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# ANTIBACTERIAL POTENTIAL OF THE HIMALAYAN ORCHIDS

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**Abstract**

The present paper describes and discusses the antibacterial potential of the Himalayan Orchids with reference to the nine species namely *Aerides multiflora* Roxb.,*Crepidium acuminatum* (D.Don) Szalch., *Dendrobium amoenum* Wall. ex Lindl., *Flickingeria macraei* (Lindl.) Seidenf., *Habenaria intermedia* D.Don, *Pholidota articulata* Lindl., *Rhynchostylis retusa* (L.) Blume, *Smitinandia micrantha* (Lindl.) Holttum, and *Vanda testacea* (Lindl.) Rchb.f. These orchids were also screened for their alkaloid test. Also, the test microorganisms (bacterial species) *Bacillus subtilis, Bacillus megaterium, Escherichia coli, Klebsiella pneumoniae, Micrococcus luteus, Salmonella typhi,* and *Staphylococcus aureus* are described for their biochemical, cultural characteristics, and pathogenicity.

## Introduction

ALTHOUGH THE therapeutic applications of orchids are discussed in the Atharvaveda, one of the four Vedas, (the oldest Sanskrit scripture) (Kaushik, 1985) and the post-vedic literature, no practical work has ever been done previously to explore the antibacterial/antimicrobial activities of the orchids in general or that on the Himalayan orchids or the other Indian orchids in particular (Gaur, 1999; Ghanaksh and Kaushik, 1999a, 1999b, 2007; Hegde and Ingalhalli, 1998; Kataki, 1986; Kaushik, 1983, 1985, 1988, 2009, 2013; Singh *et al.,* 2009).

The aim of the planned work was to examine the therapeutic orchids being used in Ayurvedic system of medicine for their antibacterial potential. These were also screened for presence of alkaloids. In the qualitative tests that were carried out, all the orchids studied for the presence of alkaloids showed positive results. The species studied for their antibacterial potential and presence of alkaloids were *Aerides multiflora* Roxb. (=*A. affine* Wall. ex Lindl.), *Crepidium acuminatum* (D.Don) Szalch. (=*Malaxis acuminata* D.Don., *Microstylis wallichi* Lindl.), *Dendrobium amoenum* Wall. ex Lindl., *Flickingeria macraei* (Lindl.) Seidenf., (=*Dendrobium macraei* Lindl.), *Habenaria intermedia* D.Don [=*Kryptostoma intermedium* (D.Don) Olszewski & Szlach.], *Pholidota articulata* Lindl. (=*P. griffithii* Hook.f.), *Rhynchostylis retusa* (L.) Blume (=*Epidendrum retusum* L.), *Smitinandia micrantha* (Lindl.) Holttum (=*Saccolobium micranthum* Lindl.), and *Vanda testacea* (Lindl.) Rchb.f. [(=*Vanda parviflora* (Lindl.)].

## Material and Methods

Several petriplates poured with the appropriate medium were incubated and the mean of the active inhibitory zone from edge to edge and from filter paper disc to the clear zone was recorded. However, in case of *Aerides multiflora* and *Rhynchostylis retusa,* well diffusion method was used instead of filter paper discs (Ghanaksh and Kaushik, 1999a, 1999b). In case of *Vanda testacea,* the active inhibitory zone of 1.25 times diluted alcoholic extract was measured 1.67 times, 2.5 times, and 5 times dilutions. But the undiluted alcoholic extract could not be measured. So, it was presumed that the inhibitory zone of undiluted extract of *V. testacea* would have certainly been a higher value than that of 1.25 times dilution of the extract of *V. testacea.*

As stated under the test microorganisms used, *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Micrococcus luteus, Salmonella typhi,* and *Staphylococcus aureus* were employed as test organisms. *Dendrobium amoenum* could not be tested against *Escherichia coli; Flickingeria macraei* against *Bacillus subtilis* and *Escherichia coli; Pholidota articulata* against *E. coli* and *Salmonella typhi; Smitinandia micrantha* against *E. coli, Staphylococcus aureus,* and *Salmonella typhi* could not be tested. But the studied species revealed their antibacterial potential against the bacteria studied by placing filter paper discs impregnated with different concentrations of alcoholic extracts and tetracycline and control discs of alcohol and water respectively, and incubating inoculated petriplates to develop the bacterial lawns.

*Test Microorganisms*

***Bacillus subtilis***

It is a type species (*Bacillus* of family Bacillaceae). It belongs to Group 18 'Endospore-forming Gram positive Rods and Cocci' of 9th edition of 'Bergey's Manual of Determinative Bacteriology' published in 1994 (Kaushik, 1996, 2000, 2009; Kaushik and Kaushik, 2018) which

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is equivalent to the part 15 of 'Endospore forming rods and cocci' according to 1974 edition of 'Bergey's Manual of Determinative Bacteriology' which was published two decades earlier to the 1994 edition. They are found in dust, milk, soil, water, and hay and often have been referred to as hay bacillus. They are Gram positive, rod shaped and 1.5-3.0 µm × 0.5-0.8 µm in size, spore forming, size of the spore 1.0-1.5 × 0.6-0.9 µm, oval in shape, motile by means of peritrichate flagella (flagella on entire surface of the cell). But the variant may be capsulated and non motile. Growth does not occur in anaerobic glucose broth. Nitrate is reduced. Casein and gelatin are hydrolysed. Germination is by lateral emergence of bacillary rods splitting spore case. Growth on nutrient agar at $37^0$C at pH 7.1. The colonies are rounded; irregularly spreading, 3.5 mm in diameter, slightly convex with a dark centre and lighter periphery, and the surface is finally granular.

*Pathogenicity*

Generally, all the species of *Bacillus* are not pathogenic but occasionally they cause serious infections in wounds, conjunctivitis, iridochroiditis (inflammation of both iris and choroid), and panopthalmitis (inflammation of all the tissues of the eyeball). It is fetal to mice when injected intraperitonealy; also produce haemorrhagic necrotic lesions in skin of rabbits. Sometimes outbreaks of food poisoning have been attributed to *Bacillus subtilis.* It is of industrial importance too as it can be used to produce bacitracin, a cyclic dodecapeptide antibiotic active against many Gram positive and certain Gram negative bacteria.

*Drug Susceptibility*

*B. subtilis*is susceptible to penicillin, tetracycline, and streptomycin.

***Bacillus megaterium***

The width of *B. megaterium* cell is about 1.5 µm, but in carbohydrate containing media the width may reach upto 3.0 µm. The spores are ellipsoidal, not distending the cell. It can utilize glucose and many other carbohydrates anaerobically.The cultures of *B. megaterium* were only used for *Aerides multiflora* and *Rhynchostylis retusa* antibacterial potential.

***Escherichia coli***

The *Escherichia* is a genus of Gram negative bacteria of Enterobacteriaceace. The genus was named after Escherich who was first to describe the colon bacterium under the name *Bacterium coli commune.* The old generic name is now obsolete since many years. It is normally found in human and animal intestine. Once *E. coli* passes out in faeces, it survives in the exterior environment for only some days. In the 9th edition of 'Bergey's Manual of Determinative Bacteriology' of 1994, it has been placed under Group 5 'Facultatively anaerobic Gram-negative rods' subgroup- I Family Enterobacteriaceace (Kaushik,1996, 2000, 2009; Kaushik and Kaushik, 2018). *E. coli* has been the principal guinea pig of the microbiologists and has been studied more extensively (Singleton and Sainsbury, 1988) than any other species. *E. coli* is a Gram negative, straight rod measuring 1-3 × 0.4-0.7 µm arranged singly or in pairs. It is peritricate (flagella are distributed uniformly over the surface of the cell) and motile. The capsule and fimbriae are met within many strains. The spores are never formed in *E. coli.*

*Cultural Characters*

The growth of the *E. coli* occurs aerobically and anaerobically on a wide range of media *e.g.* nutrient agar, EMB agar, and Mac Conkey's agar. The optimum temperature for its growth is $37^0$C but it can grow from 10 to $46^0$C. The colonies are large, thick on nutrient agar/$37^0$C/24 hr and are 1-3 mm in diameter, grayish white, moist, smooth, opaque or partially translucent discs. This description applies to *smooth* form seen on fresh isolation which is easily emulsifiable in saline. The *rough* forms give rise to colonies with irregular dull surface and often auto-agglutinable in saline. The strains isolated from pathological conditions are beta haemolytic on blood agar. The colonies of *E. coli* on Mac Conkey's medium are bright pink due to fermentation of lactose. The growth is largely inhibited on selective media such as DCA or SS agar used for the isolation of *Salmonella.* The growth of *E. coli* in broth is generally turbid with heavy deposits which disappears on shaking.

*Biochemical Factors*

*E. coli* ferments glucose, lactose, mannitol, maltose, and many other sugars and produces acids and gas. Typical strains do not ferment sucrose. Generally four biochemical tests are employed as indole, methyl red (MR), Voges Proskauer (VP), and citrate utilization test generally referred to as 1MViC. The *E. coli* is indole and MR positive and VP and citrate negative ($1MViC^{++--}$). It does not liquefy gelatin and no $H_2S$ is formed. Also, it does not split urea and does not grow on KCN medium.

*Toxin Production*

Some strains of *E. coli* are known to produce two types of exotoxins, enterotoxins, and three types of

haemolysins. The *E. coli* strain causing diarrhoea, is due to enterotoxins. Two types of enterotoxins have been identified, heat labile and heat stable. A strain of *E. coli* may either produce one or both type of enterotoxins. Enterotoxin production is under the genetic control of transmissible plasmid (ent. plasmid). The heat labile toxin (LT) is a large molecular weight ($5\times10^6$ D) protein which is inactivated by heating at $60^0$C in 10 minutes. It resembles the cholera enterotoxin antigenically as well as its mechanism of action in producing fluid accumulation in the intestinal lumen by stimulating the adenylcyclase cyclic adenosine monophosphate (cAMP) system. The heat stable (ST) toxin is a low molecular weight, non antigenic toxin which appears to stimulate fluid secretion in the gut through the mediation of cyclic guanosine monophosphate (cGMP). In the litigated ideal loop, ST induces fluid accumulation more rapidly than LT. *E. coli* is known to produce three types of haemolysins. The first type is heat labile toxin, separable from cells by filteration and is lethal for experimental animals. The second type is closely associated with the bacterial cells and cannot be separated by filteration. Third, the enteropathogenic *E. coli* strains of procine origin, produce an alpha haemolysin which is under control of transmissible plasmid.

*Pathogenicity*

The four main types of clinical syndromes caused by *E. coli* are:1, Diarrhoea; 2, Urinary tract infection; 3, Pyogenic infection, and 4, Septicaemia.

1. Diarrhoea

*E. coli* is a dominant bacteria of human intestine and is usually non-pathogenic in this location. There are three groups of *E. coli* which causes diarrheal disease particularly in infants and also in adolescent children and adults. These have been called enteropathogenic *E. coli*, enterotoxigenic *E. coli,* and enteroinvasive *E. coli.*

Enteropathogenic *Escherichia coli*

The diarrhoeal disease caused due to enteropathogenic *E. coli* (EPEC) has been observed throughout the world. EPEC causes several serious institutional outbreaks of diarrhoea in babies less than 18 month old. Pathogenic mechanism of EPEC diarrhoea is obscure. Essential feature of EPEC is colonization in duodenum, jejunum, and upper ileum by pathogenic strains. EPEC strains generally do not produce heat labile or heat stable enterotoxin. They also do not invade the tissues. The identified enterotoxin may have been probably responsible for fluid accumulation.

Enterotoxigenic *Escherichia coli*

They include the strains that form the heat labile or heat stable enterotoxins and cause diarrhoea. These are known to be the major cause of diarrheal illness in children of developing countries and are the main cause of diarrhoea in travellers. ETEC sometimes cause a disease indistinguishable from cholera in infants, young children, and adults.

Enteroinvasive *Escherichia coli*

Some strains of *E. coli* isolated from the stools of children and adults with dysentery like diseases do not produce enterotoxins, but invade the intestinal epithelial cells, as do dysentery *Bacilli*. These have been called as enteroinvasive *E.coli* (EIEC). Many outbreaks due to EIEC have been mistaken as shigellosis.

2. Urinary Tract Infections

*E. coli* and other coliforms account for the majority of naturally acquired urinary tract infections. Those acquired in hospitals following instrumentation, are usually caused by other bacteria such as *Pseudomonas* and *Proteus*. Infection may be precipitated by urinary obstructions due to prostatic enlargement and calculi ore pregnancy. About 5-7% of the pregnant women have been reported to have urinary infections without any symptoms. Such a symptomatic bacterium, undetected and untreated, may cause asymptomatic infection later in pregnancy, pylonephritis and hypertension in women, as well as prematurity and prenatal deaths of the foetus. While infections of lower urinary tract seems to be the ascending infections caused by faecal coliforms. Polynephritis is probably due to haematogenous infections.

3. Pyogenic Infections

*E. coli* may cause superficial infections such as wound infections and abscesses or deep infections such as peritonitis, cholecystitis, and meningitis. *E. coli* is a common cause of meningitis in new born infants but is much less in older patients.

4. Septicaemia

It has been often reported the *E. coli* and other coliforms and *Pseudomonas aeruginosa* have replaced *Staphylo cocci* as commonest cause of septicaemia in many hospitals. This syndrome of Gram negative septicaemia consists of fever, hypotension, and disseminated intravascular coagulation.

***Klebsiella pneumoniae***

*Klebsiella* is a genus of Gram negative bacteria of the Enterobacteriaceae. In 9$^{th}$ edition of 'Bergey's Manual

of Determinative Bacteriology' published in 1994 (Kaushik, 1996, 2000, 2009; Kaushik and Kaushik, 2018), it has been placed under Group 5 of 'Facultatively Anerobic Gram-Negative Rods'. *Klebsiella pneumoniae* formerly known as *Bacillus mucosus capsulatus* and frequently called Friedlander's bacillus as it was first isolated by Friedlander from vital cases of pneumonia in 1888. It is interesting that some strains of *K. pneumoniae* also carry on nitrogen fixation. They are small, non-motile, non-sporing, capsulated, Gram negative rods with rounded ends. The strains occur in the intestine and respiratory tract of man and animals and may be pathogenic causing several diseases, varying greatly in size, 0.6 to 2.0 µm in length and 0.32 to 1.5 µm in breadth. They usually occur in pairs but also singly and in short chains.

*Cultural Characteristics*

*Klebsiella pneumonia* grow on nutrient agar between $12^0$C to $43^0$C. The optimum temperature being $37^0$C. On nutrient agar medium with pH 7.2 organisms show grey, large, mucoid, convex, disc like or dome shaped colonies with varying degree of thickness. It shows no growth at $10^0$C. *K. pneumoniae* can be grown on selective media like Mac Conkey's agar and blood agar. *K. pneumoniae* ferments sugar (glucose, lactose, and mannitol) with production of acid and gas. It causes hydrolysis of urea and is able to synthesize all amino acids needed for their growth. *K. pneumoniae* can survive at room temperature for weeks and even for months. It gets destroyed if heated to a temperature of $65^0$C for one hour.

*Pathogenicity*

Only the capsular strains of *K. pneumoniae* are pathogenic. It may cause pneumonia, urinary tract infections, and pyogenic infections. The virulence of *Klebsiella* is due to their capsules. The capsules of *K. pneumonia* are composed of very fine fibrils. The capsules are commonly carbohydrates and contain only one monomer.

Pneumonia

Davidson in 1971 regarded pneumonia as a term used to describe inflammation of lungs. The pneumonia is of different types depending upon etiological agent. The pneumonia caused by *Klebsiella* is known as Friedlander's pneumonia. It occurs in middle age and older people who have been underlying medical problems such as alcoholism, chronic bronchopulmonary disease, and diabetes. The patients of pneumonia discharge a chocolate coloured, thick bloody sputum. The necrosis and abscess formation usually occur in the upper lobes.

Urinary Tract Infection

*Klebsiella* also causes urinary tract infections.

*Drug Susceptibility*

The property of resistance to drugs shown by *K. pneumonia* is due to high frequency of mutations. The strains of *Klebsiella* contain R factor which is responsible for the drug resistance as reported by Pyatkin in 1967. It also shows resistance to tetracycline. Benzylpenicillinis the drug of choice because bacterium is fully sensitive to it. Ampicillin is given for curing urinary tract infections. It is also important to note that medicines should always be taken under supervision of a qualified physician.

*Outstanding Features*

*K. pneumoniae* include strains, sometimes called *K. aerogenes* and *K. edwardsii.* It is indole negative, shows no growth at $10^0$C, melezitose negative, and pectate negative. Some strains carry out nitrogen fixation. Strains occur in the intestine and respiratory tract of man and animal and may be pathogenic causing bowine mastitis, equine metritis, ozaena, pneumonia, and nosocomial urinary tract infection. Gas is produced from lactose at $44.5^0$C and these are MR negative, VP positive, and urease positive.

***Micrococcus luteus*** *(= M. lysodeikticus= Sarcinapelagia)*

This is a yellow pigmented species of bacteria. Generally, it does not produce acid from glucose or mannose. Most strains are sensitive to lysosome and the bacterium is VP negative.

***Salmonella typhi***

*Salmonella* is a genus of Gram negative bacteria of the Enterobacteriaceae. In 'Bergey's Manual of Determinative Bacteriology' (1994, 9th edition, cf Kaushik, 1996, 2000, 2009; Kaushik and Kaushik, 2018). The genus *Salmonella* has been placed under group 5 of' Facultatively anerobic Gram-negative rods' and Sub group 1, Family Enterobacteriaceae. The *Salmonella typhi* was described by Eberth in 1880 as typhoid bacillus in tissue and was isolated by Gaffky (1884). Earlier, it was known as Eberth-gaffky bacillus or *Eberthella typhi* after its discoverer. Salmon Smith in 1885 discovered causative agent of hog cholera (*Salmonella cholerasuis)* which at present is known as *Salmonella typhi.* The generic name *Salmonella* was proposed by Theobald Smith and was accepted by International Committee. *Salmonella typhi* formerly also known as *S. typhosa.* The *Salmonella* are Gram negative rods 2-4 µm in length and 0.6 µm in breadth. They are

motile with peritrichate flagella. They do not form capsules or spores but have fimbriae. *Salmonella* from faeces can survive for weeks in water and for months in soil under favourable conditions but are normally killed by routine chlorination of water supplies, by pasteurization and many common disinfectants.

*Cultural Characteristics*

They are aerobes or facultative anaerobes. The temperature for their growth ranges from $15^0$C to $41^0$C. It grows well in nutrient agar medium. On Mac Conkey's agar, it is non lactose fermenter and colourless. The colonies are large, 2-3 mm in diameter, circular, low convexed, and smooth on nutrient agar. On Wilson and Blair's medium, colonies are first greenish and then develop a black center which enlarges to fill the entire colony after 24 to 48 hr incubation. The *S. typhi* ferments glucose, mannitol, and maltose forming acid but no gas. The thermal death of *S. typhi* is at about $56^0$C. They die within a few hours on drying. On moist soil, they can survive for six weeks or longer.

*Pathogenicity*

These are parasites of humans. The infection takes place due to consumption of dirty water, contaminated food *etc.* The infected person spreads the organism through urine and stool.

Typhoid Fever

Typhoid fever is an infection of lymphatic system and other tissue. The disease begins with the invasion of mucosal epithelium and rapid movement of the pathogens to lymphoid tissue associated with gastrointestinal tract. The invading pathogen multiplies in the lymphoid tissue, moves into the blood and spreads throughout the body. Blood culture remains positive for only a short period as the bacilli become localized in various types of tissue. The typical typhoid symptoms are headache, fever, malaria, spleen enlargement, and constipation resulting from necrosis of lymphoid tissue and liver. Necrosis of Peyer's patches (flat patches of lymphatic tissue situated in the small intestine but mainly in the ileum, they are the seat of infection in typhoid fever and also known as 'aggregated lymph nodules'. The term Peyer's patches is after Johann Conrad Peyer, Swiss anatomist) in the intestine produces haemorrhage. The larynx, gall bladder, and bone marrow may also be foci of infection. If the intestine wall becomes perforated, the infecting pathogens enter the peritoneal cavity, causing peritonitis, and death.

*Drug Susceptibility*

The *Salmonella* is susceptible to ampicillin or chloramphenicol. In 1972, it was seen in Mexico that some strains of *S. typhi* are resistant to ampicillin. The combination of trimethoprim-sulphamethoxazole proved effective against *Salmonella*. The bacterium shows preference for gall bladder and antibiotics are not effective in this organ. In such cases, removal of gallbladder is required.

***Staphylococcus aureus***

*Staphylococcus* is a genus of Gram positive, facultative anaerobic bacteria. In 'Bergey's Manual of Determinative Bacteriology' of 1994, 9th edition, the genus has been placed under the Group 5 'Facultatively Anaerobic Gram Negative Rods' Sub Group 1. Family Enterobacteriaceae (Kaushik, 1996, 2000, 2009; Kaushik and Kaushik, 2018).

It was Robert Koch who in 1878 observed micrococcus like organisms in pus. Pasteur, in 1880 cultured these cocci in liquid media. One year after in 1881 Orgaston, a surgeon found the bacterium being constantly present in the pus of acute and chronic abscesses. The microorganism was cultivated by him in eggs and he found it to be pathogenic. Julius and Rosenbach in 1884 made a thorough study of the microorganism. He also raised the pure culture of *Staphylococci* on solid media and categorized them on the basis of production of pigments.

*Morphology*

*Staphylococcus aureus* is spherical in shape, 0.8 to 1.0 µm in diameter. They are non capsulated, non-sporing, non-motile, arranged in cluster, and Gram positive. The formation of clusters is because cell division in the cell of this bacterium occurs in three planes, with daughter cells tending to remain in close proximity.

*Cultural Characteristics*

These are aerobes or facultative anaerobes and grow easily on nutrient agar. The optimum temperature for the growth of *S. aureus* is $37^0$C but range of temperature varies from $10^0$C to $40^0$C and optimum pH is 7.4 to 7.6. On nutrient agar, the colonies formed are rough, thick opaque, smooth, raised even confluent. They produce golden yellow pigment which develops best at room temperature. It produces uniform turbidity in Mac Conkey's broth. On nutrient agar, pigmentation is enhanced when 1% glycerol monoacetate or milk is incorporated in the medium. The pigment formed is lipoprotein. On blood agar, a wide zone of beta homolysis (formation of clear zone) is seen around the colonies. *S. aureus* ferments a large number of sugars which includes glucose, lactose, sucrose, maltose, and mannintol producing acid but no gas. Coagulase positive

strains ferment mannintol but gives negative test in case of non-pathogenic strains.

*Enzymes Produced*

*S. aureus* produces free coagulase, bound coagulase, hyaluronidase (spreading factor), and deoxyribonuclease.

1. Free coagulase

It is a filterable and heat labile enzyme and is antiphagocytic in action. It brings about clotting of human and rabbit plasma. It along with coagulase reacting factor present in the plasma converts fibrinogen into fibrin.

2. Bound coagulase

It is a heat stable protein which does not require coagulase reacting factor and fibrinogen does not get converted into fibrin. This is a component of the cell wall. This enzyme gets liberated upon autolysis of the cell.

3. Hyaluronidase

This enzyme as reported by Schwabaker *et al.*, 1945 is produced by 93.6% of coagulase positive strains. It was Duran Deynals who observed that invasive character of *S. aureus* is due to hyaluronidase which helps in spreading of infection.

4. Deoxyribonuclease

This enzyme is produced by coagulase positive strains.

*Pathogenicity*

*S. aureus* pathogenic strains are coagulase positive, manitol fermenter, cause beta hemolysis, produce golden yellow pigment, liquefy gelatin, and produce phosphatase. The bacterium produces following toxins. The haemolysins produced by it are alpha, beta, gamma, and delta. Alpha haemolysin brings about lysis in rabbit and sheep blood cells at $37^0$C in one hour. Gamma haemolysin causes rapid haemolysis of blood cells of human, rabit, and sheep. Alpha and delta haemolysin are produced by the strain found in human and cause human infections. Van de velde in 1894 demonstrated the production of toxin leucocidin which was found to be enough powerful to kill both human and rabbit leucocytes. Enterotoxin is another toxin which is responsible for *Staphylococcal* poisoning nausea, vomiting, and diarrhoea within six hr of taking contaminated food. It is heat stable but is antigenic and is neutralized by antitoxin. *S. aureus* has also been held responsible for skin infections, furunculosis (a condition due to affliction of boils), and sycosis (barbers itch). The furunculosis is a deep seated septic hair follicle in which the hair root is completely destroyed and comes out as the core of the boil. Multiple boils are given the name conglomeration of the boils. Sycosis is the chronic folliculitis due to *S. aureus* which affect the hairy zone of the body which can be beard region, neck, scalp, legs, arms, and pubic regions.

*Himalayan Orchids Used as Test Material*

*Aerides multiflora* Roxb., *Crepidium acuminatum* (D.Don) Szalch., *Dendrobium amoenum* Wall. ex Lindl., *Flickingeria macraei* (Lindl.) Seidenf., *Habenaria intermedia* D.Don, *Pholidota articulata* Lindl., *Rhynchostylis retusa* (L.) Blume*, Smitinandia micrantha* (Lindl.) Holttum, and *Vanda testacea* (Lindl.) Rchb.f. were all collected from the outer ranges of the Central Himalayas and were used as the test materials. The plant materials were crushed and the alcoholic extract of each orchid species was used for analysis. In all cases, fresh material was used except *Flickingeria macraei* and *Habenaria intermedia* which were taken as dried samples.

*Screening for Orchid Alkaloid*

All the species of orchids which were used as test material to study their antibacterial/antimicrobial potential were screened for the alkaloid test. The alcoholic extract of the orchids was tested for alkaloids using following reagents.

Dragendroff's Reagent

For this, 0.85 gm of Bismuth nitrate was mixed in 50 ml of acetic acid. The solution was named as X. Then 20 gm of KI was mixed in 50 ml of distilled water and named as solution Y. A drop of alcoholic extract of the particular orchid was placed on a clean slide and the colour was noted. One drop of each solution *i.e.* X and Y was added into the drop of the extract. It was noted that the spot became bright orange in colour which confirmed the presence of alkaloid.

Mayor Reagent Test

The alcoholic extract of the orchid was permitted to evaporate and the residue left after the evaporation of alcohol was taken up in water, filtered and acidified with 1% hydrochloric acid. The Mayor's reagent (Potassium iodide and mercuric chloride) or mercuric chloride iodide was added which formed precipitates.

Bouchardat's or Wagner's Reagent

To the alcoholic extract of orchid, Wagner's reagent or Bouchardat's reagent (Potassium iodide and Iodine) was added which gave dull coloured precipitates. The precipitation occured because the heavy metals

combine with alkaloids forming their salts which get precipitated.

Silicotungastic Acid Test

5% silicotungstic acid reagent (having silicon dioxide and tungsten trioxide in 5% tannic acid) was added to the alcoholic extract. This resulted in the formation of precipitates which confirmed the presence of alkaloid.

*Preparation of Alcoholic Extract*

As alkaloids are generally soluble in alcohol, so an alcoholic exude extract was prepared. For this, 100 gm of the plant drug material was washed with tap water and then with sterilized distilled water. The plant material was crushed in a mortar with a pestle or waring blender and mixed with 250 ml of alcohol. The mixture was covered and placed for 24-48 hr at normal room temperature. After 24 hr of alcoholic treatment, the extract was filtered through a muslin cloth and then refiltered through Whatman's filter paper 42. The filterate was concentrated by evaporation at room temperature till 80 ml of filterate remained. This concentrated extract was kept in screw tight-bottles, and was referred to as stock solution. Dilutions of the stock solutions were prepared using sterilized distilled water. Besides the undiluted stock solution, solutions were diluted 5 times, 2.5 times, 1.67 times, and 1.25 times. Sterilized paper discs were dipped into the solutions 30 minutes before placing these over petriplates.

*Growth Media*

Nutrient agar and Mac Conkey's agar were the media used. To prepare nutrient agar, the ingredients were beef extract (5 gm), peptone (5 gm), agar (15 gm), and distilled water (1000 ml). The pH was maintained at 7±1 after sterilization. Some microbiologists also add 0.5% Sodium chloride w/v (*i.e.* 5 gm per litre) to maintain osmotic pressure.The ingredients for the Mac Conkey's agar were peptone (20 gm), bile salts (5 gm), lactose (10 gm), neutral red (5 ml), agar (15 gm), and distilled water (1000 ml). The final pH was maintained at 7.4.

*Unit Solution of Tetracycline*

A unit solution of tetracycline was prepared for the comparison of antibacterial potential exhibited by the crude alcoholic extract of the orchids used as test material. For this, a tetracycline capsule 250 mg, of strength 2,50,000 was dissolved in 100 ml of sterilized distilled water, according to Pharmacopoea of India. Vol. II$^{nd}$, Q-Z Appendix. Ministry of Health and Family Welfare, Govt. of India. Controller of Publications, Delhi, page 88 (1985). 4 ml of this solution was taken and mixed with 96 ml of sterilized distilled water. Now 50 ml of the solution so prepared was made upto 100 ml by the addition of sterilized distilled water and finally 0.02 ml (one unit) solution was absorbed by a pair of paper discs of 5 mm diameter.

*Inoculation*

To each culture tube with 24 hr old bacterial culture, 3 ml of sterilized distilled water was poured and shook properly to make a homogenous solution of bacterial suspension. The medium already stored in the flasks (200 ml) was melted in the water bath and was shaken for complete homogenization of the medium. These flasks were then cooled upto 42-45$^0$C (above 42$^0$C).The bacterial suspension prepared was then added into the medium flasks and were shaken thoroughly to ensure the thorough distribution of the respective test organisms in the respective flask. The culture media inoculated with the inoculum was now transferred to the petriplates (25 ml of culture medium in each) and kept on a plane surface for solidification of medium into uniform thickness of 4 mm, the bottom of the petriplate was labelled with the name of the microorganism.

*Placing Impregnated Discs*

Kirbi-bauer disc diffusion test (Placement of Extract Soaked and Other Discs) also adopted by Kaushik (2003), Kaushik and Chauhan (2009), Kaushik and Kishore (1991), and Kaushik and Goyal (2011) was followed. The various dilutions of absolute alcohol and alcoholic orchid plant extracts *i.e.* 5 times, 2.5 times, and 1.25 times and undiluted alcoholic crude extract of the orchid plant were taken and the filter paper discs were dipped into the solutions, 30 minutes before their placing. The absorption capacity of a pair of paper discs is 0.02 ml which was measured separately. Generally, paper discs of diameter of 1.25 cm (1/2 inch) are recommended but for the present work, paper discs of diameter of 5 mm were taken for the sake of convenience. 5 pairs of filter paper discs were used for each petriplate; of these, 4 pairs of the discs were placed on the four corners of the petriplate and one pair was placed in the centre. The central pair was impregnated in alcoholic dilutions which served as control for the alcoholic extract of the orchid. Out of the 4 pairs of filter paper discs placed in the corners, one was for the alcoholic extract of the orchid (undiluted), one for the extract control (absolute alcohol), one for the antibiotic tetracycline (one unit solution), and one for antibiotic control (sterilized distilled water), and same was repeated for the solutions of different concentrations.

*Incubation*

The seeded petriplates with a uniform thickness of 4

ml medium were incubated at 37$^0$C for 24 to 48 hr on placing filter paper discs.

*Zone of Inhibition*

The zone of inhibition (diameter) was measured in millimetres. For this, the total diameter of the clear zone formed as a result of inhibitory effect of the alcoholic extract of the active principal or tetracycline or some other chemical impregnated in the filter paper disc, was measured to find out the effective zone of the inhibition the diameter of the filter paper disc was substracted from the total zone diameter of the inhibition.

## Results

11 species of the orchids were studied for the presence of alkaloids and their antibacterial potential. All of these gave positive test for alkaloids. Over 100 tables were prepared on the basis of observations recorded to measure the active inhibitory zone of undiluted alcoholic extract of the orchids and that of several dilutions against bacterial species. Inhibitory effect of one unit strength of the antibiotic oxy-tetracycline, which was used as control, was measured for the sake of comparison between two therapeutic agents. The list of observations made regarding active inhibitory zone against the bacterial species used as test microorganisms are given below.

***Aerides multiflora*** Roxb.

This is one of the two species of the orchids (the second species was *Rhynchostylis retusa*) where cups were used instead of filter paper discs. So, the exact comparison was a bit difficult but, no doubt, the antibacterial potential of both of these species was proved *in vitro*.

The undiluted alcoholic leaf extract produced inhibitory zones of diameter 4.67 mm, 4.03 mm, 5.50 mm, and 5.17 mm against *Bacillus megaterium, B. subtilis, Micrococcus lutea,* and *Staphylococcus aureus,* respectively and that for one unit strength of oxy-tetracycline (an antibiotic) turned out to be 20.40, 20.11, 20.50, and 20.20 in the same order of bacterial species. The inhibitory effect of oxy-tetracycline was 4.3 times, 4.9 times, 3.7 times, and 3.9 times, respectively.

***Crepidium acuminatum*** (D.Don) Szalch.

The undiluted alcoholic extract of *Crepidium acuminatum* showed the effective inhibitory zones of the diameter 3.30 mm, 1.97 mm, and 0.80 mm against *Klebsiella pneumoniae*, *Salmonella typhi,* and *Staphylococcus aureus,* respectively while one unit strength of tetracycline showed the inhibitory zones of the size of 9.3 (to 11.1) mm, 9.83 (to 12.1) mm, and 12.5 (to 11.5) mm diameter against the bacterial species in the aforesaid order which is about 2.81 times , 4.98 times, and 15.6 times more in comparison to the alcoholic extract of the pseudobulbs of *C. acuminatum .*

***Dendrobium amoenum*** Wall. ex Lindl.

The undiluted alcoholic shoot extract of *Dendrobium amoenum* against test organism *Bacillus subtilis, Klebsiella pneumoniae*, and *Staphylococcus aureus* produced effective zones of inhibition of diameter 2.10 mm, 2.04 mm, and 0.44 mm while one unit strength of tetracycline produced the inhibitory zones 11.33 mm, 11.23 mm, and 14.21 mm in the same order of the bacterial species. Also, the four aqueous dilutions of alcoholic extract of the aforesaid orchid species *i.e.* 5 times, 2.5 times, 1.67 times, and 1.25 times were used to study the antibacterial effect of the said dilutions against the aforesaid microorganisms. The least inhibitory effect of the undiluted extract was shown against *Staphylococcus aureus* but in the other two bacterial species, better and sharp zones were observed.

***Flickingeria macraei*** (Lindl.) Seidenf.

The undiluted alcoholic extract of shoot and pseudobulb of *Flickingeria macraei* showed active inhibitory zones of 2.6 mm, 3.1 mm, and 2.0 mm diameter against *Klebsiella pneumoniae*, *Salmonella typhi,* and *Staphylococcus aureus*, respectively. The inhibitory zones produced by the tetracycline of one unit strength was 11.1 mm, 11.5 (to 12.1) mm, and 12.1 (to 11.5) mm, respectively against the above said bacterial species in the same order. The effect of tetracycline was about 4.26 times, 3.70 times, and 6.05 times, respectively for the order of bacterial species. Several aqueous dilutions of the alcoholic extract of the shoot *i.e.* 1.2 times, 1.67 times, 2.5 times, and 5 times were also used which against*, Klebsiella pneumoniae* showed of 2.5 mm, 1.82 mm, 1.13 mm, 0.77 mm, and 0.46 mm diameter active inhibitory zones.

***Habenaria intermedia*** D.Don

The undiluted extract of root tubers of *Habenaria intermedia* produced effective inhibitory zones of diameter 4.75 mm, 4.3 mm, 5.1 mm, 2.7 mm, and 3.8 mm, respectively against the cultures of *Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae*, *Salmonella typhi,* and *Staphylococcus aureus,* while that of antibiotic tetracycline were 12.5 (to 11.7) mm, 14.0 mm, 14 mm (to 11.1) mm, and 14.5 (to 12.1) mm against the aforesaid bacterial species in the same sequence. The effective inhibitory zones produced by 1.25 times, 1.67

Table 1. Antibacterial potential of tetracycline and undiluted alcoholic extract of the orchids.

| Test organism | Effective zone of inhibition (diameter) in mm | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Tetracycline | *Aerides multiflora* | *Dendrobium amoenum* | *Flickingeria macraei* | *Habenaria intermedia* | *Crepidium acuminatum* | *Pholidota articulata* | *Rhynchostylis retusa* | *Smitinandia micrantha* | *Vanda testacea* |
| *Bacillus subtilis* | 11.7 | Cup was used instead of filter paper disc not comparable | 2.07 | - | 4.75 | - | 2.26 | Cup was used instead of filter paper disc | 1.5 | 2.45 |
| *Escherichia coli* | 14.0 | -do- | - | - | 4.3 | - | - | -do- | - | Above 3.4 |
| *Klebsiella pneumoniae* | 11.1 | -do- | 2.05 | 2.6 | 5.0 | 3.30 | 3.28 | -do- | 1.03 | Above 3.0 |
| *Salmonella typhi* | 11.5 | -do- | 1.22 | 3.1 | 2.7 | 1.93 | - | -do- | - | Above 2.4 |
| *Staphylococcus aureus* | 12.1 | -do- | 0.48 | 2.0 | 3.8 | 0.80 | 1.26 | -d0- | - | 2.45 |

times, and 5 times aqueous dilutions of the aforesaid alcoholic extract of the root tubers were 2.0 mm, 1.75 mm, 1.5 mm, and 1.3 mm, respectively against *Bacillus subtilis*; 3.75 mm, 3.2 mm, 2.9 mm, 3.7 mm, 3.2 mm, 2.15 mm, 1.95 mm, and 1.7 mm. The increasing dilutions show decreasing effectives zones of inhibition in all the five bacterial species with the dilutions in the same order.

***Pholidota articulata*** Lindl.

The alcoholic extract of the shoot of *Pholidota articulata* was tried for its growth inhibitory effect against test microorganisms *Bacillus subtilis, Klebsiella pneumoniae,* and *Staphylococcus aureus*. The diameter of effective inhibitory zones formed in case of undiluted extract was 2.25 mm, 2.23 mm, and 1.22 mm, respectively for the aforesaid bacterial species and that of tetracycline against the said microorganisms was 11.73 mm, 9.7 mm, and 14.37 mm, respectively. The efficacy of the extract was, however, noted to be directly proportional to its concentration. Higher the level of aqueous dilution lesser was the effective inhibitory zone diameter. It was 1.90 mm, 2.13 mm, and 1.90 mm for 80% alcoholic shoot extract diluted with water; and 1.25 mm, 0.60 mm, and 0.75 mm with 20% aqueous dilutions in the above shown order.

***Rhynchostylis retusa*** (L.) Blume

The inhibitory zone formed due to cups of undiluted alcoholic extract of *Rhynchostylis retusa* was 4.13 mm, 6.48 mm, 5.86 mm, and 4.40 mm in diameter against *Bacillus subtilis, Micrococcus lutea,* and *Staphylococcus aureus,* respectively and 18.73, 21.00, 20.80, and 19.30 due to oxy-tetracycline of one unit strength for the bacterial species in the same order. The oxy-tetracycline in comparison to the undiluted alcoholic leaf extract was 4.4 times, 3.2 times, 3.5 times, and 2.3 times, respectively for the bacterial species mentioned in the aforesaid order.

***Smitinandia micrantha*** (Lindl.) Holttum

The undiluted alcoholic extract of the leaf of this orchid showed inhibitory zones of diameter 1.53 mm, 1.03 mm, and nill against the test microorganisms *Bacillus subtilis, Klebsiella pneumoniae* and *Staphylococcus aureus,* respectively. 4 dilutions of alcoholic extract of leaf of this orchid *i.e.* 5 times, 2.5 times, 1.67 times, and 1.25 times were also tested. The inhibitory zones formed were of the diameter 0.80 mm, 0.94 mm, 1.10 mm, and 1.4 mm respectively for these dilutions against *Bacillus subtilis*; and 0.42 mm, 0.52 mm, 0.74 mm, and 0.81 mm respectively against *Klebsiella pneumonia* but no significant effect was noted against *Staphylococcus aureus.*

***Vanda testacea*** (Lindl.) Rchb.f.

The inhibitory zones formed by the filter paper discs impregnated with 1.25 times, 1.67 times, 2.5 times, and 5 times aqueous dilutions of alcoholic extract of the leaf were 2.35 mm, 2.15 mm, 1.95 mm, and 1.85 mm, respectively against the cultures of *Bacillus subtilis.* Against *Escherichia coli* such zones formed in the dilutions of the same order were 3.4 mm, 2.95 mm, 2.65 mm, and 2.55 mm, respectively. Against *Klebsiella pneumonia,* the inhibitory zones formed by such dilutions of alcoholic extract were 3.0 mm, 2.75 mm, 2.4 mm, and 2.25 mm, respectively. The inhibitory zones given by the aforesaid dilutions in the same order against *Salmonella typhi* were 2.4 mm, 2.2 mm, 1.9 mm, and 1.75 mm, respectively. Against *Staphylococcus aureus* the aforesaid dilutions produced effective inhibitory zones of diameter 2.4 mm, 2.25 mm, 2.1 mm, and 1.85 mm diameter while the undiluted extract showed the zone of 2.45 mm size *i.e.* almost of the size of that produced by 1.25 times aqueous dilutions.

## Discussion

The orchids are being used in Ayurvedic system of medicine since Vedic period. The Sanskrit literature is full of information on usage of orchids as therapeutic plants (Kaushik, 1983, 1985, 1988, 1996, Kaushik and Dhiman, 1997). Keeping all that in mind, an objective was aimed to explore the antibacterial potential of such therapeutic orchids and to screen them for the presence of alkaloids. All the orchids studied gave positive test for alkaloids. Their antibacterial potential against the bacterial species has been described. The undiluted alcoholic extract as well as the aqueous dilutions of the extract were tested for the effective inhibitory zones against the cultured bacterial species; *Bacillus megaterium*, *B. subtilis*, *Escherichia coli, Klebsiella pneumoniae*, *Micrococcus luteus*, *Salmonella typhi*, and *Staphylococcus aureus.* It was not possible to test all the orchids chosen against all the bacterial species mentioned here.

In *Vanda testacea* undiluted extract could not be tested, therefore, that has been considered above the first dilution *i.e.* 1.25 times. In case of *Aerides multiflora* and *Rhynchostylis retusa,* cup wells were dug in the petriplate instead of filter paper discs.

The results have been generalized in, and all the orchid species studied possess antibacterial potential indicated by the formation of effective inhibitory zones. The effects of *A. multiflora* and *R. retusa* alone were studied against *Bacillus megaterium* and *Micrococcus luteus*. Although based on the study of 9 species of

the orchids, this work most probably is the first attempt of its kind to prove *in vitro* antibacterial potential of this most fascinating group of the plant kingdom. Ghanaksh and Kaushik (2007) studied antibacterial potential of seven orchid species, which are *Aerides multiflora*, *Coelogyne gardneriana*, *Dendrobium farmeri*, *Luisia trichorhiza, Oberonia pachyrachis*, *Rhynchostylis retusa*, and *Vanda cristata* against 5 bacterial strains *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi,* and *Staphylococcus aureus*.

Very little is known about phytochemistry of the orchids. The cells of the orchids are rich in mucilage and require treatment with chloral hydrate, to remove it out (Kaushik, 1983) for structural studies. He has given two separate chapters on 'Orchids in Ancient Indian Literature' and 'Medicinal Value of the Orchids' in the monograph referred above. The orchids are being used in numbers of combinations as in Ashtavargchuran, Jeewanio Darhko Mahakshay, and Chywanprash Linetus perhaps because of the aforesaid characteristics of the orchids. Moreover, they are used singly also (Kaushik 1983, 2013). Kaushik *et al.* (2017) mentioned the use of orchids *Flickingeria macraei and Vanda rouxburghii* with *Argavadhiyadhyaya* group of plants, a chapter named after *Cassia fistula*, that comprise the 3rd chapter of Charak Samhita, where the orchids have been used to treat chest pain. Kaushik *et al.* (2017) makes a mention of message of Acharya Charak, the great physician of ancient India *A medicine in the hands of an incompetent physician becomes a poison, whilst a poison in the hands of a gifted physician becomes a powerful medicine*.

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