broth microdilution assay and disc diffusion assay method. A complementary in-silico study was also employed to understand the molecular interaction between conessine and New Delhi metallo-β-lactamase 1 by molecular docking. It was observed that, the extract obtained with 5% di-ethylamine containing methanol contains maximum amount of conessine (Approx. 3%). In-vitro antibacterial assay showed that, the same extract exhibited maximum anti-bacterial properties when compared with the other extracts (2.5% and 10% di-ethylamine) which directly corroborates with conessine content. Molecular docking experiment also revealed strong antibacterial action (docking score: -8.1) compared to tetracycline (docking score: -7.5) by blocking with the crucial amino acid residues of New Delhi metallo-β-lactamase 1 protein which may be responsible for antibacterial action. From the current findings it can be concluded that, the extract obtained from methanol containing 5% di-ethylamine is ideal solvent for better recovery of conessine from H. antidysenterica bark with better antimicrobial action.*

I. INTRODUCTION

Bacterial infection has been a longstanding challenge in human history, causing a wide range of illnesses and posing threats to public health and not less to domestic animals. Bacterial diseases can affect various parts of the body, causing illnesses such as pneumonia, tuberculosis, urinary tract infections, and skin infections [1]. Antibiotics are drugs designed to kill or inhibit the growth of bacteria, and they have been instrumental in treating bacterial infections since many decades. However, the excessive use of antibiotics in medicine, agriculture, and livestock has led to the development of antibiotic-resistant strains of bacteria. Antibiotic resistance occurs when bacteria evolve to withstand the effects of antibiotics, rendering these drugs less effective or entirely ineffective [2]. The ability of bacteria to transfer genetic material containing resistance mechanisms to other bacteria accelerates the spread of antibiotic resistance [2,3]. Treatment of antibiotic-resistant infections often requires more expensive and prolonged therapies, increasing healthcare expenses. Though synthetic drugs are somewhat successful in the eradication of drug resistant bacterial infections but not free from concern like unwanted side effects and toxicity [4]. One of the most common side effects associated with the consumption of synthetic is disturbing the gut microbiota eventually leads to gastrointestinal malfunctioning and later organ toxicity due toxic metabolite production during metabolism [4,5].

On the other hand, plants have long been recognized as a rich reservoir of bioactive compounds, offering a diverse array of secondary metabolites that exhibit remarkable medicinal properties [6]. There are unlimited opportunities to explore numerous natural products, plant extracts and herbal medicines exist in the nature and look for safer option to eradicate various dieses including microbial infections.

Numerous plant secondary metabolites are found imperative in controlling range of microbial infections [7]. Alkaloids are one such category of phytochemicals which are extracted from medicinal plants exhibited promising efficacy in controlling microbial infection [8]. *Holarrhena antidysenterica* is such medicinal plant which is commonly known as Kutaj or Kurchi, abundantly found in India and other Asian countries especially in Himalayan ranges [9]. According to the classical Ayurveda, the plant holds numerous medicinal importance including antidiarrhoeal, antidysenteric, anti-anthelmintic, stomachic, febrifugal, digestive and tonic properties. Herbal preparations from *H. antidysentrica* bark have been used extensively in treatment of gastric disorders such as amoebic dysentery from ancient era [10]. It is exported in the form seed powder, bark powder, Kutajakwatha, Kutaja Prapati Vati and as herbal dietary supplement. The bark of this plant is rich in alkaloids and few of the key ingredients show potent amoebicidal effect [9,10]. Within this, Conessine, one of the major steroidal alkaloids reported from this bark is responsible for amoebicidal action. This is a major constituent of *H. antidysenterica*, responsible for numerous pharmacological activities [11, 12]. Despite, there is limited literature available related to conessine content in *H. antidysenterica* stem bark extracts till the date.

Therefore, in the present research, we presented a detailed account of the extraction process, purification method, and characterization of the conessine-enriched extract.

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Furthermore, we elucidate the antimicrobial potential through comprehensive *in-vitro* assays against clinically relevant pathogenic bacterial strains. The findings of this study are expected to contribute significantly to the growing body of knowledge on natural antibacterial herbal extracts and may pave the way for the development of new and effective therapeutic interventions.

II. Materials And Methods

Plant materials

Fresh barks of *H. antidysenterica* was purchased from Dhanvi Agro-Herbal Pvt. Ltd., Kota, Rajasthan, India and dried in shade followed by grounded into coarse powder (#40).

Extraction of conessine from H. antidysenterica bark

Approximately 100 gm of powdered H. antidysenterica bark was extracted by reflux extraction technique using methanol as a solvent with addition of varying percentage (2.5%, 5% and 10% v/v) of di-ethyl amine (DEA) separately. Extraction was achieved by continues reflux for 4 hrs. at 55 ºC. The process was repeated for three times with freshly prepared solvents. After 4 hrs. liquid extract was combined and concentrated in vacuum to get semisolid extract.

Isolation and characterization of conessine

Extract was prepared in bulk and approximately 50 g of extract was adsorbed on 100 g of 100-200 mesh silica gel. Column was packed with 500 g of 100-200 mesh silica gel in chloroform. Elution of column was started with 2 L of $CHCl_3$ and 250 mL each fraction was collected. Polarity was increased with increment of 5.0% methanol. Similar fractions were pooled based on the TLC and the fractions collected from 25.0% - 40.0 % methanol conc. and evaporated. Pooled fractions were combined and re-chromatographed on 230- 400 mesh silica gel. Column was eluted with $CHCl_3$ and polarity increased with 5% methanol in chloroform (total vol. 500 ml) and collect the fraction of 100 ml each. The fraction collected from 25.0 % methanol in chloroform was pooled and concentrated fraction to minimum, and kept overnight to obtain the compound (~70.0 mg). TLC mobile phase = Toluene: Ethyl acetate: Dimethylamine: 6.5:2.5:1. Detection = Spray with Dragandorff's reagent. Isolated compound was characterized further by ^{1}H and ^{13}C NMR experiments.

Estimation of conessine content in H. antidysenterica extract

Conessine content in the H. antidysenterica extract was quantified by using HPTLC densitometric method. Briefly, chromatographic separation was performed pre-coated HPTLC silica gel plates and spotted the samples by dissolving in HPLC grade methanol. Extracts and standard were prepared in 1000 ppm concentration and 5 µL extract and standard were spotted on TLC silica plate 60 F254 (Merck, Germany) as 6 mm band at 8 mm from the bottom of the plate with the help of Linomat automated band applier to CAMAG HPTLC system. The system was programmed through vision CATS software (CAMAG, Switzerland). The plates were developed in the twin throw chamber (CAMAG, Switzerland) saturated with the mobile phase (Toluene: Ethyl acetate: Dimethylamine /6.5:2.5:1) 20 min before band application. The HPTLC plates were derivatized with Draggendorff's reagent and visualized in the visible and UV366 spectrum using TLC Visualizer (CAMAG, Switzerland) and scanned using the TLC plate scanner (CAMAG, Switzerland).

Assessment of antibacterial properties of different extracts of H. antidysenterica bark

Determination of minimum inhibitory concentration (MIC)

Different conessine containing extracts obtained from H. antidysenterica was subjected to determine the minimum inhibitory concentration by broth dilution method [13]. The bacterial strains were cultured overnight at appropriate temperature in nutrient agar. The test strains were suspended in nutrient broth to give a final density of 5 x 10^5 cfu/ml and these were confirmed by viable count. The test extracts were dissolved in 10% DMSO were first diluted to the highest concentrations (500 µg/mL) and then serial dilutions were made with nutrient broth in the concentration range from 1.56 to 500 µg/mL The MIC was determined as the lowest concentration of test sample showing inhibition of growth in the broth.

Disc diffusion method

Antibacterial tests were then carried out by the disc diffusion method [14] using 100 µl of suspension containing 108 cfu/ml of bacteria spread on nutrient agar. Sterile paper discs (6 mm in diameter) were impregnated with MIC concentration of extracts and placed on the inoculated agar. The inoculated plates were incubated at appropriate temperature for 24h. The antibacterial activity was evaluated by measuring the zone of inhibition against the test organisms.

Molecular docking

Molecular docking was performed between conessine and New Delhi metallo-β-lactamase 1 considering antibacterial target protein using Auto Dock vina software, and data visualization using Discovery Studio. The receptor molecule which was downloaded from the protein data bank (PDB) was further prepared with the Discovery Studio. Ligand molecules were prepared with the ChemDraw Ultra 8.0. Finally, docking experiment was performed in AutoDock Vina. Tetracycline was taken as standard inhibitor and for each molecule 10 iterations was given and the best one was considered as output.

III. Results

Isolation and quantification of conessine content

Conessine was obtained as a creamy white powder exerts melting point of 125-126 ºC. Further the compound was subjected 1H and 13C NMR and the data obtained was aligned with the reported literatures (Fig. 1).

On the other hand, HPTLC analysis revealed that significant difference between the conessine content in the various extracts of H. antidysenterica. It was observed that, extract obtained 5% DEA in methanol yields maximum conessine content (2.9 ± 0.22 %) followed by 10% DEA in

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methanol yields (1.1 ± 0.04 %) and the lowest yield with 2.5% DEA in methanol (0.7 ± 0.11 %). The chromatographic pattern of different conessine extract is given in the (Fig. 2).

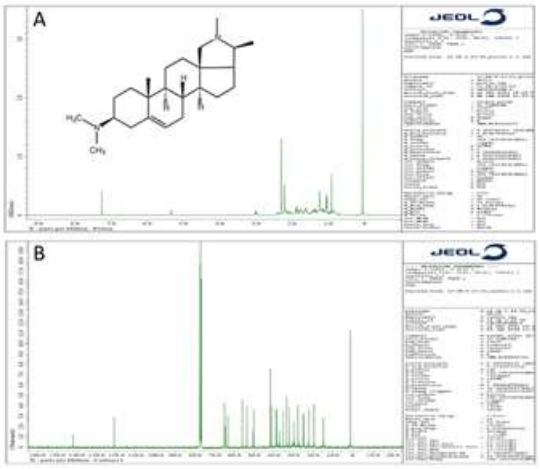

Figure 1: (A) 1H NMR and (B) 13C NMR of isolated conessine.

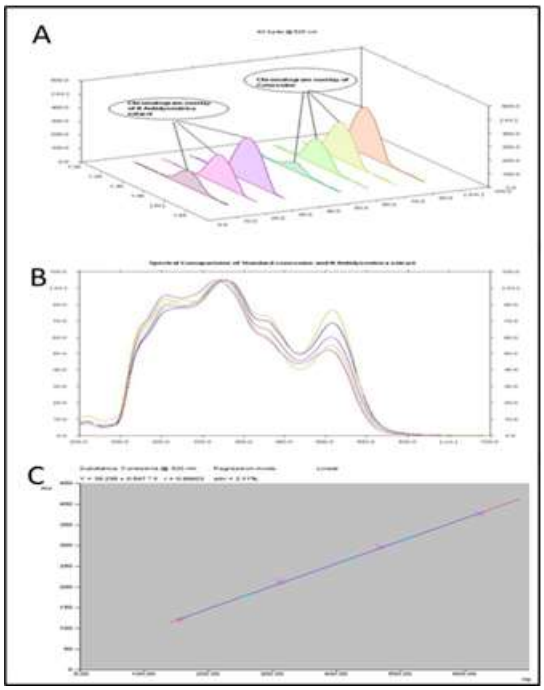

Figure 2: HPTLC analysis of conessine content. (A) Chromatogram overlay; (B) Spectral overlay; (C) Linearity of standard conessine.

Antibacterial properties of extracts containing various content of conessine

Antibacterial potential of different extracts of H. antidysenterica obtained was subjected to determination of minimum inhibitory concentration (MIC) against gram +ve and gram -ve bacterial strains and found that, extract obtained from 5% DEA in methanol exhibited lowest MIC value against *Staphylococcus aureus* (125 µg/mL), *Bacillus cereus* (250 µg/mL), *Escherichia coli* (125 µg/mL), *Salmonella typhi* (62.5 µg/mL) respectively indicates the most potent extract compared the other tested samples. On the other hand, extract obtained with 2.5% DEA exerts high MIC value compared to other extracts (Table 1).

TABLE 1: Comparative antibacterial properties of different extracts of *H. antidysenterica*

Extracts / standard	MIC Value (µg/mL)			
	S aureus	*B cereus*	*E coli*	*S typhi*
S1 (2.5% DEA)	>250	>250	>250	>250
S2 (5% DEA)	125	250	125	62.5
S3 (10% DEA)	>250	>250	>250	>250
Tetracycline	1.56	1.56	6.25	3.12

Zone of inhibition (ZOI) of the extracts were tested using the MIC value against the same microorganisms and observed that extract obtained from 5% DEA in methanol exhibited maximum ZOI against all the microbial strains which is also aligned with the content of conessine in the extracts. A comparative analysis of ZOI is represented in the Table 2.

TABLE 2: Comparative antibacterial properties of different extracts of *H. antidysenterica*

Extracts / standard	Zone of Inhibition (mm)			
	S aureus	*B cereus*	*E coli*	*S typhi*
S1 (2.5% DEA)	10	14	9	8
S2 (5% DEA)	19	23	21	17
S3 (10% DEA)	16	18	14	12
Tetracycline	21	27	29	23

In-silico molecular interactions of conessine with antibacterial target protein Delhi β-lactamase

In-silico interactions of conessine with the bacterial protein target New Delhi metallo-β-lactamase 1 was assessed by molecular docking studies. It was observed that, conessine blocks the binding pocket of the protein by forming van der Waals force interactions with amino acid residues ALA243, LYS242, PRO241, ASP199 and with TRP168, LYS181, PHE240 by for p-Alkyl interactions. The interactions of tetracycline were considered as standard inhibitor and found that it forms H-bond interactions with LYS216, SER217, LYS211, ASN220; p-Alkyl bonding with Val73; and van der Waals force interactions with SER251, ASP212, HIS250, LEU218, HIS189, ASP124 respectively. However, the docking score for conessine and tetracycline was obtained -8.1 and -7.5 respectively.

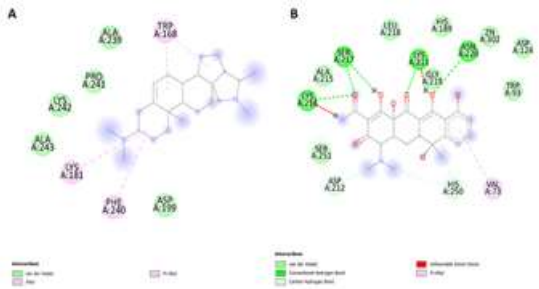

Figure 3: *In-silico* interactions of New Delhi metallo-β-lactamase 1 with (A) Conessine and (B) Tetracycline

IV. DISCUSSION

Holarrhena antidysenterica is often employed to clear excess of pathogenic heat or dampness heat from the body in

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cases of diarrhea and dysentery. It is used to address digestive issues, including abdominal pain, bloating, and irregular bowel movements. In traditional Chinese medicine, it is used to address conditions related to microbial imbalances, supporting the body's natural defense mechanisms [9-11]. Conessine is reported as the main antibacterial phytoconstituent present in the bark extract of this plant [9, 10]. In this study we observed that the antibacterial property of the extract is directly proportional with the conessine content.

It is known that the extracts are prepared from different solvents, reported to contain 0.3-0.5% of conessine content so far which is not sufficient enough to produce potent antibacterial efficacy. Other alternate classical methodologies of extraction of alkaloids are available which use acid-base treatment but the process is complicated, involves number of expensive reagents and multiple steps [15]. Moreover, the yield of the total extract is less since only alkaloids containing fraction is separated. Due to these limitations, available processes are not viable for large scale production of conessine.

The present research discloses a simplified process for preparing Holarrhena antidysenterica extract with high conessine content (Approx. 3% w/w). The prime advantage of the present invention is that it is a simple, efficient and commercially viable and cost-effective method of preparing H. antidysenterica extract from its bark with high concentration of conessine content as well as higher yield, when compared with the other available classical methodology of isolation of alkaloids. This study also indicates the inhibition of New Delhi metallo-β-lactamase 1 protein may be one of the mechanisms of antibacterial action.

V. CONCLUSION

From the findings of the current study, it can be concluded that the solvent composition containing 5% DEA in methanol may be recommended as the best solvent for better recovery of conessine from H. antidysenterica bark. However, the scaleup and further validation may give a concrete and much clear conclusion of the study.

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