



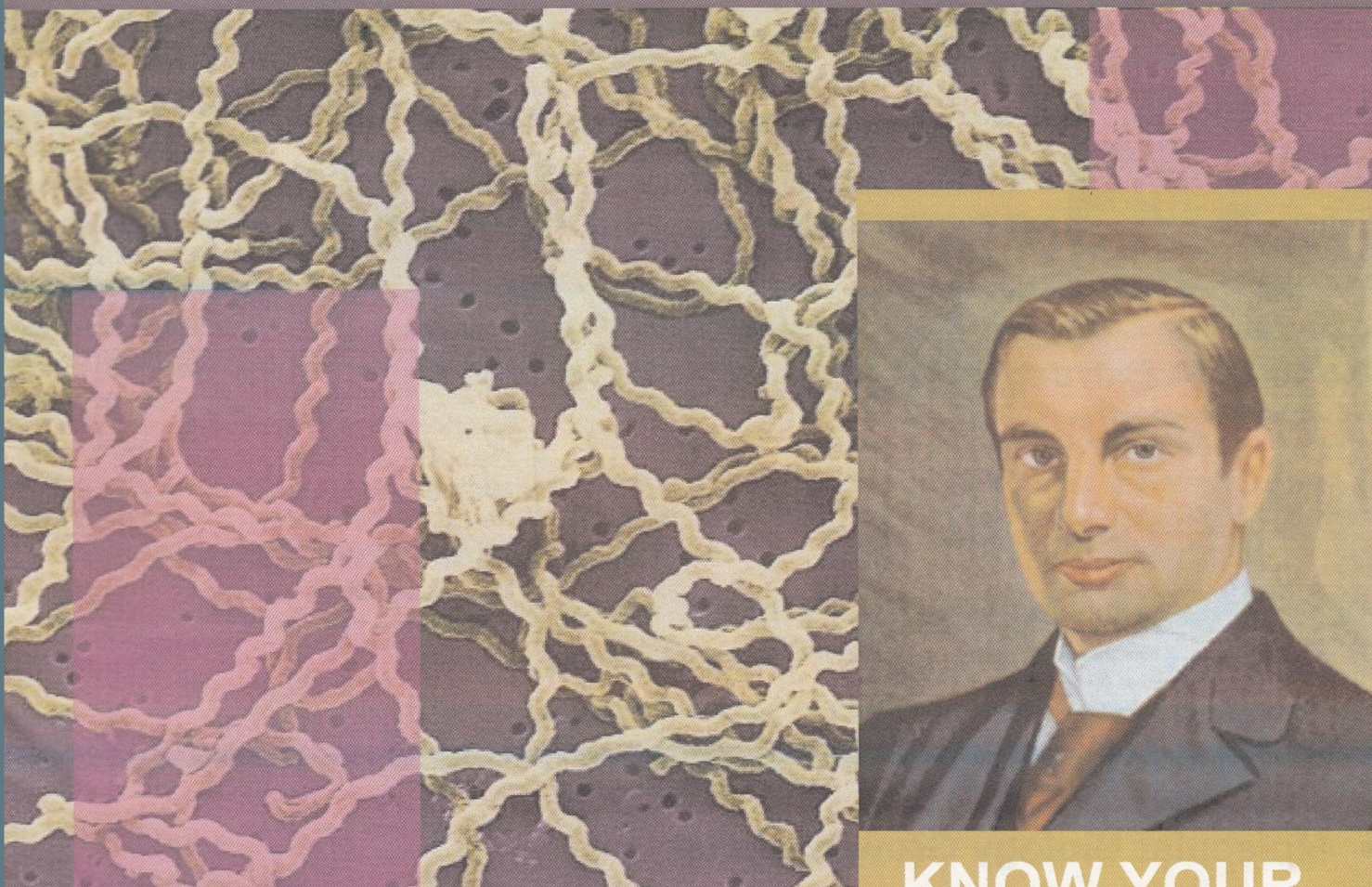
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BULLETIN

OF *Haffkine Institute*

Leptospirosis



**KNOW YOUR
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हाफकिन प्रशिक्षण, संशोधन व चाचणी संस्था

आचार्य दोंदे मार्ग, परळ, मुंबई - ४०० ०१२.

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Haffkine Institute for Training Research and Testing, Mumbai

Acharya Donde Marg, Parel, Mumbai - 400 012.

Tel: 022 - 24160947, 24160961, 24160962.

Fax : 022 - 24161787

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I, Dr. Abhay Chowdhary, hereby declare that the particulars given above are true to the best of my knowledge and belief.

Dated: 10 January 2011

(-Sd)
Dr. Abhay Chowdhary
Editor-in-Chief
Bulletin of Haffkine Institute &
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Towards a new era of the Bulletin of Haffkine Institute

Greetings from Haffkine institute for a happy, prosperous, healthy and infection free New Year.

It's my great pleasure and honour to serve the science community across the globe through this academic organ of Haffkine institute. Due to certain inadvertent circumstances, this publication was ceased in the year 1984. After a long hibernation, today, on the auspicious occasion of the 111th Haffkine day celebration, we are pleased to present before you our learned readers, this wonderful peer-reviewed journal in a brand new avatar.

One of the first few peer-reviewed scientific journals of the bygone 'Bombay' era, the Bulletin of Haffkine Institute is now open to a plethora of topics, from conventional to the state of art in the arena of Biomedical Sciences. It is expected to provide a platform of the young researchers to showcase current research. The bulletin also aims to share the space with the key opinion makers to enlighten the scientific community.

The inaugural issue talks about different methods and strategies to control pathogens: parasitic, viral and bacterial. These preliminary works when translated on a wider platform, may lead to a better diagnosis or a novel purity of research, through this current issue basically highlights the research works carried out at

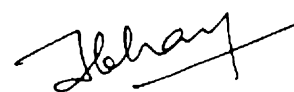
the Haffkine Institute. We are sure that in the coming times many of our fellow researchers carrying out research in different institutes will use this bulletin as an ideal platform to share their works. I take this opportunity to invite my fellow scientists from the corporate world, too, to partake in this scientific feast for the brain.

It is absolutely essential to remember all those who have contributed to bring this journal to life and thank all those who have supported us to re-launch this journal: be it scientific, financial and more importantly, moral support.

We look forward to your contributions in the form of research papers, case reports, brief communications as well as constructive communications, feedback and comments to make this journal an exemplary reading experience.

Hope you have a good time reading this journal!

Warm regards



Dr. Abhay Chowdhary
Editor-in-Chief

Ayurveda: Ancient yet Ageless

Prof. R D Lele[†]

"The Science of life shall never attain finality. Therefore, humility and relentless industry should characterize your every endeavour and approach to knowledge. The entire world consists of teachers for the wise and enemies for the fools. Therefore, knowledge, conducive to health, longevity, fame and excellence coming even from an unfamiliar source should be received, assimilated and utilized with earnestness". Charak Samhita Viman Sthan 8, 14.

These words of wisdom should continue to guide the 600,000 practitioners of Ayurveda in India today, as well as 400,000 practitioners of modern medicine who may not have familiarity with our glorious ancient scientific heritage. According to Ayurveda (Science of Life) - The aim of medicine is to promote health- "Swasthsya Swasthyarakshanam", increase immunity "Vyadhikshamatva" and to cure disease "Aturasya vyadhi Parimokshaya"

"The physician will try to cure the disease that are curable, adopt palliative measures in cases where palliation is the only remedy that can be offered, and give up a case which is beyond all medical treatment and mostly those which are more than a year's standing".

"There is in the world no substance that may not be used as medicine, in this or that manner for this or that purpose".

"Even an acute poison is converted into an excellent medicine, by the right method preparation and administration. While, even a good medicine may act as an acute poison of improperly administered.

"There is no substance which is absolutely of good or bad qualities. Hence, our concern should be to select such substances that possess more of the required good qualities".

Today, our understanding of health and disease has been profoundly extended by advances in molecular cell biology and recombinant DNA technology. We now appreciate that homeostasis is maintained among the 40 trillion cells in the human body through constant communication with each other through signalling molecules (proteins, peptides, amino acids, nucleotides, steroids, retinoids, eicosanoids and small molecules of diffusible and dissolved gases such as nitric oxide and carbon monoxide). All cells have receptors (on the cell membrane, in the cytoplasm, in the nuclear membrane) which recognize the signalling molecules, whose binding to the receptors trigger signal transduction to produce a specific response. Molecular recognition is a fundamental feature of all biological processes encompassing ligand-receptor, enzyme-substrate and antigen antibody reactions. A receptors is a protein to which a ligand or a drug binds to activate or suppress a signal. Thanks to recombinant DNA technology, most of the important signalling molecules and their receptors have been cloned and are now available for research using radioactive ligands. It is now possible to image the distribution and function of receptors on the living human body including the brain. The technique of whole body autoradiography microimaging in small animals, and PET studies in humans provide direct quantitative information about the distribution and site of action of drugs.

Molecular pharmacology provides a new interface

between Ayurveda and modern medicine. Based on empirical wisdom Charak, Shushruta and Vagbhata described 700 herbal drugs with their properties and clinical effects. Based on clinical effects, 50 categories of drugs have been described- such as appetizers, digestive stimulants, laxatives, anti-diarrheal, anti-haemorrhoid, anti-emetic, anti-pyretic, anti-inflammatory, anti-puritic, anti-asthmatic, anti-epileptic, anti-helminthic, haemopoietic, haemostatic, analgesic, sedative, promoters of life, promoters of strength, complexion, voice, semen and sperm, breast milk secretion, fractures and wound healing, destroyers of kidney stones etc. Using modern techniques, these categories of Ayurvedic drugs could provide novel molecular probes. It is now possible to explore the mechanism of action of ayurvedic drugs in terms of current concepts of molecular pharmacology.

Radiolabeling of herbal drugs:

On 25th May 2009 in my Homi Bhabha Birthday Centennial lecture at BARC in Mumbai, I pleaded the urgent need for application of radiotracer technology for the validation of Ayurvedic Herbal drugs. Every new molecule introduced in modern therapeutics is first labelled with C-14 to study its absorption, bio distribution & excretion in small animals. The only tritium labelling effort of a Chinese herbal drug Gen Sing was published in French in 1992. Haffkine Institute's new project with financial support from DST and DBT has been launched in collaboration with BRIT (Board of Research in Isotope Technology). Tritium-labeling of Arjun Sal an important Ayurvedic drug has been successfully accomplished and its bio-distribution in small animals is being studied. Labeling Tulsi leaves with C-14 has also been achieved. Feeding C-14 urea as a manure in plants will be used as a novel approach for radiolabeling of various parts of the plant- bark, leaves, flowers etc. and the C-14 radio-labelled plant parts will be fed to small animals and whole body autoradiography will be performed to study the bio-distribution of the herbal drugs. To begin with, forty single drugs described by Vagbhata will be taken for study. Details on autoradiography will be found in Reference 1.

Medhya Rasayanas:

Ayurveda has described 10 herbal drugs as Medhya Rasayanas- Amalaki, Ashwagandha, Bramhi, Jatamansi, Jyotishmati, Kavach Bcej, Mandukparni, Shankhapushpi, Vacha, Yashtimadhu.

A transgenic mouse model of Alzheimer's disease has now become commercially available. At birth these animals are absolutely normal. Within three months they develop all the changes – amyloid plaque deposition amyloid angiopathy. Tau protein, loss of acetylcholine

[†] (affiliation)

neurons, hypometabolism and hypoperfusion in parieto-occipital regions etc. and the animals die within the next 6 months.

All these changes can be non-invasively shown by small animal PET/CT with optical imaging without sacrificing the animals. This facility is now available at ACTREC, new Mumbai where a collaborative research project of Haffkine Institute is approved for studying the effect of the 10 medhya rasayans- single and in combinations in 40 mice: first 3 months to assess the preventive potential and next 6 months to assess curative potential. At present there is no effective treatment for Alzheimer's disease yet \$ 15 billion are spent annually on its treatment. If this study provides validation of Medhya Rasayans for prevention of Alzheimer's disease, a world market of \$ 15 billion will be available to India. Details on small animal PET will be found in Reference 1.

Bioavailability of Ayurvedic herbal drug:

Bioavailability of Ayurvedic herbal drugs is a totally neglected subject. Devasagayam's group at BARC used the inverted loop of rat intestine to study the intestinal absorption of *Terminalia Arjuna* extracts as well as the active principle Baicalein. Almost 15% of the baicalein (4 mg/ mL) was recovered from the serosal surface as monitored by HPCL. Both aqueous and methanolic extracts of *T. Arjuna* were absorbed. Haffkine Institute is establishing this facility where 40 single herbs described by Vagbhata will be studied for absorption: (duodenum, jejunum, ileum, colon). This "blind spot" in herbal drug research is frustrating for clinicians who wish to translate the laboratory in vitro data to clinical application. The poor bioavailability of oral curcumin and resveratrol are important illustrative examples.

High through-put screening for mechanism of action:

In my Haffkine Oration 2009, I have listed over 90 classes of receptors, transporters, ion channels and intracellular signalling molecules into component subtypes and subgroups, selective agonists, selective antagonists and specific radioligands of choice for mechanism based screening of Ayurvedic drugs.

As an illustrative examples Sukh Dev (1992) in collaboration with a group in USA studied *Triphala*^[2]. Using I-125 cholecystokinin (CCK) as ligand and mouse pancreatic membrane as receptor they showed affinity of three Ayurvedic herbal extracts – *Terminalia Chebula* (96% ligand displacement) *Terminalia bellerica* (91%) and *emblica officinalis* (76%) showing that "Triphala" constituents act on CCK receptors. It is surprising that in the last two decades no further efforts were made in this direction. Haffkine Institute has launched a DBT – supported project of mechanisms-based screening of the 40 single drugs described by Vagbhata.

Charak States: "A single drug may have many appellations owing to its diverse actions just as a man is able to perform various actions". Many popular Ayurvedic drugs such as *Ashwagandha*, *Bramhi*, *Guduchi*, *Katuka*, *Shatavari* etc. have multifarious properties ascribed to them. Obviously their molecular targets are shared by many cell systems and cell membrane components such as phospholipase A2, phospholipase C, Adenylyl Cyclase and cAMP adenosine

receptors, eicosanoids, ion channels and neuroreceptors Dopamine, Serotonin, NE, GABA etc. SAPK2 (stress-activated protein kinase) is an enzyme highly activated by bacterial lipopolysaccharides and cytokines. Many Ayurvedic Rasayan drug act by blocking this enzyme and prevent down stream activation of NFkB. Dahanukar and Thatte made pioneering contribution by showing immunomodulatory action of *Amlaki*, *Ashwagandha*, *Guduchi*, *Haritaki*, *Pipalli* and *Shatavari*, all of which are now shown to suppress NFkB activation, and regulate chronic dysregulated NFkB pathway.

The Ayurvedic concept of Disease as Dissonance due to Atiyoga (excessive interaction), Ayoga (absent interaction) and Mithya yoga (erroneous interaction) between signalling molecules and their receptors is a great stimulus for research in system Biology.

Chemoprevention: future approach in Medicine:

Chemoprevention of infection (Tuberculosis, viral infection including HIV), malignancy, neurodegenerative disorders (Alzheimer's, Parkinson's disease) metabolic syndrome (diabetes, hypertension, atherosclerosis) should be the major focus of future Ayurvedic drug research. Oxidative stress, due to Reactive Oxygen species (ROS) and reactive nitrogen species (RNS) is normally taken care of by natural protective mechanisms (superoxide dismutase SOD, catalase, glutathione peroxidase, glutathione (GsH) and anti-oxidants : Vit E (tocopherol and tocotrienols), Vit. C, Vit. A (Carotinoids) and flavonoids. This protective mechanism is overwhelmed in many disease processes characterized by excessive oxidative stress. Devasagayam's group at BARC have shown the effectiveness of 15 Ayurvedic anti-oxidants at various levels- prevention of radical formation scavenging of primary and secondary radical, breaking chain initiation & propagation, repair of lipid membrane and repair of DNA and other cellular constituents^[3].

By similar scientific validation of all other Ayurvedic drugs, apart from health benefits, a large share of global drug market will flow to India. Current global herbal market of US \$ 70 billion is growing annually at 10–15%, Global nutraceutical market is US \$ 142 billion.

What we need is vision and imaginative thinking and swift action as outlined, in the four Haffkine Research Projects to capture our share in this huge market.

✉ – CR

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Leptospirosis: A Re-Emerging Disease

Deepak Y Patil^{1*} and Ranjana A Deshmukh¹

ABSTRACT

A number of leptospirosis outbreaks have occurred in the past few years in various places such as Nicaragua, Brazil and India. It is a direct zoonotic disease caused by spirochetes belonging to different pathogenic species of the genus *Leptospira*. Human infection results from accidental contact with carrier animals or environment contaminated with leptospires. Majority of leptospiral infections are either sub clinical or result in very mild illness and recover without any complications. Only a small proportion of cases develop severe leptospirosis. The disease has also been a diagnostic enigma for the microbiology laboratories across the country. Since isolation rate of the causative organism from clinical specimens is low, serological techniques remain the cornerstone of diagnostics. Although the basic principles of prevention such as source reduction, environmental sanitation, more hygienic personal practices, chemoprophylaxis and immunization etc., are same everywhere, there is no universal control method applicable to all epidemiological settings. Hence, there is a need to develop a comprehensive strategy to combat this emerging public health threat. A comprehensive overview of the disease is hereby presented and discussed.

Keywords: Leptospirosis, Nicaragua, Brazil, India, *Leptospira*

INTRODUCTION

The power of water has been highlighted over the past year, following the Boxing Day tsunami and the devastation wreaked on New Orleans by hurricane Katrina. However, water can represent a danger in other ways; for example, in the transmission of Leptospirosis.”

~Richard A Collins^[1]

Leptospirosis has been recognized as an important emerging global public health problem because of its epidemic proportions and increasing incidence in both developing and developed countries^[2]. Due to its nonspecific symptoms that mimic better-known diseases, leptospirosis has been frequently under-diagnosed and under-reported. However, with better reporting and improved diagnostic techniques, increasing cases of leptospirosis are being detected; and leptospirosis is now considered as an emerging disease^[3]. Leptospirosis, the infection caused by leptospires, is the most widespread zoonosis in the world. It has wide geographical distribution and occurs in tropical, subtropical and temperate zones and may be prevalent in both urban and rural settings. Annual incidence is estimated from 0.1–1 per 100000 in temperate climates to 10–100 per 100000 in humid tropics. A disease incidence of more than 100 per 100000 is encountered during outbreaks in high risk groups. Worldwide prevalence rates remain underestimated, most likely as result of the protean nature of the disease^[4].

ETIOLOGY

Leptospirosis is primarily a contagious disease of animals, occasionally infect humans and is caused by pathogenic spirochete of the genus *leptospira* that traditionally consist of two species *L. interrogans* and *L. biflexa*. The former includes all pathogenic serovars and the latter includes the saprophytic strains. *Leptospira* strains have been divided into 23 sub-groups of which 2 belong to saprophytic leptospires. Each sub-group consists of several strains designated as serovars. More than 260 host adopted leptospiral serovars are naturally carried

by more than a dozen species of rodents, wild and domestic animals in moderate to highly conducive abundantly available variety of hosts, resulting in very successful perpetuation of this organism^[5].

EPIDEMIOLOGY

Leptospirosis has a wide geographical distribution and occurs in tropical, subtropical and temperate climatic zones. In the developed world, the incidence has come down substantially and most cases that occur now are associated with recreational exposure to contaminated water. In contrast, the incidence seems to be increasing in developing countries. Some countries such as Thailand, where leptospirosis is under surveillance, have recorded an increase in the incidence of leptospirosis. Most countries in the South East Asia region are endemic to leptospirosis^[6]. The International Leptospirosis Society made an attempt to compile data on occurrence of leptospirosis in various countries. The data shows that on an average 10,000 severe cases requiring hospitalization occur annually all over the world. Vast majority of leptospiral infections are either subclinical or result in very mild illness and the patients may not seek medical assistance. In a small proportion of patients, severe complications may set in. In such cases, there would be clinical manifestations of multiple organ involvement and the case fatality ratio could be more than 40%. A number of leptospirosis outbreaks have occurred during the past few years in various places such as Nicaragua, Salvador and Rio de Janeiro in Brazil and Orissa, Mumbai and Andaman Islands in India. Outbreaks usually occur on the aftermath of natural calamities such as cyclones, floods and flash-floods. However, in many instances, the actual numbers may be underestimated due to underreporting of leptospirosis cases during times of severe flooding from natural disasters for example, hurricanes) For example, natural disasters including monsoons and floods increase the risk of exposure to leptospires through contact with contaminated water and mud. Leptospirosis is currently identified as a worldwide public health problem. In endemic areas, leptospirosis is a major cause of various clinical syndromes such

¹Nodal Laboratory for Re-emerging Diseases, Haffkine Institute, Mumbai, India; *Corresponding Author

as jaundice, renal failure, myocarditis and atypical pneumonia. A multi-centric study in India showed that leptospirosis accounts for about 12.7% of cases of acute febrile illness attending hospitals. Leptospirosis is the cause of a significant proportion of cases of non-hepatitis A–E jaundice, non-malarial febrile illnesses and non-dengue haemorrhagic fever in South East Asian countries. In many tropical endemic areas, a significant proportion of the population is exposed to leptospires. High seroprevalence rate is found among healthy population of many endemic areas such as Andaman Islands, Seychelles Island, rural areas around Chennai etc. High seroprevalence has also been observed among some of the primitive tribes living in the jungles. Even in subtropical and inter tropical areas, seroprevalence could be high among certain occupational groups such as farmers^[7].

ANIMAL RESERVOIRS OF LEPTOSPIRES

Although, leptospirosis is typically a zoonotic disease, leptospires can also infect humans. These bacteria can infect and colonize a wide variety of wild and domestic animals. However, the rat is typically the reservoir of leptospires. Certain serovars of leptospires are typically associated with particular animal species; however, other leptospiral serovars which do not normally reside in a particular animal can produce very severe disease in that animal^[8]. For example, the serovars Canicola, Icterohemorrhagiae, Grippityphosa, and Pomona commonly replicate in dogs. The serovars Hardjobovis, Pomona, Grippityphosa, and Icterohemorrhagiae frequently replicate in cattle and deer^[9]. Serovar Hardjo infection is usually less severe than serovar Pomona in cattle because serovar Pomona is more adapted to pigs. Pigs are frequently host animals for the serovars Pomona, Bratislava, Canicola, Tarassovi, and Icterohemorrhagiae. Pigs can excrete very large amounts of leptospires in the carrier state (i.e., chronic leptospiral colonization of the renal tubules) and can be important source of human infection. Rats and mice are commonly colonized by the serovars Ballum and Icterohemorrhagiae. These serovars can be problematic for humans dealing with rodent infestations. Sheep can be hosts for the

serovars Pomona, Grippityphosa, Bratislava, and Hardjo. Approximately 15–20% of abortions in sheep and goats are due to leptospiral infection. Finally, horses can be the host animals for the serovars Pomona, Bratislava, Canicola, Icterohemorrhagiae, and Sejroe. Horses are rarely infected by leptospires and the disease is usually mild^[3].

RISK GROUPS AND RISK FACTORS

Occupational and recreational exposures account for 30–50% of leptospirosis cases. Typically, individuals who work outdoors or with animals are at risk of the disease. Previously, most cases were due to occupational exposure. However, more cases are occurring due to recreational exposure; although, occupational exposure is still a significant concern^[8]. Prime examples of high-risk occupational groups include pet shop owners, veterinarians, veterinary technicians, agricultural workers, abattoir workers, plumbers, meat handlers, meat inspectors, hunters, laboratory staff, butchers, herders, meat carriers, coal miners, construction workers, fish industry workers, military personnel, civil emergency personnel, sewer workers, and garbage collectors.^[8, 10]

As for recreational exposures, high-risk recreational exposures for leptospirosis include adventure traveling to the tropics, cave explorers, camping, hiking and riding trail-bikes through contaminated puddles, swimming, canoeing and kayaking, fishing, water skiing and windsurfing, and other outdoor sports played in potentially infected water^[3]. In addition, urban dwellers are also at increased risk because of potential exposure to infected rats. Also, children can be at risk by playing in contaminated water or having contact with infected animals.

TRANSMISSION OF LEPTOSPIRA

Indirect transmission: It can occur through contact with soil, water, food or bedding that has been contaminated with leptospires, usually from the urine of infected wildlife. Leptospires are adept at burrowing through tissues, therefore transmission can even occur through intact skin that is soft or macerated from moisture, although it is more likely to occur through broken skin or a mucous membrane. Dogs

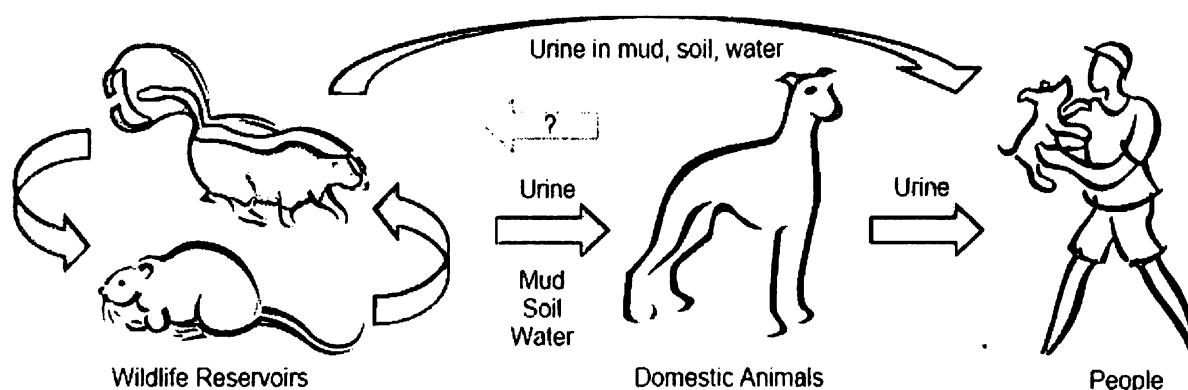


Figure 1: Transmission of Leptospirosis ^[11]

can become infected from simply walking through a puddle in which an infected skunk or raccoon has urinated. Humans can become infected from swimming in similarly contaminated lakes or ponds.

Direct transmission: It involves skin or mucous membrane contact with urine, venereal discharge or tissues from an infected animal. Because people have such close contact with their pets, this is an important mode of transmission from these animals, although in general it is less common than indirect transmission. The bacteria can occasionally be transmitted by bites, although leptospires are generally not shed in saliva^[11].

PATHOGENESIS

Once leptospires penetrate the skin or mucous membranes of a host, they enter the bloodstream and begin to multiply rapidly. The bacteria then invade other tissues including the kidney, liver, spleen, central nervous system, eyes and genital tract, and continue to multiply. Following development of a humoral immune response, the organisms are cleared from most tissues, but frequently persist in the proximal renal tubules where they are inaccessible to serum antibodies. The bacteria can survive and replicate in this location for weeks or even years in a reservoir host, resulting in leptospiuria.

The mechanisms by which leptospires exert their damaging effects are unclear. The bacteria cause severe vasculitis and endothelial damage, which can lead to coagulation disorders. Damage to the renal tubules commonly leads to renal insufficiency or failure. It is suspected that hepatic necrosis is initially caused by interference with cellular enzyme systems. Acute or chronic recurrent uveitis may occur due to the presence of leptospires in the aqueous humour and/or cross-reactivity of leptospire antibodies with ocular tissues. Although leptospirosis is a relatively commonly reported cause of abortion in cattle and swine, reproductive problems associated with leptospirosis are not commonly reported in other species, including humans, but they can occur^[11].

ANTIGENS AND IMMUNE RESPONSE

Leptospires have a complex antigenic structure. The somatic antigen is genus specific. The surface antigen is a polysaccharide and is serovar specific. The outer membrane is a potent immunogen—lipopolysaccharide in nature. It is the major antigen and the target of antibody and complement-mediated bactericidal activity. Antibodies directed against it are protective in nature. Flagellar antigen is composed of genus and serotype specific antigens. Some serovars, e.g. *L. Icterohaemorrhagiae*, have an additional Vi antigen associated with virulence. The immunological response to leptospires is both humoral and cell mediated; after the entry of the organism into the host, both the B- and T-cell dependent areas

are stimulated. The initial elimination is done by phagocytosis. Most of the leptospires are digested in the vacuoles of macrophages. The phagocytic activity of the polymorphonuclear cells is enhanced by opsonizing antibodies. Cell-mediated immunity plays a role in preventing renal localization. The antibody response is classical, with peak IgM levels appearing first, quickly followed by IgG antibodies, which persist longer than IgM. High IgM levels can be observed during the first two months of the disease. IgG response in leptospirosis is often erratic and occasionally is not detected. Heterologous, i.e. genus-specific, antibodies appear first but decline faster; homologous, i.e. serovar specific; antibodies appear later and persist longer. Recovery from infection is possible after the appearance of lytic and opsonic antibodies and phagocytic clearance of leptospires from blood and tissues^[6].

SYMPTOMS OF LEPTOSPIROSIS

In humans, the incubation period of leptospirosis ranges from 2 to 21 days with a mean of 10 days. *Leptospira* infections can be asymptomatic or symptomatic depending on host susceptibility and the serovar involved. Of the symptomatic illnesses, 90% are self-limited, flu-like illnesses, but 5–10% of infections can result in multi-organ damage, including liver failure, renal failure, and hemorrhagic pneumonitis. Mortality among those with severe disease can range from 5–25%. The classic clinical syndrome has two phases: a septicemic and an immune phase. It is in the immune phase that organ-specific damage and more severe illness is seen. The typical presenting signs of leptospirosis in humans are fever, headache, chills, conjunctival suffusion, and myalgia (particularly in calf and lumbar areas). Less common signs include a biphasic fever, meningitis, photosensitivity, rash, and hepatic or renal failure^[12].

DIFFERENTIAL DIAGNOSIS OF LEPTOSPIROSIS

Because leptospirosis has nonspecific symptoms that can mimic more familiar diseases, a careful case history needs to be taken. Special attention to occupational and/or recreational exposures to potentially contaminated animals and environments should be considered^[3].

LABORATORY DIAGNOSIS OF LEPTOSPIROSIS

Laboratory diagnosis is broadly divided into two categories: those that give direct evidences and those that give indirect evidences. The direct evidences include either demonstration of leptospires or its DNA and isolation of organism from clinical specimens. Detection of specific antibodies to leptospires (serological diagnosis) is indirect evidence^[7]. Different techniques for laboratory diagnosis of leptospirosis are shown in Figure 2

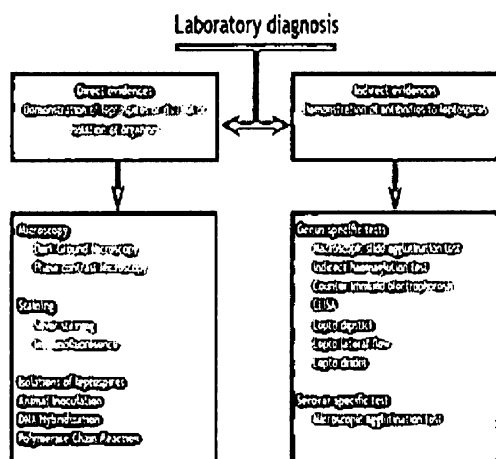


Figure 2: Laboratory diagnosis of leptospirosis ^[7]

LABORATORY SAFETY

Standard laboratory safety procedures are necessary to work with leptospires. The main concern with leptospires is accidents which may damage the skin (cuts, abrasions, punctures, and bites) and expose the eyes. Leptospires can be destroyed by desiccation, acid, phenolic compounds, disinfectants and antiseptics, and heat. Prophylactic antibiotics should be given if there is a possible exposure. Because of potential exposures to other infectious agents, laboratory workers should be vaccinated against hepatitis B and rabies^[3].

TREATMENT

Leptospirosis in all its forms is amenable to treatment with antibiotics. Leptospires are susceptible in laboratory tests to all clinically useful antibiotics, except chloramphenicol and rifampicin. Leptospires are not usually cultured and tested for susceptibility in individual cases. Resistance in clinical use has not been reported, although failures of treatment have rarely been suggested. The antibiotics most usually recommended are penicillin, at high doses, unless the patient is hypersensitive to penicillin, in which case erythromycin is used. Tetracycline is used but has disadvantages and is contraindicated for people with renal insufficiency, for children and for pregnant women. Doxycycline is recommended for treatment and short-term chemoprophylaxis. Penicillin should be administered as early as possible, during the leptospiraemic phase, parenterally in very ill patients. No antibiotic can reverse the destructive effects of leptospirosis in tissues and organs, but penicillin was found to have beneficial effects, reducing mortality and duration of illness in severe leptospirosis, when given intravenously, even at a late stage. Antibiotic treatment may be accompanied by a Jarish-Herxheimer reaction (a transient increase in fever and severity of symptoms immediately following treatment), which does not contraindicate administration of antibiotics^[6].

PREVENTION AND CONTROL

Prevention of leptospirosis in all situations is not

possible, because it is widespread in so many animals and places all over the world. The best that can be done is to limit the effects of leptospirosis on humans and the animals they depend on. This involves identification of sources, containing them and eliminating them or their effects. Indications that leptospirosis occurs comes from correct diagnosis, which depends on laboratory expertise and quality assurance. Hospital, clinical or practitioner records can point to known or suspected leptospirosis. Clustering of cases can suggest a common source. The prevalent serovars can likewise suggest animal reservoirs. A history of contact with animals or with surface waters during occupational or recreational travel or pursuits is also important.

The best way to avoid leptospirosis is to keep away from animals and areas that may be contaminated by their urine. People whose occupation, travel or hobbies involve risks should know of the disease and how to avoid it. The main groups at risk are dairy farmers, slaughter-house workers, meat inspectors, veterinarians and meat carriers in food industries; people who work habitually in wet occupations (rice farmers, sugar cane harvesters, drainers, sewer workers, miners); adventure travelers (cave exploration, white water rafting, water sports) and military or civil emergency personnel. People should be aware of the dangers and be dissuaded from swimming in rivers or pools suspected to be contaminated. Education through industry or community self help groups can raise awareness and prevent infection in humans and the animals that they keep.

Even in urban centers, civil emergencies that break drains and sewers from which rats and effluents emerge break the spatial barrier between them and urban dwellers. Rat control in and around food storage and preparation areas, crop storage areas, stables, milking sheds, intensive animal production installations and dwellings is difficult but will remove a major source of leptospirosis for humans and domesticated animals. All the people involved in high-risk activities should wear protective clothing and need to adopt a reasonable standard of hygiene. Impervious knee-high boots, aprons, gloves, face masks or eye protection should be used wherever indicated^[6].

ACTIVE PROTECTION – CHEMOPROPHYLAXIS AND IMMUNIZATION

Chemoprophylaxis

Doxycycline can prevent leptospirosis, if given before and during exposure. Prolonged administration is not recommended.

Immunization

Vaccines have been developed for use in man. Existence of a large number of serovars makes it difficult to develop a universally effective vaccine.^[13]

Leptospire have evolved ways to evade host immune response.^[14] Because of the ability of pathogenic leptospire to translocate between mammalian cells quickly and reach blood stream and their ability to enter and initiate programmed cell death in kidney fibroblasts rapidly^[15], it is difficult for immunologists to develop effective and safe leptospirosis vaccines. Currently the molecular and cellular studies on leptospirosis vaccines have been focused on bacterial motility, lipopolysaccharides (LPSs), lipoproteins, and potential virulence factors. Various attempts for development of leptospirosis vaccines are summarized in the 'Leptospirosis Vaccine Ontology Database' available online.^[14] Various recombinant leptospiral proteins have been constructed and tested for their usefulness as vaccine candidates. Amongst these, OMPs, lipoproteins and virulence factors have generated considerable interest. *Leptospira* external membrane protein LipL32 cloned and expressed in mycobacterial vectors have been found to be protective to hamsters. In 2002, Palaniappan described a 130 kDa immunoreactive leptospiral immunoglobulin-like protein LigA. LigA as well as another immunoglobulin-like protein LigB were found to be immunogenic^[1]. It has been shown that LigA DNA vaccine gives significant protection against leptospirosis in hamsters. Inactivated and attenuated vaccines for leptospirosis have been described for more than 50 years. These vaccines have been tested in cattle and some of these are currently being used. Some of these vaccines have been tested in humans as well. At present, the only leptospirosis vaccine licensed for use in humans is being produced in Cuba. However, there is still an interest in inactivated and attenuated vaccines. Recently the focus is on newer vaccines such as DNA vaccines. At least two DNA vaccines, one encoding haemolysis-associated protein (Hap1) and the other endoflagella gene (flaB2) have been tested. These DNA vaccines require more extensive investigations^[16].

CONCLUSION

In view of dependence of human life on animal resource and due to changing life styles, leptospirosis has become a fast re-emerging zoonosis. Climatic and environmental conditions, host availability, population density and behavioral factors favor high transmission of the disease. The non-specific manifestation of the disease, limited laboratory capacity and lack of transversal policy, all contributed to underreporting of cases hence undermining the true prevalence of the disease. The high case loads observed during outbreaks lead to increases in the number of cases requiring secondary and tertiary care with a burdening of National Health Systems. Leptospirosis however is preventable. Environmental control programs (rodent control, drainage of flooded areas and occupational hygiene) need to be implemented. There is a need to gain a better understanding on the community beliefs, practices and attitudes towards leptospirosis, modes of

transmission and to increase its awareness. Effective surveillance is the key to the early containment of the disease. There is a need to develop epidemiology at the community level and improved diagnostic facilities which should be rapid, specific, simple and affordable. The strategy to combat the disease needs a strong public health structure, effective risk communication, epidemic preparedness and rapid response.

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*Address Correspondence to:

Dr. Deepak Y Patil
Nodal Laboratory for Re-emerging Diseases
Haffkine Institute
Acharya Donde Marg, Parel
Mumbai 400012, India

e-mail: patildeepak8@gmail.com

Limiting Dilution: A Cheaper Alternative for Detecting Viral Load of Human Immunodeficiency Virus-1

Sweta T. Kothari¹* and Ranjana A Deshmukh¹

ABSTRACT

Introduction: Viral load can be mainly estimated using two assay types, culture based and molecular-biology based. Culture based assays estimate direct infectious viral counts while molecular-biology assays are indirect, measuring nucleic acid levels. While methods such as PCR have advantages of detection of drug-resistance mutations, can be extrapolated to sequencing and have a high throughput, they are attached to considerable expense. Cell-culture methods are however, equally sensitive for viral load detection and are cost-effective. The present study aimed at developing a H9 cell-culture based assay for evaluation of viral load. This was correlated with Real-Time RT-PCR for HIV.

Materials and Methods: Ten samples representing all three HIV-disease stages were evaluated using both assays. The 'limiting-dilution' principle was used for infection and cultivation of HIV in H9 cells and the released p24 was measured using antigen capture ELISA. For the RT-PCR, viral nucleic acid was extracted and subjected to amplification. Long-terminal repeats of HIV were used for generating primers for the RT-PCR.

Results: The results showed a significant correlation ($p < 0.05$, $r = 0.9$) between the two assays. The culture-based method was also able to pick up two samples that were 'undetectable' by RT-PCR.

Conclusion: The standardization of this method can therefore be a suitable alternative to measure HIV viral load in monitoring drug therapy, disease progression, and clinical trials of various anti-retrovirals. Most importantly, this method gives a diagnostic tool to the millions of infected individuals who cannot afford the expensive molecular-biology methods.

Keywords: HIV-1, viral load, limiting dilution, H9 cells, p24 antigenemia

INTRODUCTION

Tremendous advances have been made in combating the Human Immunodeficiency Virus-1 (HIV-1) infection in developed countries. Combination of anti-retroviral therapy (ART) to suppress the virus replication and reduce mortality, along with CD4⁺ cell counts and HIV-1 viral load monitoring, has been an excellent mode to combat progression to AIDS^[1]. Unfortunately, the benefits of ART have made limited progress in the developing or resource-poor countries, where estimation of HIV-1 viral load using molecular methods is beyond reach of the common population.

Scaling up of methods to estimate viral load in such countries is difficult because of inadequate laboratory capacity; many programs have minimized laboratory monitoring in an effort to accelerate widespread availability of HIV treatment^[2]. Measurement of HIV-1 RNA levels which is done routinely to monitor HIV-infected patients in high-income countries was not recommended for use in resource-limited settings in the 2003 World Health Organization (WHO) guidelines^[3]. Equipment to determine the viral load is often unavailable or sits unused. Where available, viral load testing is prohibitively expensive: a single viral load test costs anything between Rs. 3000–5000/-^[4].

In HIV-1 infected adults, CD4⁺ cell counts and viral load act as predictive markers determining the clinical outcome of the infection and anti-retroviral therapy strategies. However, CD4⁺ cell counts fall naturally with age in uninfected children; even after adjusting for age, the CD4⁺ cell counts in HIV-1 infected children appear to be less effective in predicting

disease outcome. Although CD8⁺ lymphocytes are believed to be important in the control of HIV-1 infection, only a few studies have examined the prognostic significance of the number of peripheral CD⁺ cells^[5].

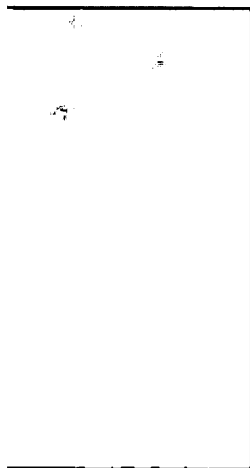
MATERIALS AND METHODS

Due clearance was obtained from the Institutional Ethical Committee at Haffkine Institute before the initiation of the study.

Sera samples from 10 HIV-1 infected individuals, none on anti-retroviral therapy, were taken for this study. The principle of limiting dilution was employed to estimate the viral load. The sera samples were passed through a 0.22 μ m membrane filter (Millipore, India). Each sample was then diluted ten-folds, viz. from undiluted sample to a factor of 10^{-6} . Two hundred microliters of each sample were inoculated onto the H9 cell suspension (Human T cell derivative, procured from National Center for Cell Sciences, Pune). H9 cell suspension was used at a density of 10^6 cells per millilitre, in RPMI 1640 medium (Invitrogen, India). This growth medium contained the basal RPMI 1640 medium, with 2.0 mM Glutamine, 4.5 g/L Glucose, 1.5 g/L Sodium bicarbonate, 1.0 mM Sodium pyruvate and 10.0 mM HEPES buffer. The medium was fortified using 10% Fetal Bovine Serum.

The virus was allowed to be adsorbed onto the cells for 60 minutes. The cells were then centrifuged at 10,000 rpm for 10 minutes, and re-suspended into fresh growth medium. This ensured the removal of soluble, virus non-associated p24 antigen. The infected H9 cells were incubated at 5% CO₂ at 37°C, and growth was monitored for 10 days. On

¹Department of Virology, Haffkine Institute, Mumbai, India; *Corresponding author



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measured the viral load in terms of HIV-1 RNA copies per millilitre in the samples.

Sample No.	Viral load/mL		Correlation Co-efficient
	p24 Antigen Assay	Taqman® RT-PCR	
HIV-01	5	Undetectable	R = 0.9 p<0.05
HIV-02	5×10^2	0.169×10^2	
HIV-03	5×10^4	2.17×10^4	
HIV-04	5×10^4	1.39×10^4	
HIV-05	5×10^2	4.08×10^2	
HIV-06	5×10^2	3.34×10^2	
HIV-07	5×10^2	2.25×10^2	
HIV-08	5×10^4	0.5×10^4	
HIV-09	5	Undetectable	
HIV-10	5	Undetectable	

Table 1: Correlation of Two Methods of Viral load determination

DISCUSSION

The ability to accurately determine viral and infected cell burden is essential in understanding the natural history of HIV-1 infection, predicting disease progression, and assessing the efficacy of various therapeutic drug regimens and vaccines.

Although CD4+ cell counts are the best-known and most broadly used surrogate marker for AIDS, the levels of CD4+ cell counts do not always correlate with the disease state^[8]. Efforts to quantitate viral load in infected individuals have been complicated by the paucity of infected cells, viral particles, and expressed viral components. Endpoint dilution cultures have been used to quantify HIV-1 viral particles in plasma and infected peripheral blood mononuclear cells^[9, 10].

Resources vary significantly by country, human resources and infrastructure that might be readily available in major cities, such as trained personnel, clean water, and electricity, may not be found in more rural areas. Reference laboratories that have the personnel, equipment, and infrastructure to perform CD4+ cell counts and viral load testing may lack the resources to purchase the kits.

In such countries with limited resources there is an urgent need for an affordable, reliable diagnostic test for HIV infection. Nucleic acid tests, the 'gold standards' against which other diagnostic tests are measured, are expensive and technically challenging^[11-13], making their use in developing countries problematic.

It is with this backdrop that we planned the present study of evaluating an alternative method for detecting viral load, using susceptible cell suspension of H9, rather than co-cultivation with peripheral blood mononuclear cells. We observed a significant correlation between the two methods, as has been observed in other studies^[14, 15], while other groups have obtained contrasting results^[16].

There are some obvious drawbacks in the assay

developed in the present study. The p24 antigenemia method was less sensitive, meaning that it could detect the load only in the exponential factor of 10, and not exact. This method can therefore be used for estimating the efficacy of an ART regimen, where it is important to know if the viral load is under control or declining. Additionally, the detection of p24 antigen from infectious particles is time consuming and requires higher sample volume as compared to Real Time RT-PCR. In contrast, the main advantages of this assay are that it requires routine laboratory set up and is rather inexpensive (Rs. 250–1000/-). The p24 assay can thus be used for the routine monitoring of viral load in conjunction with the Real Time RT-PCR at fixed intervals as a gold standard.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.



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*Address Correspondence to:

Dr. Sweta T Kothari
Department of Virology
Haffkine Institute
Acharya Donde Marg, Parel
Mumbai 400012, India

e-mail: dr.sweta.kothari@gmail.com
mailto:as_bobade@yahoo.com

Evaluation of *In Vitro* Anti-Tuberculosis Activity of Extracts of *Asparagus racemosus*, *Solanum surattense*, *Cinnamomum verum*

Suvarna Joshi¹, Forum Shah², Pooja Bansod², Swati Vaish², Soumen Roy¹, Shrikant V Athlekar¹ and Abhay S Chowdhary^{1*}

ABSTRACT

Introduction: Tuberculosis (TB) affects approximately 1.8 million people each year. The emergence of TB as a co-infection in HIV disease, has added to the gravity of the situation. The emergence of MDR/XDR TB has pressed for the need to develop novel, safe, efficacious and cost-effective anti-TB agents.

Materials and Methods: Extracts from the commercially procured plant materials were made using various solvents in the Soxhlet's apparatus. Phytochemical composition was determined by HPTLC. Median Cytotoxicity (CC₅₀) was estimated by MTT assay for determining an appropriate range of plant extract concentrations to be use for anti-TB activity. Anti-TB activity was established using MTT microplate assay and Agar Dilution method.

Results: Preliminary Phytochemical analysis demonstrated flavanoids, tannins, steroids and essential-oils as the primary constituents in the extracts. Aqueous extract of *Asparagus racemosus*, *Solanum surattense*, *Cinnamomum verum* inhibited H37rV strain of *M. tuberculosis* with IC₅₀ values of 60 mg/ml, 12 mg/ml, 18.5 mg/ml while their methanolic counterparts had IC₅₀ of 20 mg/ml, 35 mg/ml, 33 mg/ml respectively. No growth was observed on the slants during agar dilution test at concentrations of 100mg/ml and 200mg/ml of all the plant extract (aqueous and methanolic) showing inhibition of *Mycobacterium tuberculosis* H37rV strain.

Conclusion: The aqueous extract of *Solanum surattense* showed effective *in vitro* anti-TB activity (IC₅₀=12 mg/mL) followed by methanolic extract of *Asparagus racemosus* (IC₅₀=20 mg/mL). These extracts however must be subjected to further specific evaluations to confirm their anti-TB activities.

Keywords: *Mycobacterium tuberculosis* (H37rV), Anti- TB activity, HPTLC, MTT assay, Agar Dilution

INTRODUCTION

Tuberculosis (TB) remains one of the most prevalent causes of death in developing countries, due to a single infectious bacterial agent—*Mycobacterium tuberculosis*.

Currently, one third of the world's population is infected with *M. tuberculosis* and each year there are 2–3 million deaths worldwide caused by the disease. The emergence of Multi Drug-Resistant (MDR) and Extensively Drug-Resistant (XDR) TB is the main issue in case of the treatment of TB. The available drugs are either no more effective or are too expensive. There is, therefore, an urgent need for new, inexpensive TB drugs which are more effective and with less side effects^[1].

The practice of using medicinal plants for the treatment of various diseases started since the dawn of civilization^[2, 3, 4]. Naturally occurring biologically active plant products are used as an alternative strategy to prevent the spread of diseases^[5, 6].

Asparagus racemosus is a perennial branched climbing herb found all over India, especially in tropical and sub-tropical parts and in Himalayan region. It is widely utilized as a traditional remedy for a wide range of bacterial and fungal

infections. The root and their different extracts have shown alterative, demulcent, aphrodisiac, antiseptic and anti-cancer activity. It also acts as a nutritive tonic. Different scientific studies have proved the efficacy of it in a number of physical and mental ailments^[7].

Different parts of plant *Solanum surattense* are used in the treatment of the diseases like fever, asthma, tuberculosis, etc. The plant extract of also possesses insecticidal and larvicidal and anti-malarial property^[8].

Cinnamomum verum (Cinnamon) has been used to treat diarrhea and other problems of the digestive system. It has been used traditionally for respiratory disorders such as cough, cold, whooping cough, bronchitis and asthma. Essential oil of this plant is known to be active against, MTB *in vitro*^[9].

Thus this study was undertaken so as to focus studying the anti-mycobacterial activity of *Asparagus racemosus*, *Solanum surattense* and *Cinnamomum verum*.

MATERIALS AND METHODS

Plant Collection

Plant material of *Asparagus racemosus* (Shatavari), *Solanum surattense* (Kantakari),

¹Department of Clinical Pathology, Haffkine Institute, Mumbai, India; *Corresponding author

²Department of Biotechnology & Bioinformatics, Pad. Dr. D. Y. Patil, University, Navi Mumbai, India

Cinnamomum verum (Dal Chini; Cinnamon) were commercially procured.

Plant extracts

Aqueous and methanolic extracts of the plant material were prepared using a Soxhlet apparatus. The solvent fraction was evaporated using a vacuum rotary evaporator. The aqueous and methanolic extracts of *Asparagus racemosus* are coded as SAS and SMS, *Cinnamomum verum* is coded as CAS and CMS and *Solanum surattense* as KAS and KMS respectively.

Assessment of phytochemical constitution of the plant extracts

Phytochemical compositions of the plant extract were determined by High Performance Thin Layer Chromatography (HPTLC)^[10].

Assessment of median cytotoxicity of plant

The median cytotoxicity of plants extracts to MDCK cell line was assessed using the MTT assay. Here the reduction of MTT to formazan is indicative of the viable cells^[11].

Screening for the anti- TB activity

Anti-TB activity of the plant extracts was evaluated by following techniques:

MTT assay

The median inhibitory concentration was evaluated by MTT assay, as only the viable MTB cells convert tetrazolium to purple formazan^[12, 13].

Agar dilution test

The plant extracts in the desired concentration were incorporated in Middle brook 7H11 agar supplemented with OADC. A loopful of H37Rv strain of MTB containing 3×10^8 cfu/mL was then streaked onto the slant. These slants were incubated for 4 weeks at 37°C. Activity was evaluated on the basis of growth or no growth as observed in the slant^[14].

RESULTS

High Performance Thin Layer Chromatography (HPTLC)

Phytochemical evaluations were carried using HPTLC. SAS and SMS similarly KAS and KMS showed the presence of alkaloids, flavanoids,

steroids, glycosides, tannins & essential oils while CAS and CMS showed the presence of terpenoids, phenols and essential oils.

Cytotoxic Evaluation of The Plant Extracts

Cytotoxic assay performed to check CC_{50} . The least toxic plant to cells was SAS and SMA whose CC_{50} is at 230 mg/mL. CAS and CMS demonstrate CC_{50} at 70 mg/mL and 90 mg/mL respectively. KAS showed CC_{50} at 110 mg/mL and KMS showed CC_{50} at 100 mg/mL (Figure 1).

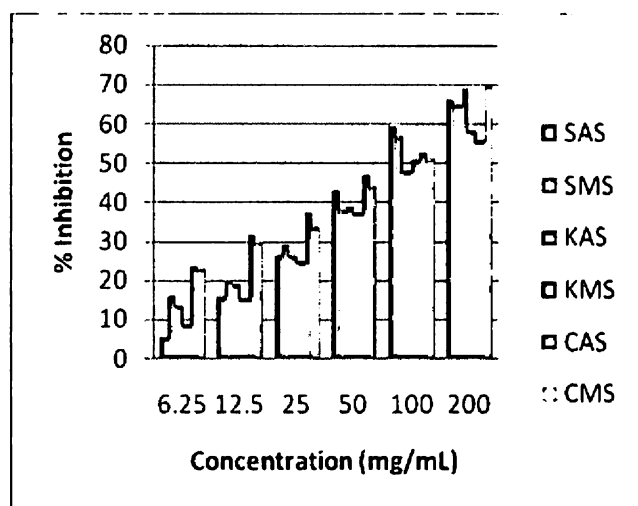


Figure 1: Percent cytotoxic inhibition of various plants extracts

Anti-TB Activity of The Plant Extracts

Anti-TB MTT assay was performed where KAS extract showed the best results with median inhibitory concentration IC_{50} at 12 mg/mL and KMS at 35 mg/mL. CAS exhibits the IC_{50} at 18.5 mg/mL and CMS at 33 mg/mL while SAS and SMS showed the IC_{50} at 60 mg/mL and 20 mg/mL respectively (Figure 2).

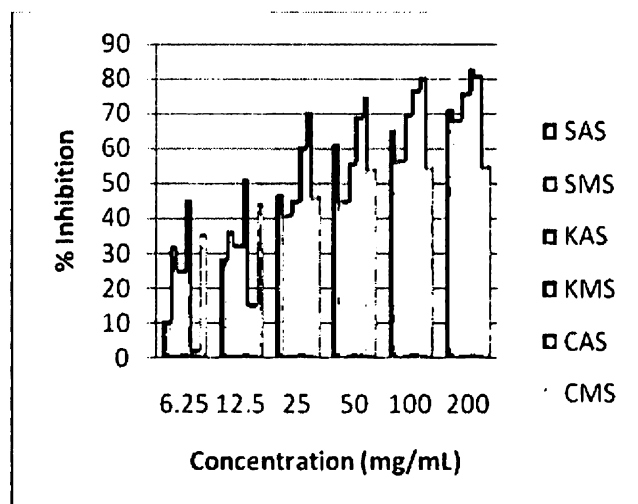


Figure 2: Percentage inhibition of the plant extracts to H37Rv strain of *Mycobacterium tuberculosis*.

Agar Dilution Test

No growth was observed at concentration of 100 mg/mL and 200 mg/mL in SMS, KAS, KMS, CAS, CMS. Growth was seen in 100 mg/mL in SAS (Table 1).

Plant Extracts	Concentrations (mg/mL)	
	100	200
DMSO	-	-
Rifampicin	+	+
Control TB Growth	-	-
SAS	-	+
SMS	+	+
KAS	+	+
KMS	+	+
CAS	+	+
CMS	+	+

Table 1: Agar Dilution Technique

Key : (+) → inhibition (MTB didn't grow); (-) → no inhibition (growth of MTB)

DISCUSSION

Plants are staging a comeback and ayurvedic renaissance is happening all over the globe. The herbal products today symbolize safety in contrast to the synthetic drugs that are regarded as unsafe to the human and environment. Anti TB activity of plant extracts *Asparagus racemosus*, *Solanum surattense*, *Cinnamomum verum* were studied. Preliminary Phytochemical analysis demonstrated flavanoids, tannins, steroids and essential-oils as the primary constituents in the extracts. Aqueous extract of *Asparagus racemosus*, *Solanum surattense*, *Cinnamomum verum* inhibited H37rV strain of *M. tuberculosis* with IC₅₀ values of 60 mg/mL, 12 mg/mL, 18.5 mg/mL while their methanolic counterparts had IC₅₀ of 20 mg/mL, 35 mg/mL, 33 mg/mL respectively. No growth was observed on the slants during agar dilution test at concentrations of 100mg/mL and 200mg/mL of all the plant extract (aqueous and methanolic) showing inhibition of *Mycobacterium tuberculosis* H37rV strain. These extracts however must be subjected to further specific investigations by isolating the compound showing anti-TB activity. Fractionation and identification of the active compound shall help us in increasing the efficacy and activity and decreasing its toxicity levels. Development of safe and more efficacious antimycobacterial agents in the era of MDR and XDR –TB becomes imperative. Thus the treasure

of knowledge which imbibes in our traditional systems of medicine must be complemented with contemporary scientific analysis.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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*Address Correspondence to:

Dr. Abhay Chowdhary
Department of Clinical Pathology
Haffkine Institute
Acharya Donde Marg, Parel
Mumbai 400012, India
e-mail: haffkineinstitute@gmail.com

Biological Control of Mosquito Larvae by Cyclopoid Copepods

Saroj Bapna^{1*}, Harshad Mayekar¹, Rahul Gosavi¹, Sahil Kulkarni¹, Mrunal Ghag¹, Meera Ramaiya¹ and Abhay Chowdhary¹

ABSTRACT

Introduction: Biological control of mosquitoes is an attractive alternative to chemical pesticides for suppression of mosquito pests and disease vectors. Copepods are tiny aquatic Crustaceans that prey upon mosquito larvae. In the present investigation predatory potential of two species of cyclopoid copepods *Macrocyclus distinctus* and *Mesocyclops leuckarti sensu lato* were tested as potential biological control agents of *Aedes aegypti* (Diptera: Culicidae) in laboratory microcosms.

Materials and Methods: Cyclops were collected from Picchola Lake, Udaipur, and Rajasthan and maintained in the laboratory using a Protozoan culture.

In the first set of experiment predation preference of single adult female copepod was assessed against mosquito larvae in presence and absence of alternative food. In the second set, predation was determined in relation to different densities of mosquito larvae with respect to volume of water and container size.

Results: The results of laboratory experiments suggest that both the species of copepods preyed on first larval instars of *Aedes*. However in presence of alternative food the preference for larvae was significantly reduced. Prey density, container size and volume of water were also found to be affecting the predation efficacy.

Conclusion: Since we do not have consistent evidence of acceptable mosquito population reduction brought about by biological agents in a wide range of habitats, both *Macrocyclus distinctus* and *Mesocyclops leuckarti sensu lato* can be good candidates for controlling container breeding *Aedes* larvae. The fact that copepods are globally distributed, mass production is simple and inexpensive and compatible with commonly used larvicides, augments their practical use.

Keywords: *Aedes aegypti* Mosquito, biological control, copepoda, *Macrocyclus distinctus*, *Mesocyclops leuckarti sensu lato*

INTRODUCTION

Mosquitoes are important pests that affect human health and well-being throughout the world. Biological control is an attractive alternative to chemical pesticides, particularly in view of the current restrictions on pesticide use and habitat management, and continuing problems with chemical resistance.

Although a variety of biological control agents ranging from viruses to fish have been tried for mosquito control, we still do not have consistent evidence of acceptable mosquito population reduction brought about by biological control agents^[1]. Copepods are tiny crustaceans that are ecologically very different from other aquatic invertebrates that prey on mosquito larvae. Past studies report various species of Cyclopoids (Crustacea: Copepoda), as mosquito bio-control agents^[2-4]. A notable success in the control of *Aedes* vector of dengue was reported in Vietnam with copepods of the genus *Mesocyclops*^[5]. In this study we have reported the predatory potential of two locally available copepod species *Macrocyclus* and *Mesocyclops* for the control of *Aedes aegypti* mosquito larvae.

MATERIALS AND METHODS

Collection and culture of copepods

Cyclops were collected from Picchola Lake, Udaipur, Rajasthan. Cyclops was maintained in the laboratory using a Protozoan culture^[6,7]. Identification was made as per previously reported methods^[8] and reconfirmed by Dr. Boxshall from the British Museum, London.

Mosquito larvae

Newly hatched first instar mosquito larvae were obtained from the mosquito colony maintained in the Department of Zoonosis at Haffkine Institute. Continuous culture of *Aedes* larvae was carried out in a plastic tray with de-chlorinated tap water until pupation and they were fed on dog biscuits and yeast powder in a 3:1 ratio. Pupae were placed in screened cages for adult emergence. Adults were fed on 10% glucose solution and a restrained guinea pig was offered as a blood source twice a week. *Aedes aegypti* females were allowed to lay eggs in a container half filled with water. A strip of filter paper was added inside the perimeter of the container for oviposition of *Ae. aegypti* females. The eggs were used for continuous supply of first stage larvae in the experiment.

Predation trials

Laboratory experiments were conducted at room temperature ($30^{\circ} \pm 2^{\circ} \text{C}$) under a photoperiod of 12:12 (Light: Dark) hrs. To evaluate predatory potential of both these species of Cyclops, different experiments were conducted using various combinations of mosquito larvae and Cyclops species.

Experiment 1

In the first set of experiment a single adult female was placed with 50 first-instar mosquito larvae in a plastic bowl ($10 \times 10 \times 5 \text{ cm}$) containing 100 mL of de-chlorinated tap water at room temperature. The numbers of surviving larvae on each day were recorded for two days (24–48 hrs). To assess copepod feeding

¹Department of Zoonosis, Haffkine Institute, Mumbai, India; *Corresponding author

in the presence of an alternate food source, identical trials were conducted under similar conditions with addition of 10 mL protozoa suspension per container from the stock protozoa culture.

Experiment 2

Predation capacity of individual copepod females was determined in relation to different densities of first instar larvae of *Ae. aegypti* (10, 20, 40, 60, 80 and 100 larvae). The larvae were placed in a 24 well plate (1.6 cm diameter) with a single adult female copepod and 3 mL of de-chlorinated water per well. Larval mortality was recorded at 24 hrs. Similar tests were conducted in 250 mL containers with 100 mL of de-chlorinated water.

Six replicates and two controls (containers with larvae without copepods) were conducted for each experiment.

RESULTS

Both the species of copepods preyed on first and early second instar larvae of *Aedes* mosquito (Figure 1). A single adult female of *Macrocyclus distinctus* and *Mesocyclops leuckarti sensu lato* caused 48.98% and 33.72% mortality of 50 first instar larvae of *Ae. aegypti* respectively, in a 48 hour test period (Cumulative mortality of 24 and 48 hrs). When alternative food was added to the containers, mortality rates declined to 20.64% and 14.12% respectively (Table 1).

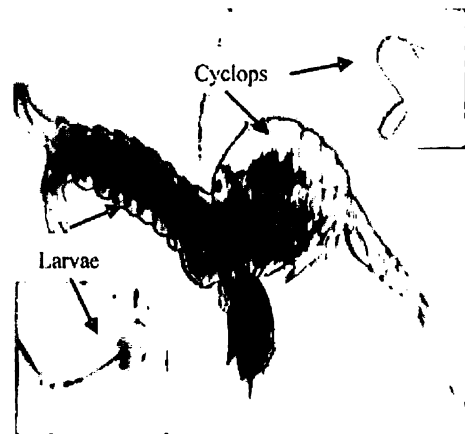


Figure 1: Cyclops devouring Mosquito larvae

Figure 1: Cyclops devouring Mosquito larvae

In the experiments with different larval density in a 24 well plate, average percent mortality of first instar larvae was observed to be 67.65% and 53.04% for *Macrocyclus* and *Mesocyclops* respectively (Figure 2). When a similar experiment was conducted in containers with 100 mL water the average larval mortality was reduced to 44.12% in case of *Macrocyclus* as compared to 23.02% for *Mesocyclops*, in a 24 hour test period (Figure 3).

Species of Copepodes	First stage Larvae (L1)	Average daily mortality of larvae (n)		Cumulative mean % Mortality
		24 hours	48 hours	
<i>Macrocyclus distinctus</i>	L1	18.12 ± 7.98	6.28 ± 1.69	48.8
	L1 + Food	08.19 ± 1.14	2.13 ± 1.76	20.64
<i>Mesocyclops leuckarti</i>	L1	15.19 ± 5.56	5.17 ± 1.11	40.72
	L1 + Food	06.35 ± 1.96	1.85 ± 0.87	15.3
Control	L1	00.00 ± 0.06	0.83 ± 0.03	1.66
	L1 + Food	00.12 ± 0.08	0.43 ± 0.02	1.1

Table 1: Average mortality (Mean ± SD) of first instar *Aedes aegypti* larvae exposed to *Macrocyclus* and *Mesocyclops* in presence and absence of alternative food

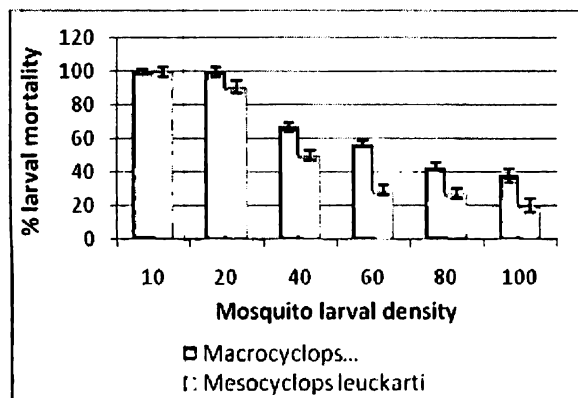


Figure 2: Average mortality (Mean ± SD) of first instar *Aedes aegypti* larvae exposed to one female copepod in different larval densities in 24 well plate with 3.0 mL water per well

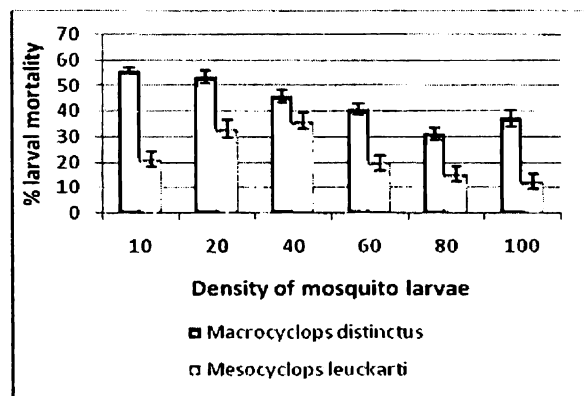


Figure 3: Predation capacity (Mean ± SD) of individual *Macrocyclus* and *Mesocyclops* on different *Aedes* larval densities in 100 mL water

DISCUSSION

In this study, we demonstrated that both *Macrocyclus* and *Mesocyclops* adult females preyed on first stage larvae of *Ae. aegypti*. In addition, presences of alternative food in containers with mosquito larvae drastically reduce predation of mosquito larvae. Our results suggest that both these species did not have a feeding preference for mosquito larvae if algae and protozoa were available. Reduction of predation rates of copepods on mosquito larvae in the presence of abundantly alternative food has been reported previously^[3, 9].

Prey density and container size also affect predation rate. When a single copepod of *Macrocyclus* and *Mesocyclops* was placed in a 24 well plate with 3 mL water, the predation was significantly higher as compared to larger containers with higher volume of water. Similar numbers of larvae were killed only when larval densities were more. When volume of water was high, *Mesocyclops* lack the ability to locate the prey in larger containers. Such a suggestion has been supported by Marten^[10] who observed larval escape from predation when volume of water was high. On the other hand *Macrocyclus* was observed to be an aggressive predator and often kill most of the larvae it encounters even when it is too satiated to eat. When mosquito larvae are numerous, *Macrocyclus* eat only a small part of each larva and thus the effective predation rate was comparatively higher than *Mesocyclops*.

Since *Macrocyclus* thrive well in laboratory containers, they can be successfully employed as a control measure for container breeding *Aedes* mosquitoes. Though it is unusual for copepods to get into man-made containers on their own, when introduced they can thrive regardless of the supply of mosquito larvae in the container for a longer period and attack newly hatched larvae.

In the practical sense, both these species *Macrocyclus distinctus* and *Mesocyclops leuckarti sensu lato* can be good candidates for use as mosquito control agents as copepods are globally distributed, mass production is simple and inexpensive and they survive even under extreme conditions without being killed by several pesticides commonly used for mosquito control. Further tests are required to determine the effectiveness of this predator in large bodies of natural habitat. Since Cyclops can remain in a dormant state, they may be encapsulated to use as and when required.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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*Address Correspondence to:

Dr. Saroj Bapna
Department of Zoonosis
Haffkine Institute
Acharya Donde Marg, Parel
Mumbai 400012, India

e-mail: sarojbapna@rediffmail.com

Study of Hepatoprotective Activity of *Cymbopogon Citratus* Against Hepatotoxicity Induced by Carbon Tetrachloride in Female Wistar Rats

Monu Ojha¹, Pushkar Athlekar¹, Pallavi V Ansulkar¹, Amruta Naik¹, Mrunal Ghag², Sandeepan Mukharjee³ and Abhay Chowdhary^{1,2,3*}

ABSTRACT

Introduction: The ancient literature reports *Cymbopogon citratus* for its various uses in folk medicine including its hepatoprotective activity. In the present study the hepatoprotective and antioxidant effects of methanolic extract of *Cymbopogon citratus* was investigated against hepatotoxicity induced by carbon tetrachloride in female Wistar rats.

Materials and Methods: The hexane-dichloromethane-methanol extracts were subjected to preliminary phytochemical analysis and HPTLC analysis. The methanolic extract was further investigated for its antioxidant activity by DPPH radical scavenging, total phenolic content and Reducing Power by FeCl₃. A single dose of 1:1 carbon tetrachloride in olive oil (1.5 mL/Kg) was administered i.p. to induce hepatotoxicity in healthy female Wistar rats [180–250 g] and the plant extract at a dose of 500, 750 and 1000 mg/Kg was administered orally. Blood was collected on days 0, 4 and 7 for the assessment of ALT, AST and ALP.

Results: The HPTLC analysis confirmed the presence of flavonoids and terpenoids in the methanolic extract. The antioxidant assays showed IC₅₀ at 150 µg/mL and presence of 95.23 GAE/mg of extract. The reducing power of extract showed proportional increase with concentration. The methanolic extract showed reduction in hepatic toxicity with increasing concentrations.

Conclusions: The results of the present study indicated that under the present experimental conditions, methanolic extract of *Cymbopogon citratus* showed hepatoprotective abilities against carbon tetrachloride induced liver damage. High phenolic and flavonoids contents may be responsible for this activity. Further investigations are required to elucidate the exact phytochemicals and their mechanism responsible for the hepatoprotective activity of *Cymbopogon citratus*.

Keywords: *Cymbopogon citratus*, hepatoprotective, hepatotoxicity, carbon tetrachloride, female Wistar rats

INTRODUCTION

The market for herbal products is expanding and there is an urgent need for the development of potent liver protective/curative drugs because a huge world population suffers from various liver disorders^[1].

The mechanisms of liver injury are complex and interactive. In the biochemical mechanisms various chemicals are detoxified in the liver via cytochrome P-450 and conjugation. Toxic metabolites may alter plasma membrane, mitochondria, intracellular ion homeostasis, or degradative enzyme activity. Pathologic apoptosis is potentially an important mechanism of acute liver injury. The more frequent causes of acute liver failure: hypoxia/reoxygenation, liver congestion, acetaminophen poisoning, post transplant acute liver rejection, severe sepsis, viral hepatitis, and alcoholic liver disease^[2].

Popularity of herbal remedies is increasing globally and at least one quarter of patients with liver diseases use ethnobotanicals^[4]. *Cymbopogon citratus* (DC.) Stapf. belonging to the family *Poaceae* is an ayurvedic herb. The lemon grass consists of various poly-phenolic compounds like flavonoids, terpenoids and alkaloids, having anti-oxidant activities. It has been shown that when rats were pre-treated with the aqueous extract of lemon grass and then induced with paracetamol for oxidative stress, the serum levels have decreased in the catalase enzyme and lipid peroxidation end products were also reduced in the alkaline phosphates (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels indicating

protection to the liver imparted due to its anti-oxidant activity^[3, 5]. Hence there is a need to screen various other extracts of lemon grass for its most potent activity.

The main aim of this study was to investigate the effect of crude methanolic extract of *Cymbopogon citratus* on female Wistar rats using CCl₄ as a hepatotoxicity inducer.

MATERIALS AND METHODS

Plant Material:

Cymbopogon citratus purchased from local market was washed, shade dried and then powdered. Soxhlet extraction technique was carried out using 100% n-hexane-dichloromethane-methanol (non-polar to polar) in succession and percent extractive values were calculated.

Phytochemical Analysis:

The crude extracts were examined for presence of different phytoconstituents such as alkaloids, flavonoids, glycosides, phenolic content, terpenoids and organic acids^[6, 7].

HPTLC Analysis:

The crude extracts were subjected to HPTLC to confirm the presence of flavonoids and terpenoids. Ten percent solutions were prepared in respective solvents and were subjected to sample application using linomat 5 semi automatic sampler. Solvent system used for terpenoids, flavonoids were [n-hexane:ethyl acetate (1:1)] and [ethyl acetate:

¹Department of Biochemistry, Haffkine Institute, Mumbai, India; *Corresponding author

²Department of Zoonosis, Haffkine Institute, Mumbai, India

³Department of Virology, Haffkine Institute, Mumbai, India

formic acid:acetic acid:water (10:0.5:0.5:1.3)] respectively using vanillin-sulphuric acid detection. Densitometric evaluation of chromatogram was made using a Camag TLC scanner. Visual imprint of the TLC plate was taken using a Camag TLC visualizer and Documentation was achieved using a WIN CATS software system.

Anti-oxidant studies:

Anti-oxidant studies were carried out using DPPH radical scavenging activity, total phenolic assay and reducing power by FeCl_3 .

DPPH radical scavenging activity^[10, 11, 12]:

DPPH (2, 2'-Diphenyl-1-picryl hydrazyl) is a stable free radical with an absorption band at 517 nm and is purple in color. The color turns from purple to yellow when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H. The resulting decolorization is stoichiometric with respect to number of electrons captured. The calibration curve was carried out using standard antioxidants (L-ascorbic acid /BHT).

Total Phenolic Assay:

The total phenolic content is estimated by Folin-Ciocalteu method using Gallic acid as standard. In this method reduction products of tungsten and molybdenum oxide have a blue color that exhibits a broad light absorption with a maximum at 765 nm. The intensity of light absorption at the wavelength is proportional to the concentration of phenols. The results are expressed as Gallic Acid Equivalent, as the presence of gallic acid indicates the presence

of wide range of phenols in the sample^[13,14].

Reducing Power by FeCl_3 :

Substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm^[15].

In vivo Study:

Acute toxicity studies:

The animal study was conducted after the permission granted by the Institutional Animal Ethical Committee. Acute toxicity studies were performed as per OECD guidelines 420 using Fixed Dose Method. Nulliparous and non-pregnant female Wistar rats [180–250 g] were kept under controlled environmental conditions of temperature ($22^\circ \pm 3^\circ\text{C}$) and humidity (30–70%) with 12 hours light/dark cycle and were given access to food and water *ad libitum*^[15].

Assessment of Hepatoprotective activity:

The doses selected for the study were 500 mg/Kg b.w., 750 mg/Kg b.w. and 1000 mg/Kg b.w. based on acute toxicity results^[15–18].

Experimental Protocol (table 1):

The study was conducted for 7 days. Blood was collected on day 0, 4 and 7 via retro-orbital route under light ether anesthesia. Serum AST, ALP and ALT were estimated using Erba kits on a semi-automated analyzer (Erba). The bodyweight and feed consumption of the animals were recorded on day 0, 3, 5 and 7. Statistical Analysis was carried using ANOVA and Bonferroni's Multiple Comparison test.

Group number	Group name (5 rats/group)	Dose	Treatment/s
1	Vehicle control	10% DMSO in distilled water	DMSO + DW(vehicle)
2	Hepatotoxic control	1.5 mL/Kg b.w. of 1:1 CCl_4 in olive oil	CCl_4
3	Positive control	200 mg/Kg b.w. of Silymarin	Silymarin + vehicle
4	Plant extract dose 1	500 mg/Kg b.w.	Plant extract + vehicle
5	Plant extract dose 2	750 mg/Kg b.w.	Plant extract + vehicle
6	Plant extract dose 3	1000 mg/Kg b.w.	Plant extract + vehicle

Table 1: Animal grouping

RESULTS

Soxhlet extraction (Table 2):

Solvent used	Percent extractive value
n-hexane	3.49%
Dichloromethane	1.40%
Methanol	9.13%

Table 2: Percent extractive value of *Cymbopogon citratus* in different solvent

Phytochemical Analysis:

The Preliminary phytochemical analysis showed the presence of the following phytochemicals as expressed in Table 3.

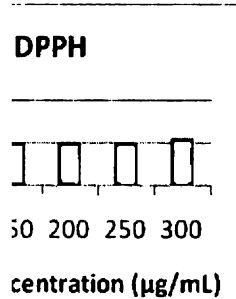
The HPTLC analysis confirmed the presence of flavonoids [blue green bands] and terpenoids [grey bands].

Test	Methanol extract	Dichloromethane Extract	Hexane extract
Phenolic	+	-	-
Flavonoids	+	+	-
Terpenoid	+	-	-
Alkaloid	-	-	-
Volatile oils	-	-	-
Glycosides	-	-	-
Organic acid	+	-	-

Table 3: Results of phytochemical analysis

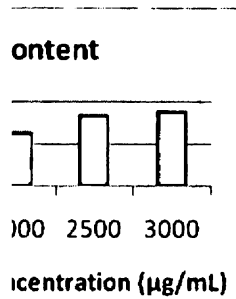
2, 3).:

methanolic extract
presence of 95.23
g power of the extract
with concentration of



f DPPH

ge inhibition of DPPH



centration (µg/mL)

content of methanolic

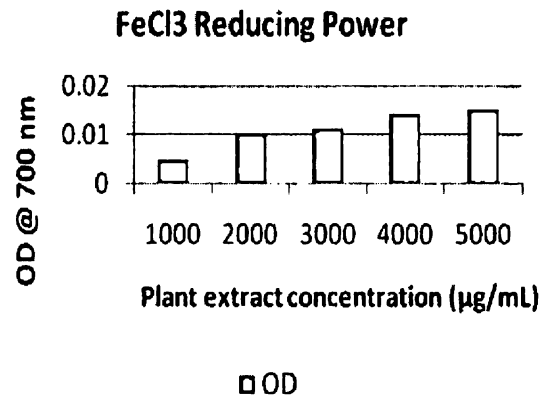


Figure 3: Graph showing reducing power of the plant extract

In-vivo study:

Acute toxicity studies:

The animals that were given a dose of 2000 mg/Kg b.w. showed impaired locomotor activity, lethargicity and piloerection after 4 hours whereas after 24 hours, no toxic signs and symptoms were observed. LD₅₀ of the plant extract was found to be greater than the dose administered to the animals since no mortality was observed.

The serum analyses were done for AST, ALT and ALP. The values are expressed as IU/L (Table 4). The data for AST, ALT, ALP and Feed consumption (data not shown) was statistically non significant, whereas significance was observed in the body weight between group 2 and 6 and also between group 3 and 6 ($p < 0.05$) (Table 5).

	ALT			ALP		
Day 7	Day 0	Day 4	Day 7	Day 0	Day 4	Day 7
181 ± 30	39 ± 19	59 ± 10	64 ± 8	175 ± 101	227 ± 136	380 ± 358
157 ± 55	75 ± 9	70 ± 23	66 ± 4	221 ± 64	282 ± 46	265 ± 32
194 ± 12	53 ± 14	71 ± 16	65 ± 11	215 ± 79	237 ± 92	266 ± 93
180 ± 48	61 ± 9	78 ± 9	58 ± 9	216 ± 69	299 ± 134	202 ± 68
196 ± 23	80 ± 12	95 ± 39	58 ± 17	248 ± 63	376 ± 64	212 ± 74
139 ± 30	58 ± 9	96 ± 26	57 ± 12	244 ± 60	270 ± 54	216 ± 60

Values of each group on day 0, 4 and 7; Values expressed as Mean ± S.D

Group	Weight (g)				
	Day 0	Day 1	Day 3	Day 5	Day 7
1	220.6 ± 10	221 ± 10	223 ± 10	220 ± 6	226 ± 6
2	213.5 ± 13	215 ± 13	209 ± 17	212 ± 13	203 ± 18
3	209.6 ± 10	210 ± 10	209 ± 10	212 ± 11	200 ± 13
4	227.1 ± 11	227 ± 11	224 ± 12	228 ± 11	203 ± 16
5	222 ± 5	223 ± 5	221 ± 6	222 ± 6	208 ± 10
6	228 ± 15	230 ± 15	228 ± 15	229 ± 18	228 ± 18

DISCUSSION

Liver disorders are a major problem globally, as large numbers of people are affected with some or the other liver problems. The hepatotoxic effects of CCl_4 are largely due to its active metabolites, trichloromethyl radical which binds to micromolecules and induces lipid peroxidative degradation of polyunsaturated fatty acids leading to the formation of lipid peroxides. This in turn gives products like malonylaldehyde that cause damage to membranes. In this study CCl_4 was used as an inducer of hepatotoxicity, which elevated the levels of serum enzymes indicating injury to the liver. The increase in ALP, AST and ALT may suggest enhanced lipid, giving rise to liver damage and failure of the antioxidant defense mechanism to prevent formation of excessive free radicals.

The serum analysis reports have shown that the serum levels of AST, ALT and ALP had increased on fourth day after induction of CCl_4 and then decreased by the seventh day.

The AST values were found to be less in the Silymarin control group indicating its protective effect. The test groups 4 and 5 also showed decreased levels of AST on day 4 whereas all the three concentrations of the plant extracts showed decreased values on the seventh day which may be due to its protective activity.

ALT levels in the serum were elevated even in the Silymarin control group on fourth day. On the seventh day, the plant extracts showed decreased levels; this trend was not observed in the Silymarin control group. The group administered with 750 mg/Kg b.w. dose showed drastic decrease in ALT values.

On day 7, the ALP values in the Silymarin control were unchanged and in case of the plant extracts, the values had decreased. The group administered with 750 mg/Kg b.w. dose showed drastic decrease in ALP values.

From the obtained results it can be postulated that 750 mg/Kg b.w of plant extract may have greater efficacy than the other concentrations. The body weight of the animals increased in all groups; there was a drastic increase in group 4 on the seventh day. The feed consumption showed a drastic increase and then a decrease was observed in all groups other than the vehicle control group and group 6.

In this study, serum analysis data were not found

to be statistically significant; however, it can be seen that the crude methanolic extract of the plant decreases the level of serum enzymes which may indicate protective activity. A previous study conducted on the aqueous extract of this plant has indicated its activity as a hepatoprotective agent. The study indicated that the dose of 100 mg/Kg b.w. had significantly inhibited elevation serum levels of malondialdehyde and catalase activity and caused pronounced increase in the level of serum reduced ascorbic acid^[3]. The hepatoprotective activity of the plant in the present study may be confirmed at the tissue level by histopathological analysis.

In this study, the difference in the hepatic markers were not found to be statistically significant in the control and experimental animals; this may be due to less leaching out of the phytochemicals in the solvent used. A varied number of solvents can be used for the purpose of plant phytochemical extraction and then the analysis of their activity can be carried out. The methanolic extract of *Cymbopogon citratus* demonstrated radical scavenging activity in the DPPH assay with IC_{50} at 150 $\mu\text{g/mL}$. In the past similar activities ranging between 40 and 68% at 33 and 50 $\mu\text{g/mL}$ were demonstrated^[18].

These were preliminary screening assays aimed primarily to assess the hepatoprotective activity of crude methanolic extract of *Cymbopogon citratus* in rodent model. Although the results are statistically non significant there is an interesting hepatoprotective activity seen in the samples which needs further evaluation especially at the molecular level.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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***Address Correspondence to:**

Dr. Abhay Chowdhary
Department of Biochemistry
Haffkine Institute
Acharya Donde Marg, Parel
Mumbai 400012, India

e-mail: haffkineinstitute@gmail.com

Synthesis of Substituted-Benzoic Acid (1-(1-Benzothiazol-2-Yl-3-(Substituted Phenyl) 1H-Pyrazol-4-Yl) Meth-(E)-Ylidene) Hydrazide as Possible Anti-Microbial Agents

Vrushali N Patil¹, Ameya G Yadav¹, Aashish L Asrondkar¹, Anil S Bobade^{1*}, Shrikant P Gajbhiye² and Abhay Chowdhary^{1,2}

ABSTRACT

Introduction: Benzothiazole derivatives are known to elicit biological activities such as anti-bacterial, anti-tubercular, anti-allergic and anti-inflammatory activities. Considerably research work has been reported that substituted benzothiazole connected to pyrazole with amide linkage is of pharmacological importance. Hence, in the present study we reported the synthesis and comparison of anti-microbial activity of various benzothiazole derivatives.

Materials and Methods: 2-amino benzothiazole was refluxed with hydrazine hydrate followed by condensation with substituted acetophenone to form hydrazone derivatives. Further, the mixture was allowed to undergo Vilsmeier-Haack reaction to form formylated pyrazolyl benzothiazole. Condensation of benzhydrazide with formylated pyrazolyl benzothiazole resulted into the title compounds. Products were characterized by elemental and spectral analysis.

Results: All the compounds were screened for their anti-bacterial activity by dilution against *Staphylococcus aureus* (NCTC 3750) and *Salmonella typhi* (NCTC 786) using Ampicillin (MIC 0.01 µg/mL) and Trimethoprim (MIC 0.01 µg/mL) as standard drug. The culture medium used in screening was Muller-Hinton broth. The compounds were screened for their anti-fungal activity by tube dilution technique against *Candida albicans* (ATCC 16404) and *Aspergillus niger* (ATCC 16404) using Miconazole (MIC 1.56 µg/mL) as standard drug.

Conclusion: The presence of amide linkage & Halogen substituents such as Chloro, Fluoro has shown excellent increase in Anti-bacterial activity but amide linkage was not beneficial as far as Anti-fungal activity is concerned. In case of Anti-bacterial screening Fluoro and Chloro derivatives showed activity up to 20 µg/mL.

The mechanistic aspect of microbiological activity for new benzothiazole moiety would be explored. Further Quantitative Structural Activity Relationship (QSAR) studies may also be conducted to improve potency efficacy.

Keywords: Anti-bacterial, anti-fungal, formylated pyrazolyl benzothiazole.

INTRODUCTION

Among the various types of known heterocyclic ring systems, benzothiazole derivatives play vital role in medicinal chemistry^[1]. Benzothiazoles possess broad spectrum biological activities such as anti-bacterial, anti-fungal, anti-tubercular, anti-allergic and anti-inflammatory activities^[2]. In recent years substituted benzothiazole and pyrazole^[3] have gained lot of prominence due to their various biological activities. These observations promoted us to synthesized a series of benzothiazolyl pyrazole^[4] derivatives to explore their potency as anti-microbial agents.

MATERIAL AND METHODS

All the solvents used in the experimental work were redistilled and dried before use. Melting point of the synthesized compounds was recorded on Thermo-nik melting point apparatus (Campbell electronics) and are uncorrected. Thin Layer Chromatography (TLC) was performed on silica gel coated plates using Iodine vapor as visualizing agents. Infra-red spectra were recorded on Hartmann-Braun, MB series (Bomem, Quebec, Canada). ¹H NMR spectra were recorded on 300 MHz instrument (Jeol Ltd, Tokyo, Japan), using DMSO solvent.

Experimental (Figure 1)

1. Synthesis of 2-hydrazino benzothiazole

Conc. HCl (6 mL) was added drop wise with stirring to hydrazine hydrate (6 mL) at 5-10°C temp. To it ethylene glycol (24 mL) and various substituted 2-Amino Benzothiazole (compound 1) 4.5 gm, (0.03 M). The mixture was refluxed for 3 hrs. Till the solid crystals appears. Cool, washed with water and recrystallized from ethanol.

IR (KBr): 3321, 3202, 1651, 1460 (C=N), 1195, 1159, 816.

¹H NMR (DMSO): δ5.04, (2H, s, NH₂), δ7.43 (1H, t, Ar-H),

δ7.60 (1H, q, Ar-H), δ7.72 (1H, d, Ar-H), δ9.34 (1H, s, NH).

Elemental Analysis: C₁₀H₁₄N₄O₃S₂

Found C (%)-54.11, **H (%)**-3.99, **N (%)**-12.71

2. Synthesis of Benzothiazol-2-yl hydrazone

2-hydrazino benzothiazole (compound 2) 1.65 gm (1.5 mmol), appropriate substituted acetophenone (2.2 mmol) and glacial acetic acid (2-3 drops) were taken in absolute ethanol (20 mL). This mixture was refluxed on water bath for 5-10 hrs (till different spot on TLC may appear). On cooling solid separates out which was filtered, washed with water and recrystallized with ethanol.

IR (KBr): 3148, 3049, 2908, 1568, 1459 (NHNH=C), 1341, 1126, 918, 937.

¹H NMR (DMSO): δ2.45 (3H, s, CH₃), δ7.22 (1H, m, Ar-H), δ7.47 (1H, d, Ar-H), δ7.76 (1H, m, Ar-H), δ11.63 (1H, s, NH).

Elemental Analysis: C₁₅H₁₁Cl₂N₃S

Found C (%)-51.23, **H (%)**-4.10, **N (%)**-13.01

3. Synthesis of Formylated Pyrazolyl Benzothiazole

Conventional Method

Benzothiazol-2-yl hydrazone (compound 3) 1.68 mL, (0.005 M) was dissolved in 6 mL of DMF and this kept in ice cold condition. To this Vilsmeier Haack reagent [(1.5 mL) of POCl₃ was added drop by drop with stirring in (6 mL) of DMF is added with stirring at room temp. for 4 hrs. then content was poured into crushed ice (previously neutralized with NaHCO₃ or liq.NH₃) solid separates out which was filter washed with water, dried and recrystallized from ethanol.

IR (KBr): 3131, 3062, 2923 (CHO), 2360, 2342, 1695, 1610, 1548, 1463 (C=N), 1356, 1207, 898.

¹H NMR (DMSO): δ7.49, (1H, m, Ar-H), δ7.63 (3H, s, Ar-H), δ8.03 (2H, s, Ar-H), δ9.75 (1H, s, >C=CH), 10.14 (1H, s, CHO).

Elemental Analysis: C₁₅H₁₁Cl₂N₃S

Found C (%)-52.69, **H (%)**-3.85, **N (%)**-11.89

¹Department of Chemotherapy, Haffkine Institute, Mumbai, India; *Corresponding Author

²Department of Bacteriology, Haffkine Institute, Mumbai, India

4. Synthesis of substituted-benzoic acid [1-[1-benzothiazol-2-yl-3-(substituted phenyl)- 1H-pyrazol-4-yl]-meth-(E)-ylidene] hydrazide

Formylated pyrazolyl benzothiazole (compound 4) 1.87 gm, (0.005 M) in round bottom flask add 0.5 mL of various substituted benzhydrazide in methanol reflux for 8 hrs. Cool Pour the content in ice cold water. Filter the solid and recrystallized from methanol.

IR (KBr): 1237 (R-CO-R), 2849 (C-H), 1681 (C=O), 1605 (C=N), 692 (C=S)

¹H NMR (CDCl₃): δ 7.1-8.4(m, H, Ar-H), 3.5(s, 2H, -CH₂)

Elemental analysis: C₂₃H₁₃Cl₃N₄S

Found C (%) -55.01, H (%) -3.05, N (%) -14.08

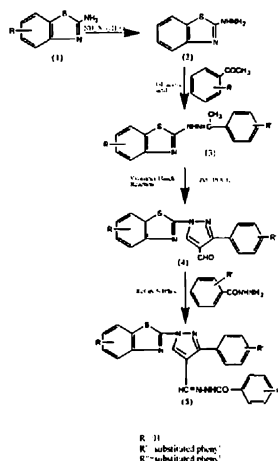


Figure 1: Schematic representation of synthesis

Anti-microbial Activity

All the synthesized compounds were screened for their *in vitro* anti-bacterial activity by tube dilution technique^[7] against *staphylococcus aureus* (NCTC 3750) using Ampicillin (MIC 0.04 μ g/mL) and *salmonella typhi* (NCTC 786) using Trimethoprim

(MIC 0.01 μ g/mL) as standard. The culture medium used in the screening was Muller-Hinton broth. The compounds were screened for their anti-fungal activity *in vitro* by tube dilution technique against *Candida albicans* (ATCC 10233) and *Aspergillus niger* (ATCC 16404) using Miconazole (MIC 6.25 μ g/mL) as standard. Sabraoud's medium was used as the growth media.

RESULTS

The results of the *in vitro* anti-microbial activity are shown in Table 1. Most of the compounds showed activity against *S. aureus*, *S. typhi*, *C. albicans* and *A. niger* at concentrations greater than 100 μ g/mL. The most active compound was found to be Compound 5e against *S. aureus*. This compound compared to the tested reference ampicillin (MIC 0.04 μ g/mL). The rest of the compounds showed varying degree of activity against test organisms.

DISCUSSION

Careful analysis of the anti-microbial activity of the compounds revealed that the pyrazole at C-2 position of benzothiazole and Azomethine (-N=CH) side chains at C-4 position of the pyrazolyl benzothiazole moiety are essential for the anti-microbial activity.

In the above series, compound 5e containing substitution of the aromatic ring with Fluoro and Chloro groups results in a marked increase in biological activity. It appears that the compounds which carry 2-Fluorophenyl and 3-Chlorophenyl group as substituents were more effective against *S. aureus* than the others.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

SD - QR

Comp. No	R	R'	R''	MIC against			
				<i>S. aureus</i> (μ g/mL)	<i>S. typhi</i> (μ g/mL)	<i>C. albicans</i> (μ g/mL)	<i>A. niger</i> (μ g/mL)
5a	H	3,4-DiCl	4-Cl	100	200	200	200
5b	H	3,4-DiCl	4-OH	100	200	200	NA
5c	H	2,4-DiOH	4-Cl	100	100	200	100
5d	H	4-F	4-OH	NA	100	200	100
5e	H	4-F	4-Cl	20	100	200	100
5f	H	4-F	4-OH	200	100	NA	100
5g	H	2,4-DiOH	4-CH ₃	100	100	NA	NA

Table 1: Antimicrobial activities of synthesized compounds against test organism (MIC)

NA: No Activity at 200 μ g/mL; Ampicillin (MIC 0.04 μ g/mL) used as standard against *S. aureus*; Trimethoprim (MIC 0.01 μ g/mL) used as standard against *S. typhi*; Miconazole (MIC 6.25 μ g/mL) as standard against *C. albicans* and *A. niger*

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*Address Correspondence to:
Dr. Anil S Bobade
Department of Chemotherapy
Haffkine Institute
Acharya Donde Marg, Parel
Mumbai 400012, India
e-mail: as_bobade@yahoo.com

Anti-Fungal Effect of *Tinospora Cordifolia* (Stem) and *Punica Granatum* (Peel) Against *Aspergillus Niger*

Shrikant Gajabhiye¹, Pratibha Chaturvedi^{2*} and Abhay Chowdhary^{1,2}

ABSTRACT

Introduction: Plants produce many secondary metabolites which constitute an important source of microbicides, pesticides and are the principal source of pharmaceutical agents used in traditional medicine. *Aspergillus niger* is fungal pathogen which induce the Aspergillosis, a disease that is related to the lungs and ear infection. In the present study an attempt was made to evaluate the anti-fungal effect of acetone and methanolic extract of *Punica granatum* (Common name: Anar; Family - Lythraceae) and *Tinospora cordifolia* (Common name: Gutuchi; Family - Menispermaceae) against *Aspergillus niger* (ATCC 16404).

Material and Methods: The plant materials of *Tinospora cordifolia* (stem) and *Punica granatum* (peel) were collected from Haffkine institute campus. The plant material were dried, powdered, weighed and soxhlet extracted in acetone and methanol separately. The prepared extracts were again weighed and the extractive values were calculated. The broth medium was prepared to test the effect of acetone and methanolic extracts of *T. cordifolia* and *P. granatum* against *Aspergillus niger* (ATCC 16404).

Results: The maximum extractive value in the present investigation was found in methanolic extract of *Punica granatum* (Peel: 11.53%) among the samples tested. All the samples showed Minimum Inhibitory Concentration (MIC) at 70 µg/mL against *Aspergillus niger* (ATCC 16404).

Conclusion: Methanolic extracts of both plants species showed higher extractive values than acetone extracts so it can be concluded that methanolic extract is more potent for anti-fungal activity against *Aspergillus niger* (ATCC 16404). Thus probably a new source was investigated to overcome Aspergillosis, a disease caused by *Aspergillus niger*.

Keywords: *Tinospora cordifolia*, *Punica granatum*, *Aspergillus niger*, Anti-fungal activity, Extractive value

INTRODUCTION

India has an extraordinarily rich flora and the wide knowledge of indigenous medicinal plants is well documented. As a result, we are investigating the potential of Indian medicinal plants as a resource for new bio-fungicides. Many of the plant materials used in traditional medicine are readily available in rural areas and are relatively cheaper than modern medicine. Plants produce many secondary metabolites which constitute an important source of microbicides, pesticides, and the principal source of pharmaceutical agents used in traditional medicine.

Aspergillus niger as a saprophyte in soil causes black mould. The main objective of this study was to investigate the inhibitory effects of different organic solvent extracts from *Tinospora cordifolia* (stem) and *Punica granatum* (peel) against *Aspergillus niger* (ATCC 16404) and to evaluate the potential application of medicinal plant based treatments to control diseases caused by *A. niger*. *Aspergillus niger* is less likely to cause human disease than some other *Aspergillus* species, but, if large amounts of spores are inhaled, a serious lung disease, Aspergillosis may occur. Aspergillosis is, in particular, frequent among horticultural workers that inhale peat dust, which can be rich in *Aspergillus* spores. *A. niger* is one of the most common causes of otomycosis (fungal ear infections), which can cause pain, temporary hearing loss, and, in severe cases, damage to the ear canal and tympanic membrane.

Guduchi (*Tinospora cordifolia*) is a large, glabrous, deciduous climbing shrub belonging to the family Menispermaceae. Guduchi is widely used in veterinary folk medicine/ayurvedic system of medicine for its

general tonic, anti-periodic, anti-spasmodic, anti-inflammatory, anti-arthritis, anti-allergic and anti-diabetic properties. The stem of *Tinospora cordifolia* is one of the constituents of several ayurvedic preparations used in general debility, dyspepsia, fever and urinary diseases. The antifungal activity of methanolic crude extract of *Tinospora cordifolia* has previously been investigated against *Alternaria solani*, *Curvularia lunata*, *Fusarium sp.*, *Bipolaris sp.* and *Helminthospori*^[1].

Pomegranate (*Punica granatum*) is a small tree up to 5 m in height. The characteristic large pomegranate fruits are and contain numerous seeds in juice containing sacs. It inhibited mycelia growth in the fungi *Botrytis cinerea* and *Fusarium oxysporum*^[2, 3]. Extracts from the peel of the pomegranate have also demonstrated anti-fungal activity. Fractionation of crude hydro-alcoholic extracts prepared from the fruit peel yields a compound (identified as punicalagin) with strong anti-fungal activity against *Candida albicans*^[2].

For both the plant species, anti-fungal assays have been carried out previously; however, antifungal activity against *Aspergillus niger* has not been reported so far. Therefore in the present study, we have taken methanolic and acetone extracts of *Tinospora cordifolia* (stem) and *Punica granatum* (peel) for anti-fungal activity assay against *Aspergillus niger* (ATCC 16404).

MATERIALS AND METHODS

The plant materials of *Tinospora cordifolia* (stem) and *Punica granatum* (peel) were collected from Haffkine Institute campus. The stem (*Tinospora cordifolia*) and peel (*Punica granatum*) were cut into small pieces

¹Department of Bacteriology, Haffkine Institute, Mumbai, India

²Department of Zoonosis, Haffkine Institute, Mumbai, India; *Corresponding author

and shade dried at room temperature for fifteen days. Finely powdered plant materials were successively extracted with organic solvent methanol and acetone using soxhlet apparatus. The extractive values of both plant species in methanol and acetone were determined to examine the suitability of appropriate organic solvent. The different extracts obtained were subsequently concentrated under reduced pressure to obtain their corresponding residues

Anti-fungal activity against *Aspergillus niger* (ATCC 16404) was carried out using the standard method^[10]. Initial dilution of the extracts was done using DMSO (Di Methyl Sulphoxide) and further anticipated dilution was carried out using sabraouds broth as a growth medium. In the mixture of diluted extract and broth medium, conidial suspension of *Aspergillus niger* (ATCC 16404) (density: $2.0 \times 10^6/\text{mL}$). Miconazole was used as the standard compound. All the tubes were kept at 28°C for incubation for 72 hrs to observe growth or inhibition.

RESULTS

The maximum extractive value in the present investigation was found in methanolic extract of *Punica granatum* (peel:11.53%) among the samples tested (Table 1).

Extract	Extractive value of <i>T. cordifolia</i>	Extractive value of <i>P. granatum</i>
Acetone	0.80%	1.7%
Methanol	0.338%	11.53%

Table 1: The extractive value of different extracts of *T. cordifolia* and *P. granatum*

All the samples showed Minimum Inhibitory Concentration (MIC) at 70 µg/mL concentration against *Aspergillus niger* (ATCC 16404) (Table 2).

Extract	Concentration			
	60 (µg/ml)	70 (µg/ml)	80 (µg/ml)	100 (µg/ml)
<i>T. cordifolia</i> Acetone	+	–	–	–
<i>T. cordifolia</i> Methanol	+	–	–	–
<i>P. granatum</i> Acetone	+	–	–	–
<i>P. granatum</i> Methanol	+	–	–	–

Table 2: Anti-fungal activity of *T. cordifolia* and *P. granatum* against *Aspergillus niger*
Key: + = growth; – = no growth

DISCUSSION

Pathogens belonging to fungal genera *Aspergillus* are ubiquitous and global in distribution^[4]. These infections, become invasive and disseminate from the primary site of infection to other parts of the body including the gut, kidney, spleen and brain.

Invasive aspergillosis is reported to be associated with a mortality rate of 55 %^[5]. Mortality due to *Aspergillus* infection in bone marrow transplant recipients was observed to be as high as 80 % despite appropriate chemotherapy^[6]. Amphotericin B, which is considered to be the drug of choice, has been found to be highly nephrotoxic and less effective in invasive Aspergillosis^[7].

The obtained results highlighted the interesting anti-fungal potency of *Tinospora cordifolia* (stem) as well as *Punica granatum* (peel) and provided scientific basis for the traditional use of this species. The result shows that both extracts have the potential to inhibit the growth of *Aspergillus niger* (ATCC 16404). The results of the present investigation is successful in identifying the nature of the bioactive principle and its solubility, which will help in further isolation and characterization of the active principle responsible for antifungal activity. Thus a new source to combat Aspergillosis, a disease caused by *Aspergillus niger* has been investigated.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

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*Address Correspondence to:

Dr. Pratibha Chaturvedi
Department of Zoonosis
Haffkine Institute
Acharya Donde Marg, Parel
Mumbai 400012, India

e-mail: pratibha.c@rediffmail.com

Chemosensory Basis of Mosquito Oviposition Behaviour in *Aedes Aegypti*

Harshad Mayekar¹, Saroj Bapna¹, Meera Ramaiya¹ and Abhay Chowdhary^{1*}

ABSTRACT:

Introduction: Mosquito oviposition is a complex behaviour involving sensory modalities of vision, olfaction, contact chemosensation and mechanosensation. In order to choose a site suitable for egg-laying, a female mosquito must assess its surroundings taking into account several parameters. This is largely achieved by the sensillae/hair present on the body of the mosquitoes. We have tried to investigate the presence of chemosensillae on female *Aedes aegypti* mosquito to understand their function in oviposition behaviour.

Materials and Methods: Chemosensillae were stained using silver staining protocol which differentiates porous chemosensillae from the aporous mechanosensillae. These chemosensillae appear brown in colouration as the stain penetrates the epicuticle.

Results: Peg-shaped, and bristle-shaped chemosensillae were observed on the antennae while slender chemosensillae found on the 5th tarsomere of the meso-thoracic leg.

Conclusion: Chemosensillae play a crucial role for detecting volatiles in the life-cycle of mosquitoes. Chemosensillae on the antennae are likely to be olfactory and useful in the detection of volatiles which would aid in foraging for a suitable site for egg-laying. However contact chemosensillae such as those observed on the tarsomere would help in probing the actual surface where eggs are to be deposited. It is only after this final assessment that the female decides to lay eggs on the probed surface. Knowledge of these sensory abilities of the organism can help in developing strategies like ovitraps to control their populations.

Keywords: Sensillum, olfaction, contact chemosensation, oviposition, chemosensillae

INTRODUCTION

Vector control remains a primary strategy to control mosquito-borne diseases. Oviposition/egg-laying plays a significant role in the population dynamics of mosquitoes [1]. Understanding the sensory basis of the chemical cues perceived by the mosquito during oviposition would aid in their control measures.

Sensory cues play a crucial role in survival, propagation and also the disease transmission cycle of mosquitoes. These physical and chemical cues from the environment are detected by structures known as sensillae. While aporous mechanosensillae detect physical cues like wind-direction, balance during flight, pressure etc; volatiles are detected by chemosensillae. These are porous for chemicals to enter through them. Upon entering the sensillum, the molecule binds to its specific receptor which further sends a signal to the nervous system. After processing the stimulus an appropriate action is elicited [2].

Chemosensillae may be further classified based on the distance from which a stimulus is perceived. Perception of a chemical from a further distance is known as olfaction whereas that from close quarters/touch is known as contact chemoreception. This may also be termed gustation [3].

This interaction with the surrounding via sensillae becomes important during oviposition as the female mosquito forages for a suitable site for laying eggs. In order to ensure maximum survival of larvae to adulthood, a female mosquito must choose a site which is free from predators, has enough nutrition and lesser competition amongst sibling species [4].

In *Aedes aegypti* mosquitoes, eggs are laid in instalments and on a damp surface just above the water level [5]. The sensillae present on the different body-parts have been accounted for in literature [6]. We have attempted to investigate the presence of chemosensillae, which are likely to be involved in oviposition, by the simple technique of silver staining.

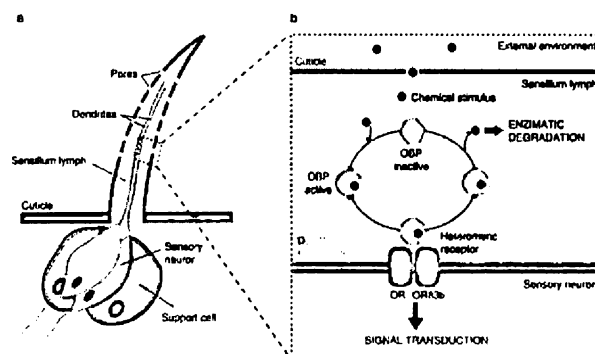


Figure 1: a) Porous nature of a chemosensillum b) Signal transduction cascade on arrival of a stimulus Adapted from A Sanchez-Gracia et al [7]

MATERIALS AND METHODS

The method of silver staining used was first employed in *Drosophila* by Venkatesh and Singh [8]. The protocol was modified at Life Sciences Department, Sophia College, Mumbai for mosquitoes [9]. Ionic silver diffuses into the epicuticle and aggregates into larger grains and is known as argentaffate layer [10]. Only chemosensillae which are porous get stained by this technique as compared to mechanosensillae which are aporous.

¹Department of Zoonosis, Haffkine Institute, Mumbai, India

***In vitro* susceptibility of Influenza A viruses to Amantadine Hydrochloride**

Sandeepan Mukherjee¹, Soumen Roy², Ritwik Dahaké¹ and Abhay Chowdhary^{1,2*}

ABSTRACT

Amantadine and Oseltamivir is the mainstay in the treatment of Influenza A Virus infections. However the emergence of drug resistant strains of the Influenza Viruses is proving to be a cause of concern, as not many chemo therapeutic options are available against the Virus. In the present study we carried out the *in vitro* susceptibility assay on the circulating strains of Influenza A viruses to (Amantadine HCl). (H1N1/Pune/2009 & H3N2/Pune/2010) at an initial Hemagglutination (HA) titre of 1:8 were seeded separately on in 24 well plates. The viruses were allowed to adsorb for 1 hour, following which Amantadine HCl in various test concentrations were added with the medium. The system was incubated for 5 days and the virus titre was estimated by. The test viruses were inhibited at different concentrations of Amantadine HCl. Influenza A (H1N1) showed complete inhibition at 5 µg/mL and above. Influenza A (H3N2) showed complete inhibition at 1 µg/mL and above. The results indicate the possibility of of the Influenza Virus strains used in the study to Amantadine HCl. However genetic analysis needs to be performed for confirming the same

Keywords: Amantadine Hydrochloride, Influenza A, *in vitro* susceptibility, MDCK cells, Haemagglutination Assay

INTRODUCTION

The influenza Viruses are a major cause of acute respiratory viral infections in man. Apart from vaccination, anti-virals serve as a major strategy in the control and management of influenza infections. However the emergence of drug resistant variants of influenza to both the available classes of anti influenza poses a serious concern to the chemo-therapeutic management of the infection in times to come^[1,2,3].

Some epidemiological studies into amantadine-resistance reported a low frequency of around 1% (16/2017) of resistant variants among H1N1 and H3N2 viruses in the period from 1991 to 1995^[4], but subsequently, dramatic increases appeared: 16% of H1N1 and 91% of H3N2 viruses were found to be resistant during the 2005–2006 seasons around the world. Although the estimate for the proportion of amantadine-resistant H1N1 variants was quite low, a comprehensive analysis in China showed that the frequency of amantadine-resistant H1N1 viruses had greatly increased from 28% (8/29) in the 2004–2005 season to 72% (33/46) in the 2005– 2006 season. Similar studies were performed in other countries in the 2005–2006 seasons, and the following frequencies were obtained: 45% (13/29) in Europe, 24% (4/17) in Taiwan, and 33% (1/3) in Canada^[5].

Continual Genotypic and Phenotypic monitoring of the prevalent strains of Influenza Viruses is critical to the Chemo-prophylactic management of Influenza virus infections. The present study intended to study the *in vitro* susceptibility of prevalent strains of

Influenza A viruses to Amantadine Hydrochloride (Amantadine HCl).

MATERIALS AND METHODS

Procurement of Viruses:

Influenza A H1N1/Pune/2009, Influenza A H3N2/Pune/2010 were procured from National Institute of Virology (NIV), Pune.

Maintenance of Cell Lines:

Madin Darby Canine Kidney (MDCK) cell line, procured from NIV was maintained in Minimal Essential Medium (MEM), containing 0.15% L-Glutamine, 1.0% NaHCO₃ and 10.0% Fetal bovine serum (FBS). The flasks were incubated at 37°C, in 5% CO₂ incubator for 2–3 days until a confluent monolayer was obtained^[6].

***In vitro* susceptibility assay:**

Influenza A (H1N1/Pune/2009 & H3N2/Pune/2010) at an initial Haemagglutination (HA) titre of 1:8 were seeded separately on MDCK cells in 24 well plates with MEM containing 0.15% L-Glutamine, 1% NaHCO₃ and 10% FBS. Additionally 2 µg/mL of TPCK was added for viral adsorption. The viruses were allowed to adsorb for 1 hour, following which Amantadine Hydrochloride in various test concentrations were added with the medium. The system was incubated for 5 days and the virus titre was estimated by Haemagglutination Assay.

RESULTS

The following results were obtained after performing the Haemagglutination assay on the test viruses.

¹Department of Virology, Haffkine Institute, Mumbai, India; *Corresponding author

²Department of Chemotherapy, Haffkine Institute, Mumbai, India

Amantadine HCl Concentration (µg/mL)	HA Titre	
	A (H1N1)	A (H3N2)
10.0	-	-
5.0	-	-
2.5	1:4	-
1.0	1:4	-
Virus Control	1:16	1:16

Table 1: HA titres of test and control viruses

DISCUSSION

The test viruses were inhibited at different concentrations of Amantadine HCl. Influenza A (H1N1) showed complete inhibition at 5.0 µg/mL and above. Influenza A (H3N2) showed complete inhibition at 1 µg/mL and above. It may be therefore concluded that the Influenza Viruses under this study were susceptible to Amantadine HCl. This may be useful in at least limiting virus shedding during initial phase of the disease although clinical evaluation shall be needed to establish this theory. Drug Sensitivity or Resistance patterns may however be need to be substantiated by sequence analysis of the Infuenza M2 gene.

AUTHOR DISCLOSURE STATEMENT

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*Address Correspondence to:

Dr. Abhay Chowdhary
Department of Biochemistry
Haffkine Institute
Acharya Donde Marg, Parel
Mumbai 400012, India

e-mail: haffkineinstitute@gmail.com

DR. WALDEMAR MORDECAI HAFFKINE



Dr. Waldemar Mordecai Haffkine

Born: 15 March 1860; Odessa, Russian Empire; Died: 26 October 1930; Lausanne, Switzerland

"The journey we make here upon the earth is so short. Before we know where we are, we are at the end, and called upon to answer an inner voice: 'Have you finished the work you had to do?' Happy are they who can think, yes, they have finished their work."

~ Dr. Waldemar Mordecai Haffkine

Dr. Waldemar Mordecai Haffkine—a Ukrainian scientist was a bacteriologist who worked mainly in India and developed vaccines against cholera and bubonic plague.

The son of a Jewish schoolmaster, WM Haffkine was born in the Black Sea port of Odessa, but received most of his early education in Berdiansk. Dr. Haffkine then entered the University of Odessa in 1879 to study physics, mathematics and zoology. He came under the influence of Professor Elie Metchnikoff and developed an interest in unicellular organisms. In 1883 Haffkine was awarded the degree of Candidate of Natural Sciences and became an assistant to the Zoological Museum of Odessa with the use of a research laboratory.

In 1889, he moved to Paris and started working in Pasteur's world famous laboratory. His initial work on producing a cholera inoculation was successful. He produced an attenuated form of the bacterium by exposing it to blasts of hot air. A series of animal trials confirmed the efficacy of the inoculation. In July 1892, Haffkine performed his first human test: on himself! During the Indian cholera epidemic of 1893, he travelled to Calcutta and introduced his new prophylactic inoculation. After initial criticism by the local medical bodies, it was widely accepted.

The then Governor of Bombay invited Dr. Haffkine to Bombay in 1896 after an epidemic of plague broke out in the city. He found a place to work at the JJ Group of Hospitals where he developed a plague vaccine. On the 10th January 1897, Dr. Haffkine caused himself to be inoculated with 10 cc of a similar preparation, thus proving in his own person the harmlessness of the fluid. A form useful enough for human trials was

ready by January 1897, and tested on volunteers at the Byculla jail the next month. Use of the vaccine in the field started immediately.

Recognition followed quickly. In 1898, the laboratory moved to Khushru lodge owned by Sir Sultan Shah, Aga Khan III, KCIE, head of the Khoja Mussulman community. This bungalow was fitted up at the Aga Khan's expense for Haffkine's use and about half the Khoja Mussulman community of Bombay (10,000–12,000 persons) received prophylactic inoculations under the auspices of His Highness the Aga Khan.

In 1885, the Governor left his residence and the premises were used as House of Records of the Bombay Presidency. In 1895, King Edward VII visited India as Prince of Wales, and stayed on the premises for a week. On such a historical premise, Dr. Haffkine entered on 10th August 1899. At that time it was designated as 'Plague Research Laboratory' with Dr. Haffkine as its Director in Chief. In 1906 the Institute was renamed as 'Bombay Bacteriology Laboratory'. Finally, in 1925, due to the efforts of Lt. Col. F.P. Mackie, the Laboratory was aptly named as '**Haffkine Institute**'.

After his retirement in 1914, he returned to France and settled in Boulogne-sur-Seine, and occasionally wrote for medical journals. In 1925, when the Plague Research Laboratory in Bombay was renamed the 'Haffkine Institute', he wrote "...the work at Bombay absorbed the best years of my life...". He revisited Odessa in 1927, but could not adapt to the tremendous changes after the revolution. He moved to Lausanne in 1928 and remained there for the last two years of his life.

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