STUDIES ON ANTIBACTERIAL EFFECTS OF BARK, SEED AND CALLUS EXTRACTS OF HOLARRHENA ANTIDYSENTERICA WALL.

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ABSTRACT
A procedure has been outlined for callus formation and antibacterial effects of bark, seed and callus extracts of an important medicinal shrub Holarrhena antidysenterica WALL. Explants used for callus formation were mainly seeds and both in vivo and in vitro grown plant parts like stem, nodal parts, roots and apical shoots. A friable type of callus was obtained when these explants were cultured on Murashige and Skoog (MS) culture medium containing different concentrations of 2,4-D separately and in combinations of KN. During present studies methanolic extracts of bark, seed and callus were made and tested for their antimicrobial activities against Staphylococcus aureus, Salmonella typhimurium and Escherica coli. Results showed that all three different types of extracts of Holarrhena antidysenterica possess nearly similar potential for antibacterial activity against these pathogenic bacteria.

INTRODUCTION

Plants have been used for important medicinal constituents in indigenous medical systems since ancient times. A large proportion of the drugs used in modern medicine are either directly isolated from plants or synthetically modified from a lead compound of natural origin. In plants as a result of metabolic processes many different types of organic compounds or metabolites are produced. Among different plant derived secondary metabolites alkaloids proved to be the most important group of compounds that showed wide range of antimicrobial activity (Sarkar et al., 1991; Hossain et al., 1993; Raman et al., 1997) and since long time it has been established fact that plants are safer alternative sources of antimicrobials (Pretorious and Watt., 2001; Sharif and Banik., 2006). The review of literature revealed that considerable contributions have been made on medicinal plants by many workers (Dadsena et al., 2013; Dandapat et al., 2013; Kullu et al., 2013; Kumar et al., 2013; Kumar et al., 2013a; Tabassum et al., 2013; Toppo et al., 2013; Sahu et al., 2013).

Holarrhena antidysenterica WALL. Belongs to family Apocynaceae, a deciduous shrubby 9-10 meter high tree found throughout tropical India, Burma, Sri Lanka, Pakistan, Nepal and Africa and flowers in the months of May-July. Seeds are linear or oblong concave with a long coma, are light brown in colour and shows epigeal germination. This tree is popular for its numerous medicinal properties and seeds and bark of this tree have been used in Ayurveda since long time. The stem bark which is commonly known as “kurchi” in the Indian subcontinent and as “conessi bark” in Europe is used in traditional ayurvedic medicine to treat dysentery, especially amoebic dysentery (Bhutani, 1984). Bark of Holarrhena antidysenterica is used in Ayurveda as an anti-microbial, anti inflammatory and analgesics (Kirtikar et al., 1994; Warrier et al., 1994; Sharma et al., 2004). Other useful parts used as medicine are root and leaf. The bark and the roots have been found to be an excellent remedy for both acute and chronic dysentery especially in cases where there is excessive blood with mucus and colic pain associated with stools (Ghosh, 1984). In addition the plant has been reported to possess antihelminthic, appetizing, antidiarrhoeal and astringent properties (Chopra et al., 1982). These properties are due to the presence of steroidal alkaloids conaine and aminopregnane types, the principal one being conessine. Holarrhena antidysenterica is also a rich source of other steroidal alkaloids such as kurchine, kurchimine, conessidine, holarrimine, conessidine, konkurchicine and regholarrhimmine (Radt, 1965). Holarrhena antidysenterica has been reported to be used as an immunodulating agent (Atal et al., 1986), larval growth inhibitor (Thappa et al., 1989) and against malaria and vaginitis (Hager Handbuch, 1976).

Among 1000 alkaloids isolated from different members of Apocynaceae family, around 30 alkaloids have been isolated.
from bark of *Holarrhena antidysenterica* having highest percentage ofaconesine, a steroidal alkaloid.

The systematic screening of antimicrobial plant extracts represents a continuous effort to find new compounds with the potential to act against multi-resistant pathogenic bacteria. The accumulation of phytochemicals in the plant cell cultures has been studied for more than thirty years and generated knowledge had helped in realizing use of cell cultures for production of desired phytochemicals (Evans et al., 1986, Purohit and Mathur, 1999, Castello et al., 2002). The antimicrobial activity of Apocynaceae is well documented in the literature (Reddy, 2010; Amole and Ilori, 2010; Sharif et al., 2006; Suffredini et al., 2002; Patil and Ghosh, (2010). Objective of our present investigation is to generate callus tissue and also to evaluate the antimicrobial effects of callus tissue, seed and bark extracts of *Holarrhena antidysenterica*.

**MATERIALS AND METHODS**

**Plant Material**

The fresh matured seeds and bark of *Holarrhena antidysenterica* were collected from Charchi colliery forest of Hazaribagh (Jharkhand) after the identification work done by the authentic sources (University taxonomists and local vaidyas). Seeds collected from forest were also sown in garden for plant formation. Seeds as well as different plant parts – stem, node and roots of field grown as well as *in vitro* grown plantlets were used for explants. *In vivo* explants were washed thoroughly in tap water and treated with cetavlon (1:100) for 5 minutes. This was followed by washing with running tap water and then by distilled water. Surface sterilization was carried under aseptic condition in laminar air flow cabinet and treated with 0.2% HgCl₂ for 2-3 minutes then rinsed thrice thoroughly with sterile distilled water. Small segments of 1.0 – 1.5cm length of *in vitro* grown explants were excised from one month old axenic plants and made ready for inoculation.

**Culture medium and condition**

The basal medium used was MS medium (Murashige and Skoog, 1962) supplemented with 100mg l⁻¹ (w/v) myo-inositol and 3% (w/v) sucrose and different sets of growth regulators in different concentrations and combinations. Callus initiation, induction and proliferation medium was supplemented with 2,4-D (0.5 – 5.0 mgL⁻¹), BAP (1.0 – 3.0 mgL⁻¹) separately and 2,4-D (2.5 and 1.5 mgL⁻¹) in combination with different concentration of KN (0.5 and 1.0mgL⁻¹); BAP (1.0 – 3.0mgL⁻¹); NAA (0.5mgL⁻¹) and IAA (0.5mgL⁻¹). Callus formed in this medium was white and friable and was subcultured in 5-6 wks intervals on the same medium. The pH of all types of media was adjusted to 5.8 before the addition of 0.8% agar (BDH, India). All chemicals used were of analytical grade (Sigma and Merck). The culture vials containing of media were autoclaved at 121°C and 104 kPa for 20 minutes were kept in a growth cabinet at 25 ± 1°C, under 16h photoperiod by cool white fluorescent tubes (Philips India) and with 60-65% relative humidity.

**Methodology for antibacterial effect**

**Preparation of plant extracts**

Thoroughly washed dried bark and seeds of *Holarrhena antidysenterica* were dried in shade for 15 days and then powdered with the help of a water blender. Calli derived from seed, *in vitro* grown stem, node and root were collected and dried in an oven at 45 ± 1°C for 48-60h. Dried calli were homogenized to a fine powder and stored in airtight bottles. 10gm of calli powder were mixed with 100mL of Methanol. 25gm coarse powder of bark and seeds were weighed and extracted with 250mL of Methanol. The extract was collected after filtration by using Whatman No. 1 filter paper and then evaporated below 40°C in water bath (Parekh, J. et al, 2005; Mungole, A. J. et al, 2008). A sticky material was obtained as final product. 50 mg of crude plant extract (sticky material) was mixed with 50mL of methanol. Thus a stock solution containing 50mg/ 50mL was prepared which is considered as 100% concentrated extract. In this way 75%, 50% and 25% concentrated extract were prepared (Barriada-Pereira, M., et al., 2003).

**Test microorganisms**

*In vitro* antibacterial studies were carried out against two gram-positive (*Staphylococcus aureus, Salmonella typhimurium*) and Gram-negative (*Escherichia coli*) bacteria. The bacteria were obtained from Dept. of Microbiology, Rajendra Institute of Medical Sciences Ranchi. The bacterial nutrient medium was adjusted to pH 7.4 and sterilized by autoclaving at 15 lbs (121°C) for 15 minutes. All bacterial species were incubated for 36 hrs at 37 ± 2°C. After 36h incubation, the growing bacteria were used for further tests.

**Antimicrobial activity test**

The antimicrobial susceptibility testing was done by using the agar well method to detect the presence of antibacterial activities of the samples (Perez et al., 1990; Harborne, 1998). Microbial growth was determined by measuring the diameter of zones of inhibition measured in mm and the results were recorded.

**RESULTS AND DISCUSSION**

2, 4-D was the best auxin for inducing as well as for proliferating the callus from seeds during present work. On the other hand, a high concentration of 2, 4-D was found to be toxic as it produced low amounts of callus. The callus appeared white in colour, friable in texture and lacked the potentiality of regeneration. Other explants like stem root and nodal parts were also suitable for callus induction (Graph.1).

The nature of callus, its growth rate, texture as well as colour depend on the constitution of culture media as well as combinations and concentrations of various growth regulators. After every 4-6 weeks the callus was subcultured on the same medium where it grew indefinitely (Fig. 2) and in this way in the present investigation callus was maintained up to four subcultures. In few cultures globular shaped embryoids were observed on the surface of subcultured callus on the same medium (Fig. 3).

The crude methanolic bark, seed and callus extract obtained from *Holarrhena antidysenterica* were submitted to an antibacterial screening using the agar well method against various pathogenic bacteria. In this study significant zone of inhibition (in mm) was observed on three bacteria (*Staphylococcus aureus, 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 *Staphylococcus* (Fig. 2A) whereas in case of *Salmonella* and *E. coli* it was only 6.65mm and 2.7mm respectively. *Holarrhena antidysenterica* seed extract with 100% concentration also showed antibacterial activity against *Staphylococcus* with inhibition zone of about 7.05mm; *Salmonella* with 5.50mm and *E. coli* with 3.95mm. Callus extracts with 100% concentration showed 4 mm inhibitory zone against *Staphylococcus* and its least activity was observed in *E. coli* with 3.1mm inhibition zone even in 100% concentration.

This is the first report on comparative studies on different types of extracts of *Holarrhena antidysenterica*. Thus, the study ascertains 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 Chanda, 2006). In addition microorganisms show variable sensitivity to chemical substances to different resistance levels between different strains of bacteria (Cetin and Gurler, 1989).
Previously antibacterial activity of bark extract of Holarrhena antidysenterica was studied (Ballal et al., 2001; Raman et al., 2004; Chakraborty and Brantner, 1999) and investigated alkaloids present 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. Results obtained in the present study revealed that three types of extracts of Holarrhena antidysenterica possess potential antibacterial activity against Staphylococcus, Salmonella and E. coli. Till now it has not been proven whether this effect is due to a single alkaloid or due to the mixture of alkaloids present in the stem bark of Holarrhena antidysenterica. Moreover, it has been already demonstrated that conessine is the main alkaloid present in the mixture (Panda et al., 1991). These results supported the ethno medicinal claim that Holarrhena antidysenterica contains effective bioactive compounds mainly alkaloids against bacteria causing stomach ailments and three types of extracts of this plant may serve as a valuable source of compounds with therapeutic potential.

ACKNOWLEDGEMENTS

Authors are thankful to Dr. Manju Sinha, Director, Vocational Courses for providing laboratory facilities in Department of Biotechnology, Ranchi women’s college, Ranchi University, Ranchi.

REFERENCES


