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Phytochemical and Antibacterial Investigation of Stem Bark and Callus Extracts *of Holarrhena antidysenterica* Wall

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Abstract: ***In the present investigation, the methanolic extracts of bark and callus of Holarrhena antidysenterica were evaluated for antimicrobial activity against common human pathogens and subsequently phytochemical analysis of the crude extracts was carried out to determine the active phytochemical constituents responsible for antimicrobial activity. Explant used for callus formation were mainly seeds and in vivo and in vitro grown plant parts like stem, nodal parts, roots and apical shoots. A friable type of callus was obtained when explants cultured on MS medium with 2, 4 - D separately or in combination of KN. This study also involves the bioseparation of compounds present in the crude extracts of bark and callus by TLC and HPTLC methods. Presence of peaks in the TLC and HPTLC exhibited presence of principle bioactive compound conessine in the methanolic extracts of Bark and Callus. It was observed that methanolic bark extract of Holarrhena antidysenterica was more effective in inhibiting all the two tested pathogens with zone of inhibition ranging between 2.3 mm to 10.05 mm as compared to methanolic callus extract with 1.65 mm to 4.0 mm of inhibition zone only. Phytochemical profiling of crude bark and callus extracts revealed that both the extract contain alkaloids, tannins, steroids and flavonoids. The results indicate that the methanolic bark and callus extracts might be exploited as natural drug for the treatment of several infectious diseases caused by these organisms.***

Keywords: *Holarrhena antiydsenterica*. Methanolic extracts, antimicrobial activity, phytochemical, HPTLC

1. Introduction

Plants have been used for important medicinal constituents in indigenous medical system since ancient times. The medicinal potential (antimicrobial, antioxidant, anticancer, antimalarial, immunomodulatory, etc.) attributed to plants has been linked to the presence of bioactive constituents in these plants. These bioactive substances have been found to produce definite physiological action on the human body. The majority of the species belonging to the family Apocynaceae are rich in phytochemicals which play a significant role as bioactive compounds. These bioactive substances include alkaloids, essential oils, saponin, tannins, flavonoids, terpenoids and phenolic compounds (Mungole et al, 2010).

Extraction of bioactive compounds from medicinal plants permits the demonstration of their physiological activity. It also facilitates pharmacological studies leading to synthesis of a more potent drug with reduced toxicity (Balunas, M. J., 2005). Furthermore, the active components of herbal remedies have the advantages of being combined with many other substances that appear to be inactive. However, these complementary components give the plant as a whole a safety and efficiency much superior to that of its isolated and pure active components (Royal Botanical Gardens, 2020). Presently in the developing countries, synthetic drugs are not only expensive and inadequate for the treatment of diseases but are also often with adulteration and side effects (Royal Botanical Gardens, 2020). There is therefore the need to search for plants of medicinal value.

As the demand for secondary metabolites is increasing at a very fast pace, some of the plants are increasingly at risk of extinction in their natural habitats. Besides, the production of secondary metabolites is found to be restricted to the particular plant parts, particular growth and developmental stage or under specific conditions related to the seasons, stress, and nutrient availability of the species or genus. Furthermore the extraction of secondary metabolites from the plant is very expensive with poor yield. Considering all these difficulties, in recent times *in vitro* techniques. Plant cell culture technology is the most promising approach for large - scale secondary metabolic production at the commercial level.

Holarrhena antidysenterica Wall is a medicinally important plant of Africa as well as tropical and subtropical regions of Asia (Hamilton, 2004). *Holarrhena antidysenterica* is a great economic value and continue to provide valuable therapeutic uses. It is commonly known as Kurchi in Hindi and 'Conessi or Telichery bark' in English, is a rasayan herb used in Indian system of Medicine. It belongs to family Apocynaceae (Jones, 1996). The connessi tree is popular for its numerous medicinal properties and the seeds and bark of this tree have widely used in Ayurveda and traditional Chinese medicine. Its seeds are used as anthelminthic, and its barks is reported to have antidiarrheal properties (Singh A. K. et al., 2002). In Ayurveda medicine it is used for treating anemia, jaundice, dysentery, stomach - pains, diarrhea, epilepsy and cholera (Kadir M. F et al., 2013) It is used traditionally to treat several diseases like liver complains (Huang J. et al, 2007, Kulkarni N. et al, 2014), Piles (Shankar S. S. et al., 2004), Regulate blood sugar (Swami A. et al., 2004, David E. et al., 2010), Fever (Khalil

K. A., 2013), GastroIntestinal disorders (Naik R. R. et al., 2002). The Apocynaceae is probably the most thoroughly investigated family for alkaloidal plants and about 1000 of these compounds have been isolated from its many members. Around 30 alkaloids are isolated from the bark of this plant only. The main steroidal alkaloids of *Holarrhena antidysenterica* is conessine. Previously, Hebleet al., (1976) reported the involvement of some phytochemicals in cholesterol metabolism from callus culture of *Holarrhena antidysenterica*. Phytochemicals i.e. saponins, steroids, alkaloids, tannins and flavonoids are reported to be responsible for the antidiarrheal activity of plants *Holarrhena pubscens* seeds extract tests positive for alkaloids and flavonoids, therefore these may be responsible for this activity (Kulsom Z. et al., 2020)

The well developed protocol for the bioseparation of phytocompounds could only be achieved through systematic standard evaluation using some modern analytical techniques e. g. TLC/HPTLC. The present work also deals with quantitative analysis of conessine by TLC/HPTLC method.

The aim of present work is to determine the phytochemical profile and antimicrobial activity of stem bark and callus extract. Therefore objectives of the current research also include the Extract preparation of stem bark and callus using methanol as the solvent, Determining phytochemicals present in the stem bark and callus, determining the antimicrobial activity of the plant extract against gram positive and gram negative microorganism and quantitative analysis of conessine by TLC/HPTLC method.

2. Material and Methods

Barks and explants e. g. stem, leaf of *Holarrhena antidysenterica* Wall were collected from Charhi colliery forest of Hazaribagh district of Jharkhand. The identity of these plants was confirmed by taxonomist Dr. Radha Sahu, Former Professor and Former Head, University Department of Botany (Ranchi University, Ranchi).

M S (Murashige and Skoog, 1962) medium was used as basal nutrient medium in present investigation for callus formation. Seeds as well as different plant parts stem, node, roots of field grown as well as *in vitro* grown plantlets were used as explants (Mahato et al, 2012).

Callus was dried and homozenized to fine powder and further exploited for extraction, phytochemical profiling andits antibacterial efficacy.

Preliminary tests were carried out for the presence or absence of phytoconstituents like alkaloids, flavonoids, glycosides, saponins, sterols, terpenes, tannins and resins in barks and callus extracts individually (Harborne 1998, Sazadaet al., 2009)

Cold Extraction (Maceration)
5 gm of dried callus powder was macerated with 50 ml methanol. After 15 days callus extract was filtered by Whatman filter paper 1, This methanolic extract was made concentrated through `water bath (Mahto and Mehta, 2013) and used for biotesting.

Antibcaterial activity test
Antibacterial studies were carried out against two gram positive (*Staphylococcus, Salmonella* and one negative (*Escherchia coli*) bacteria. The bacteria were obtained from Rajendra Institute of Medical Science, Ranchi. The bacteria were incubated on a nutrient agar for 24 hr at 37+_ 2 degree C (Mahato et al., 2013). The antimicrobial susceptibility testing was done by using the agar well diffusion method to detect the presence of antibacterial activities of the samples. Microbial growth was determined by measuring the diameter of zone of inhibition. The diameter of inhibition zones was measured in mm and the results were recorded. MIC values were defined as the lowest concentration of each natural product which completely inhibited microbial growth.

Phytochemical Profiling
The extracts used for TLC/HPLC investigation was prepared by cold extraction (maceration) method (Kudiet al., 1999). Various extracts were monitoring on TLC plate by spraying reagents and iodine chamber was use for detection of the components. Analysis of methanolic extract was done on HPTLC (CAMAG) (Patel and Prajapati, 2008, Garg and Bhutani, 2008, Kaur et al., 2008).

Instrumentation
Analysis was performed on 5x10 cmplates cut from 20x20 cm aluminum –backedstlica gel 60 [F. sub.254] plates. Samples were applied to the plates by means of a Lonomat - V automatic spotter with the aid of a Hamilton 100 [micro] L syringe. TLC plates were developed in a flat - bottom twin trough chamber. After spotting, TLC plate was developed in chamber using selected solvent Toluene: Ethyleactate: Diethyl amine (6.8: 2.5.1 and 6.5: 2.5: 1)

Chromatographic condition in the HPTLC
Stationary phase - Methanol pre - washed 5x10 cm aluminum - backed silica gel 60 [f. sub.254] plates (E. Mjerck. Darmstdt, Germany) Mobile phase - Toluene - Ethyl acetate - Diethyl amine (6.8, 2.5, 1 v/v). Chamber saturation - 30 min, Band width - 6 mm, Distance between tracks - 13.0 mm, Rate of spoting - 4, 6 and 8 [micro]/L/s. Distance run - 75 mm, Spraying reagent –Dragendroff reagent was sprayed after the plate was dried. The plate was then sprayed after the plate was dried. The plate was then sprayed with a 10% solution of aqueous sodium nitrate and dried in air. After 20 min, the plate was scanned at 200 - 800 nm aped 20mm/S, Slit dimensions 5.00x0.45 mm and a Temperature of 25° C. Developed plate was analyzed under UV chamber at 200 - 800 nm. HPTLC graphs of chromatogram bark, and callus extracts were obtained with TLC scanner - 3 wit Win CATS 4 software in a Pentium IV computer.

Kutajarishta (Dabur) a liquid Ayurveic medicine was used as standard conessine (Mahato and Mehta, 2013) during TLC and HPTLC.

3. Results and Discussion

Table 1: Results of phytochemical screening of bark and seed extracts (alcoholic) of *Holarrhena antidysenterica*.

S. No.	Phytoconstituents	Bark extracts	Callus extracts
1.	Alkaloids	+	+
2.	Glycosides	+	+
3.	Steroids	+	+
4.	Tannins	+	+
5.	Saponins	-	+
6.	Flavonoids	+	+
7.	Phenols	+	+
8.	Resins	-	+

Table 2: TLC Method Development of Extracts of *Holarrhenaantidy - senterica*

Scheme	Extract	Solvent System	Ratio	Rf values
Cold	Bark (Methanol)	Toluene: Ethyl acetate: Diethyl amine	6.8: 2.5: 1	0.78 0.71
Cold	Callus (Methanol)	Toluene: Ethyl acetate: Diethyl amine	6.5: 2.5: 1	0.76
Cold	Kutajarista	Ethyle acetate: Hexane: Triethyl amine	7.5: 2.4: 0.6	0.83

Table 3: HPTLC profile of methanolic extracts of *Holarrhenaantidy - senterica* bark, seed and callus

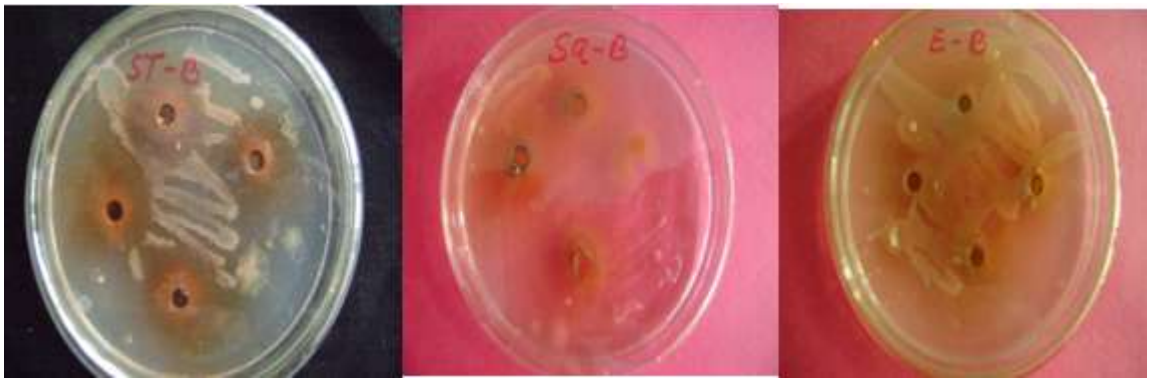
Different Extracts	No. of Tracks	No. of Peaks	Maximum Rf Value	Maximum Height	Area of Peaks	% Area of Peaks
Bark	4ul	1	0.56	17.5	347.7	100.00
	8ul	1	0.56	30.3	622.5	100.00
	12ul	1	0.56	42.1	892.7	100.00
Callus	4ul	1	0.76	69.1	2918.5	100.00
	8ul	1	0.73	124.4	5956.2	100.00
	12ul	1	0.75	67.0	3288.2	100.00

Table 4: Antibacterial activity of parts of *Holarrhena antidysenterica* (bark and callus) methanolic extracts against bacterial species tested by punch well method. Values are mean inhibition zone (mm), Mean ± Standard Error (SE), Number of observation (n) = 20

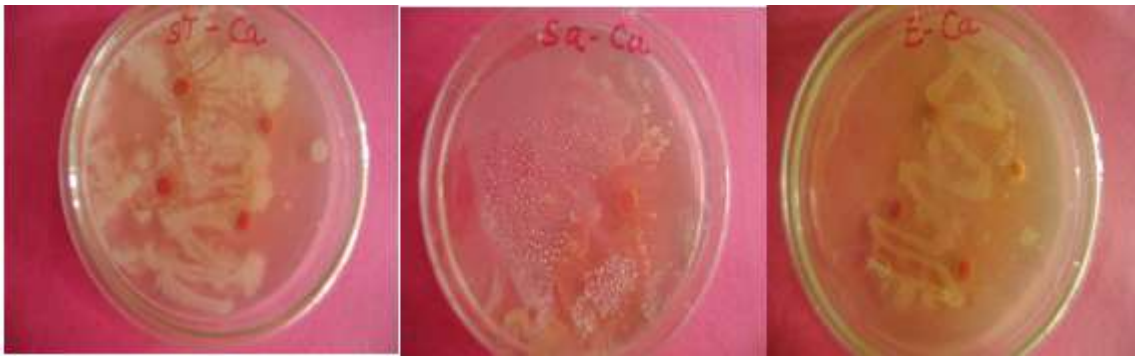
Plant Material	Bark				Callus			
	100%	75%	50%	25%	100%	75%	50%	25%
Staphylococcus	10.05 ± 0.23	7.05 ± 0.14	2.3 ± 0.15	0	4.00 ± 0.17	3.1 ± 0.18	1.65 ± 0.14	0
Salmonella	6.65 ± 0.22	4.95 ± 0.12	2.05 ± 0.12	0	3.05 ± 0.14	2.25 ± 0.11	0	0
Escherchia coli	2.7 ± 0.13	1.65 ± 0.12	0	0	3.1 ± 0.16	1.95 ± 0.10	0	0

Figure 1 (A): Bark of *H. antidysenterica*

Figure 1 (B): Callus of *H. antidysenterica*



2A 2B 2C



2D 2E 2F

2 (A - F): *H. antidysenterica* Wall. Determination of antimicrobial activity by agar well diffusion method. Figure A - C Effect of Bark extract on *Staphylococcus*, *Salmonella* and *E. coli* streak culture plate, Figure D - F Effect of Callus extract on *Staphylococcus, Salmonella* and *E. coli* streak culture plate,

4. Result and Discussion

In vitro callus formation, phytochemical analysis and antimicrobial effects of Bark and Callus extracts of *H. antidysenterica* against dysentery causing pathogenic bacteria were carried out. Best callus formation was achieved on MS with 2, 4 - D (1.5mg/L and2.5 mg/L) and 2.4 - D (2.5 mg/L) +KN (1 mg/L). This study involves preliminary phytochemical screening, the separation and identification of compounds present in crude extract of *H. antidysenterica* Bark and Callus by TLC and HPTLC methods. Further qualitative analysis of the methanolic extract prepared from *H. antidsenterica* Bark and callus revealed the presence of alkaloids, flavonoids, tannins, terpenoids. The preliminary phytochemical screening of *Holarrhena* Bark and Callus extracts showed bioactive components alkaloids, flavonoids and tannins (Table.1) This study also involves the separation and identification of compounds present in crude extract of *H. antidysenterica* Bark and Callus by TLC, HPTLC method. The solvent system was selected through trial and error method (Table.2).

The identity of the conessine in sample extracts was compared with the extract of Kutajarishta which is referred as standard. The retention factors (Rf=0.81) which lead to the conclusion that the spots on TLC plate corresponds to connessine. HPTLC method is feasible for development of chromatographic fingerprints to determine major active constituents of medicinal plants. In the present study the proposed HPTLC fingerprint method combined with digital scanning profiling was used and an attempt was made to estimate the steroidal alkaloid present in Bark and Callus extracts of *H. antidysenterica*. Different compositions of mobilephase for TLC and HPTLC analysis were tested in the order to obtain high resolution and reproducible peak. It was found that Toluene: Ethyle acetate: Diethyle amine (6.8: 2.5: 1 v/v and 6.5: 2.5: 1 v/v) were the best solvents for HPTLC fingerprint.

The crude methanolic bark and callus extract obtained from *H. antidysenterica* were submitted to an antibacterial screening using agar well method against various pathogenic bacteria. In the study significant zone of inhibition (in mm) was observed on three bacteria (*Staphylococcus aueus*, *Salmonella typhimurium* and *Escherchia coli*). During present work about 10.05 mm inhibition zone was observed in bark extract with 100% concentration, showing highest antibacterial activity against *Staphyloccus* (Fig.2A) whereas in case of *Salmonella* and *E. coli* it was only 6.65 mm and 2.7 mm respectively. Callus extracts with 100% concentration showed 4 mm inhibitory zone against *Staphylococcus* and its least activity was observed in *E. coli* with 3.1 mm inhibition zone even in 100% concentration. This is the first report on comparative studies on two types of extracts of *H. antidysenterica*. Thus, the study as certain the value of plants used in Ayurveda, which could be of considerable resistance to bacteria and Staphylococcus, the gram positive bacteria was the most susceptible one. Various workers have already shown that gram positive bacteria are more susceptible towards plants extracts as compared to gram negative bacteria (Lin et al., 1999; Parekh and Chandra, 2006). In addition microorganisms show variable sensitivity to chemical substances to different resistance level between different strains of bacteria (Certin and Gurler, 1989).

Previously antibacterial activity of bark extract of *H. antidysenterica* was studied (Ballal et al., 2001, Raman et al., 2004, Chakraborty and Brantner, 1999) investigated about the presence of alkaloids in the methanolic extract of bark, a good source of antibiotics. Plant based antimicrobial compounds have enormous therapeutic potential as they can serve the purpose without any side effects that are often associated with synthetic antimicrobials.

5. Conclusions

Results obtained in the present study revealed that two types of extracts of *Holarrhena antidysenterica* possess potential antibacterial activity against *Staphylococcus*, *Salmonel*a and *E. coli*. This result supported that *Holarrhena antidysenterica* contain bioactive compounds mainly alkaloids against bacteria causing stomach ailment and both bark and callus extracts may serve as a valuable source of compounds with therapeutic potential.

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