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Editorial

Dear Researchers and Readers

The prevailing pandemic has led to a paradigm shift in educational pedagogy and to a 'New Normal' in the execution of the teaching-learning process. This phase has been difficult for the human race with uncertainties looming over the development of a vaccine against COVID-19. It would not be out of place to appreciate the response of authors in contributing manuscripts even during this crisis when laboratory research and experimentation have been limited due to health regulations and protocols. The quality articles that we received for the present issue of the journal speaks immensely of the dedication and commitment of researchers to move on with the changing times.

The content of the present volume begins with the article on "**The deadly dozen: An overview of the top killer viruses**" by D. Syiem and Mayashree B. Syiem present a comprehensive review of viruses and emerging diseases that is being witnessed in the present times with reference to COVID-19.

Reema Vareen Diengdoh and co-authors highlight the importance of the endangered lady`s slipper orchid and how "***In vitro* seed storage of *Paphiopedilum villosum* Lind., an endangered lady's slipper orchid**" can assist in conservation of this economically important plant.

"**Colorimetric detection of Pb^{2+} ions using PVP-capped silver nanoparticles**" by Siewdorlang Diamai and Devendra P. S.Negi present a research paper on colloidal silver nanoparticles (AgNPs) synthesis using polyvinylpyrrolidone (PVP) as a capping agent and the mechanism of aggregation of the AgNPs in the presence of Pb^{2+} ions.

Casterland Marbaniang and co-authors present an in-depth discussion based on the findings on "***In-vitro* comparative studies of *Apium graveolens* L. extracts for antioxidant and anti-inflammatory activity**" wherein the plant`s secondary metabolites using various solvent extracts are compared as antioxidant and anti-inflammatory agents.

"**Photic and nonphotic cues in regulation of seasonal reproduction in birds**" by Anand S. Dixit, Debashish Khanikar and Bidisha Kataki present a review on the recent advances on the roles of photic and non-photic cues in regulation of seasonal reproduction and associated functions in birds highlighting annual variation in day length as the most consistent and reliable cue in timing life history stages including reproduction, molt and migration in most birds.

"**Multi-spectroscopic investigation on the contradictory relevance of metal nanoparticles in pharmacological milieu of protein glycation and cholinergic inhibition**" by Prayasee Baruah, Imocha Rajkumar Singh and Sivaprasad Mitra highlights how unique physico-chemical behaviour of engineered nanoparticles have brought about a large-scale revolution in many fields like medicine, industry, agriculture and technology,

simultaneously may also result in certain toxicity due to unsolicited interactions with diverse biological systems and cellular processes.

Uma Shankar in his research article "**First record of new 'invasion' in northeast India by *Centaurea cyanus* L. (Asteraceae)**" reports the first record of the herbaceous plant, Centaurea cyanus L. of Asteraceae family from northeast India with an extensive search in the floras of the region.

The present issue contains articles drawn mainly from Life Sciences and Physical Sciences and to have received papers from eminent scholars in their specialization is indeed encouraging and motivating. However, realizing the time constraints and other engagements that they are faced with to write full length articles, we intend to include short commentary by invitation from them on specific topics for future issues.

Before I conclude, I would like to place on record my gratitude to the reviewers of the articles for giving valuable comments with notable promptness. Suggestions, cooperation and contributions of the editorial members have always been a source of guidance and strength. I earnestly appeal to scholars to submit/continue submitting manuscript(s) for publication in future issues of The NEHU Journal.

Stay safe

Prof. S.R. Joshi
Editor

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The deadly dozen: An overview of the top killer viruses

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Abstract

The present review was inspired by the COVID-19 pandemic that is presently the cause of great concern worldwide. Viruses have been part and parcel of living entities since life existed on earth. Over the centuries there has been records of numerous instances of noticeable viral attacks on humans that resulted in significant number of fatalities. Earliest records of a viral infection was found in Egypt during 1580–1350 BC and amongst the oldest that infect humans are smallpox and measles viruses. Viral epidemics came to prominence when early humans started to live in communities increasing the chances of close contact. As human behavior evolved living in agrarian societies, so did viruses. Included in this review are some of the aspects of what a virus is, how a virus infects and how the viral infection is detected. Finally details of twelve different killer viruses that are the causative agents of some of the fatal diseases in humans are examined.

Keywords: Virus; Pandemic; Epidemic; Polymerase chain reaction; Nucleotide sequencing.

Introduction

A virus is a submicroscopic infectious agent that can only replicate inside living cells of an organism. Viruses cause infections and diseases to all life forms from microorganisms including bacteria and archaea, to plants and animals (Koonin *et al.,* 2006). They have over the centuries resulted in millions of deaths. Epidemics caused by viruses began when human behavior changed during the Neolithic period, around 12,000 years ago, as humans developed more densely populated agricultural communities (McMichael, 2004). Earlier, humans lived in small, isolated communities, and most epidemic diseases did not exist. However, with increasing concentration and localization of people viruses spread rapidly and subsequently became endemic. Viruses of plants and livestock also increased as humans became dependent on agriculture and farming. Humans have been battling viruses since before our species had even evolved into its modern form. Among the earliest records of a viral infection is in Egypt from the 18th dynasty (1580–1350 BC) (Drutz and Ligon, 2000). Smallpox and measles viruses are among the oldest that infect humans. Having evolved from viruses that infected other animals, they first appeared in humans in Europe and North Africa thousands of years ago. The viruses were later carried to the New World by Europeans during the time of the Spanish

Conquests, but the indigenous people had no natural resistance to the viruses and millions of them died during epidemics. Influenza pandemics have been recorded since 1580 (Potter, 2001), and they have occurred with increasing frequency in subsequent centuries. The influenza pandemic (January 1918 – December 1920, known as Spanish flu) was an unusually deadly pandemic, the first of the two pandemics involving H1N1 influenza virus in which 40–50 million died in less than a year, was one of the most devastating in history ((Johnson and Mueller, 2002). The nature of viruses remained unknown until the invention of the electron microscope in the 1930s, when the science of virology gained momentum (Brenner and Jorne, 1959; Oldstone, 2014). For some viral diseases, vaccines and antiviral drugs have allowed us to keep infections at bay, and have helped sick people recover. But we're a long way from winning the fight against viruses. In recent decades, several viruses have jumped from animals to humans and triggered sizable outbreaks, claiming thousands of lives. The Ebola outbreak (2014-2016) in West Africa killed up to 90% of the people it infected, making it the most lethal member of the Ebola family (Ebola Virus disease in West Africa, WHO, 2014). Ebola virus disease (EVD), is a viral haemorrhagic fever of humans and other primates (Ebola Virus disease in West Africa, WHO, 2014; Centre for Disease Control and Prevention Ebola Otbreaks, 2000-2014; Cordelia et al. 2017). The novel coronavirus currently driving the outbreaks around the globe, had lower fatality rates, but still pose a serious threat to public health as it is highly infectious and we don't yet have the means to combat it. In this write-up we present 12 worst killers as well as some known facts about the Covid - 19 virus that has taken the world by storm and yet to be fully understood.

The first question that comes to mind is what a virus is and how does it infect and produce disease conditions. To begin with, a virus isn't "alive" in a typical sense. By definition, viruses are the smallest and simplest entities lacking an energy-generating system and having very limited biosynthetic capabilities (Feischmann, 1996). It is just a collection of genetic material (DNA or RNA) varying in size and complexity and a small toolbox of proteins. The blueprint for the structure and functioning of a virus is contained in its genetic material. The smallest viruses may have only a few genes while the largest viruses have as many as 200 (Gelderblom, 1996; Koonin *et al.* 2015). The proteins are used to perform two selfish tasks: 1. to get inside the cells of its host and 2. to hijack that cell's own genetic machinery in order to produce thousands of copies of itself.. In size, most viruses vary in diameter from 20 - 400 nm; the largest, however, measures about 500 nm in diameter. Only large viruses can be seen under the light microscope at the highest resolution. A virus propagates by infecting a living organism (host) and it does so by "commandeering" the host cell machinery. A virus basically reprograms the host cell to become a virus factory. Since viruses contain genetic material, like cell based living entities viruses have genetic variation and can evolve to newer forms. So, even though they don't meet the definition of life, viruses seem to be in a "questionable" zone. Viruses come in different shapes and structures and are very diverse. It is estimated that there are roughly 10^{31} viruses (Microbiology by numbers, 2011). Does that mean there are 10^{31} viruses just waiting to infect us? Actually, most of these viruses are found in oceans,

where they attack bacteria and other microbes. Every kind of living organism is probably host to at least one virus. Most viruses cause disease, and they are usually quite specific about the area of the body that they attack, for example, the liver, the respiratory tract, or the blood. Common viruses include Influenza, Herpes zoster, HIV, the common cold, and the rabies virus. Although viruses vary in their sizes, shapes, and life cycles, they have a few key features in common (Flint *et al*., 2003). These include: 1. A protective protein shell, or capsid 2. A nucleic acid genome made of DNA or RNA, tucked inside of the capsid 3. A layer of membrane called the envelope (not all viruses). Replication of the viral genome is essential for inheritance and continuity. Gene expression usually involves converting DNA into messenger (what is known as transcription) RNA (mRNA) and translation of mRNA into protein. This information could get altered if the DNA/ RNA undergoes changes. Like all other organisms, viruses are subjected to genetic alterations in two primary ways which determine their infectivity. 1. Errors made during the replication process called mutations (and RNA viruses in particular are known to be bungling replicators as they undergo a lot of mutations). Since viruses reproduce in such massive numbers, eventually a "winning" combination comes up and a new viral strain is born. 2. The second way that viruses can acquire new infective capabilities is known as "reassortment" i.e. when a mammal has the misfortune of being infected with two or more (say respiratory) viruses simultaneously, then as these viruses replicate, their genome parts can be shuffled together and then recombine/exchange (de Silva *et al*., 2012). An example is H1N1 virus which combined the swine, human and an avian influenza (triple reassortment) (Newman *et al.,* 2008). That's how the swine H1N1 pandemic was born.

Thus comes the next question- how does a virus infect? For a typical virus, the lifecycle can be divided into five broad steps (Payne, 2017) (though the details of these steps will be different for each virus): 1. Attachment: The virus recognizes and binds to a host cell *via* a receptor molecule on the cell surface (the corona virus bind to the angiotensin converting enzyme-2 which serves as the receptor). 2. Entry: The virus or its genetic material enters the cell. One typical route for viral entry is fusion with the host cell membrane, which is most common in viruses with envelopes. Viruses may also trick the cell into taking them in by a bulk transport process called endocytosis. 3. Next, the viral genes are expressed to make viral proteins. This step involves copying the viral genome and making more viral proteins, so that new virus particles can be assembled. The materials for these processes (such as nucleotides to make new DNA or RNA) come from the host cell, not the virus. Most of the "machinery" for replication and gene expression is also provided by the host cell. However, certain steps, such as the copying of an RNA virus's genome, cannot be performed by host cell enzymes. In such cases, the viruses must encode their own enzymes. All viruses must encode capsid proteins, and enveloped viruses typically also have to encode envelope proteins (which often aid in host recognition). Viruses may also encode proteins that manipulate the host genome (e.g., by blocking host defenses or driving expression of genes to benefit the virus), help with viral genome replication, or play a role in other parts of the viral lifecycle. 4. Assembly: New viral particles are assembled from the genome copies and viral proteins synthesized.

During assembly, newly synthesized capsid proteins come together to form the full-sized capsid. 5. Release: The last step in the virus lifecycle is the release of newly made viruses from the host cell which then infect other cells. Different types of viruses exit the cells by different routes: some make the host cell burst (a process called lysis), while others exit through the cell's own export pathways (exocytosis), and some bud from the plasma membrane, taking a patch of it with them as they go. In some cases, the release of the new viruses kills the host cell. In other cases, the exiting viruses leave the host cell intact so it can continue turning out more virus particles.

So how are viruses detected? In the diagnostic laboratory virus infections can be confirmed by a multitude of methods. Diagnostic virology has changed rapidly due to the advent of molecular techniques and increased clinical sensitivity of serological assays. Several types of tests may be used to check for viruses (Storch, 2000): 1. Viral culture, 2. Antibody test (Antibodies are substances made by the body's immune system to fight a specific viral infection), and 3. Viral DNA or RNA detection test.

Viral culture

The method first involves virus isolation**.** Viruses are often isolated from the initial patient sample. This allows the virus sample to be grown into higher quantities and allows a larger number to tests to be run on them. This is particularly important for samples that contain new or rare viruses for which diagnostic tests are not yet developed. Many viruses can be grown in cell culture in the laboratory. To do this, the virus sample is mixed with cells (for e.g. Vera cell line), a process called adsorption, after which the cells become infected and produce more copies of the virus. One means of determining whether the cells are successfully replicating the virus is to check for changes in cell morphology or for the presence of cell death using a microscope.

Nucleic acid based methods

Molecular techniques are the most specific and sensitive diagnostic tests. They are capable of detecting either the whole viral genome or parts of the viral genome. The much talked about method of detection is the **Polymerase Chain Reaction** (PCR) based on nucleic acid amplification. This technique basically involves the use of a thermocycler (PCR machine), the genetic material RNA or DNA which is to be amplified, the enzyme and substrates required for building the new viral DNA or RNA and other ingredients like fluorescent dyes (which helps in detection). Variations of PCR such as real time PCR is a qualitative and quantitative method to determine viral loads in patient serum. **Nucleotide sequencing** is another diagnostic method that offers the full sequence of a virus genome. Hence, it provides the most information about very small differences between two viruses that would look the same using other diagnostic tests.

Microscopy based methods

Under this, **i**mmunofluorescence assay is commonly used to detect whether a virus is present in a tissue sample. These tests are based on the principle that if the tissue is infected with a virus, an antibody specific to that virus will be able to bind to it. To do this, antibodies that are specific to different types of viruses are mixed with the tissue sample. After this the tissue is exposed to a specific wavelength of light or a chemical that allows the antibody to be visualized (Cynthia *et al.,* 2009).

Host antibody detection

A person who has recently been infected by a virus will produce antibodies in their bloodstream that specifically recognize that virus. This is called humoral immunity. Two types of antibodies are important. The first called IgM is highly effective at neutralizing viruses but is only produced by the cells of the immune system for a few weeks. The second, called, IgG is produced indefinitely. Therefore, the presence of IgM in the blood of the host is used to test for acute infection, whereas IgG indicates an infection sometime in the past. Both types of antibodies are measured when tests for immunity are carried out. It can be done for individual viruses using a technique called ELISA (abbreviated for enzyme linked immunosorbent assay) but in automated panels that can screen for many viruses at once are becoming increasingly common. Virus detection by culture, PCR and serological studies have their places in the diagnosis of infection. In all instances of acute infection, virus detection using PCR is the method of choice, as antibody responses are much less informative. Indeed, in recurrent episodes, the antibody titre may not vary (Andeotti *et al.,* 2003). There are other tests like the complement fixation test and enzyme immune assays that are also used. The development of macro- and microarrays with several hundreds or thousands of probes allows for identification of viruses with only marginal homology to known taxa. Arrays present a powerful tool, allowing for broad spectrum detection of known and unknown viruses. The use of next -generation sequencing as a tool for virus detection and identification is also very much in use. Today, bioinformatics and structural analyses of putative proteins can lead to the discovery of previously unknown viruses. But this is another topic altogether.

Now that we are somewhat familiar with viruses, let us look into few viruses that are the causative agents of some of the lethal diseases in humans.

1. Marburg virus

Marburg is a highly virulent disease that causes hemorrhagic fever, with a fatality ratio of up to 88% (Brauburger *et al.,* 2012). The virus is a single stranded RNA virus first reported in 1967, when small outbreaks occurred among laboratory workers in Germany who were exposed to infected monkeys imported from Uganda. The viral outbreaks were then reported in the 1998-2000 in the Democratic Republic of Congo, as well as in the 2005 outbreak in Angola, according to the World Health Organization (WHO). Marburgvirus

genomes are approximately 19 kbp long and contain seven genes. Its infection in human was initially reported to be from prolonged exposure to mines or caves inhabited by Rousettus bat colonies. An infected individual can spread the virus by direct contact i.e., through human-to-human interaction (through broken skin or mucous membranes) with blood, secretions or other bodily fluids of infected people, and with surfaces and materials (e.g. bedding, clothing) contaminated with these fluids. The incubation period for the Marburg virus ranges from 2-21 days. The disease begins abruptly, with high fever, severe headache, muscle aches, abdominal pain, cramping and severe watery diarrhea. Nausea, vomiting and a non-itchy rash appear between 2 to 7 days after the onset of symptoms. Many patients develop severe hemorrhagic symptoms with bleeding from nose, gums and vagina. Fresh blood is also lost in vomitus. Death usually occurs due to severe blood loss and shock.

2. Ebola virus

The first known Ebola outbreaks in humans struck simultaneously in the Republic of the Sudan and the Democratic Republic of Congo in 1976 (Ebola Virus Disease in West Africa, WHO, 2014; Singh and Ruzek, 2014; Malvy *et al.,* 2019; Richardson et al. 2019). Ebola is spread through contact with blood or other body fluids, or tissue from infected people or animals. The outbreak also took place in West Africa in early 2014. Ebola virus is similar to Marburg in that both can cause hemorrhagic fever, meaning that infected people develop high fevers and bleeding throughout the body that can lead to shock, organ failure and death. The virus contains one molecule of linear, single-stranded, negative-sense RNA, 18,959 to 18,961 nucleotides in length. This viral genome codes for seven structural proteins and one non-structural protein. The fruit bats of the Pteropodidae family are natural hosts of Ebola virus. Ebola is introduced into the human population through close contact with the blood, secretions, organs or other bodily fluids of infected animals such as fruit bats, monkeys, chimpanzees, gorillas, forest antelope or porcupines found ill or dead in the rainforests. The disease causes very similar symptoms to Marburg virus. Its incubation period is 2-21 days. An Ebola infected person cannot spread the disease until they develop symptoms. Symptoms of EVD can be sudden and include: Fever, muscle pain, headache, fatigue and sore throat followed by diarrhoea, vomiting, development of rash and symptoms of kidney and liver failure. In many cases, there is bleeding from gums or blood in the stool.

3. Rabies

Rabies lyssavirus, formerly *Rabies virus*, is a virus that causes rabies in humans and animals (Abraham and Banerjee, 1976). Rabies is a preventable viral disease most often transmitted through the bite of a rabid animal. The rabies virus infects the central nervous system of mammals, ultimately causing disease in the brain and death. The vast majority of rabies cases reported to the Centers for Disease Control and Prevention (CDC) each year occur in wild animals like bats, raccoons, skunks, and foxes, although any mammal can

get rabies. Rabies virus belongs to the order Mononegavirales, are enveloped and carries a non-segmented, negative-stranded RNA genome (Anilionis *et al.,* 1981). The vaccines for pets, which were introduced in the 1920s, have helped make the disease exceedingly rare in the developed world, however the condition remains a serious problem in India and parts of Africa.

4. HIV

Today, HIV (human immunodeficiency virus), also a retrovirus (RNA) remains one of the largest pandemics in the world. HIV is a virus that attacks cells in the human immune system, such as helper T cells (specifically CD^{4+} T cells), macrophages, and dendritic cells that are vital for body to fight infection, making the infected person vulnerable to other infections and diseases. It is composed of two copies of positive-sense single-stranded RNA that codes for the virus's nine genes enclosed by a conical capsid composed of 2,000 copies of the viral protein (Muesing *et al.,* 1985; Lewis *et al.,* 1992). It is spread by contact with certain bodily fluids of an infected person most commonly during unprotected sex or through sharing injections. HIV is the same virus that can lead to AIDS (acquired immunodeficiency syndrome) which originated in non-human primates in Central and West Africa. While various sub-groups of the virus acquired human infectivity at different times, the global pandemic had its origins in the emergence of one specific strain – HIV-1 subgroup M – in Léopoldville in the Belgian Congo (now Kinshasa in the Democratic Republic of the Congo) in the 1920s (Keele *et al.,* 2006). An estimated 32 million people have died from HIV since the disease was first recognized in the early 1980s. The infectious disease that takes the biggest toll on mankind right now is HIV. Powerful antiviral drugs have made it possible for people to live for years with HIV. But the disease continues to devastate many low and middle-income countries, where 95% of new HIV infections occur. Nearly 1 in every 25 adults within the WHO African region is HIV-positive.

5. Smallpox

Smallpox is a highly contagious and deadly disease that was estimated to have infected 300 million people in the 20th Century before it became the only human infectious disease ever to be completely eradicated. Smallpox is caused by the agent referred to as the *variola* virus (VARV). *Variola* is a large brick-shaped virus, with a single linear double stranded DNA genome (Massung *et al.,* 1994). The two classic varieties of smallpox are *variola* major and *variola* minor. The occurrence of smallpox extends into pre-history, the disease emerged in human populations about 10,000 BC. The earliest credible evidence of smallpox is found in the Egyptian mummies of people who died some 3000 years ago (Barquet and Domingo, 1997). Smallpox has had a major impact on world history.

Viral transmission occurred through inhalation of airborne *Variola* virus, usually droplets expressed from the oral, nasal, or pharyngeal mucosa of an infected person. The initial symptoms of the disease included fever and vomiting, followed by formation

of sores in the mouth and a skin rash that turns into characteristic fluid-filled bumps with a dent in the center. The bumps then scabbed over and fell off, leaving scars. The last naturally occurring case of small pox was diagnosed in October 1977 and the WHO certified the global eradication of the disease in 1980 (Fenner *et al.,* 1988).

6. Hantavirus pulmonary syndrome (HPS)

Hantavirus Pulmonary Syndrome (HPS) is a severe, sometimes fatal, respiratory disease in humans caused by infection with hanta viruses (Factsheets, Centre for disease Control and Prevention, 2016). Hantaviruses belong to the bunyavirus family of viruses. They are enveloped viruses with a genome that consists of three single-stranded RNA segments designated S (small), M (medium), and L (large). All hantaviral genes are encoded in the negative (genome complementary) sense. The complete genome is 11800-13800 nucleotides long. The virus can be transmitted to humans by a direct bite or inhalation of aerosolized virus, shed from stool, urine, or saliva from a rodent. Rodent infestation in and around the home remains the primary risk for hanta virus exposure. It first gained wide attention in the U.S. in 1993, when a healthy, young Navajo man and his fiancée living in the United States died within days of developing shortness of breath. A few months later, health authorities isolated hanta virus from a deer mouse living in the home of one of the infected person. HPS has an incubation phase of 2-4 weeks. Patients develop flu-like symptoms including fever, cough, muscle pain, headache, lethargy, shortness of breath, nausea, vomiting and diarrhea. The patient could rapidly deteriorate into acute respiratory failure, characterized by pulmonary edema, as well as cardiac failure, with hypotension, tachycardia and shock.

7. Influenza

Influenza commonly known as “the flu”, is an infectious disease caused by an influenza virus (World Health Statistics, 2018). The most common symptoms are high fever, runny nose, sore throat, muscle and joint pain, headache, coughing, and feeling tired. These symptoms typically begin within two days into exposure to the virus and most last for one to two weeks. Three of the four types of influenza viruses affect humans- Type A, Type B, and Type C (Longo, 2012; Types of Influenza Viruses Seasonal Influenza, 2017). The genome of influenza type A viruses consists of eight single-stranded RNA segments, and the viral particle has two major glycoproteins on its surface: hemagglutinin and neuraminidase. The Influenza B virus genome is 14,548 nucleotides long and consists of eight segments of linear negative-sense, single-stranded RNA. The multipartite genome is encapsulated, each segment in a separate nucleocapsid, and the nucleocapsids are surrounded by one envelope. Although influenza A and B virus genomes both comprise eight negative-sense, single-stranded viral RNA (vRNA) segments, influenza C virus has a seven-segment genome. Influenza spreads around the world in yearly outbreaks, resulting in about three to five million cases of severe illness and about 290,000 to 650,000 deaths according to WHO. Approximately 33% of people

with influenza are asymptomatic. But occasionally, when a new flu strain emerges, a pandemic results with a faster spread of disease and, often, higher mortality rates. The most deadly flu pandemic, called the Spanish flu, began in 1918 and sickened up to 40% of the world's population, killing an estimated 50 million people (Andrews 2016; History.com staff, 2018). It can be difficult to distinguish between the common cold and influenza in the early stages of these infections. Influenza symptoms are a mixture of symptoms of common cold and pneumonia, body ache, headache, and fatigue. The common cold, also known simply as a cold, is a viral infectious disease of the upper respiratory tract that primarily affects the nose. The throat, sinuses, and larynx may also be affected.

8. Dengue

Dengue virus (DENV) is the cause of dengue fever (Izabela *et al*., 2010). It is a mosquito-borne, virus whose genome is about 11000 bases of positive-sense, single stranded RNA (ssRNA) that codes for three structural proteins (capsid protein C, membrane protein M, envelope protein E) and seven nonstructural proteins (Kuhn *et al*., 2002). The first reported epidemics of dengue fever occurred in 1779-1780 in Asia, Africa, and North America; the near simultaneous occurrence of outbreaks on three continents indicates that these viruses and their mosquito vector have had a worldwide distribution in the tropics. The virus appeared in the 1950s in the Philippines and Thailand, and has since spread throughout the tropical and subtropical regions of the globe. The virus has increased dramatically within the last 20 years, becoming one of the worst mosquito-borne human pathogens in tropical countries (Cucunawangsih and Lugito, 2017). Current estimates indicate that as many as 390 million infections occur each year, up to 40% of the world's population now lives in areas where dengue is endemic, and the disease with the mosquitoes that carry it is likely to spread farther as the world warms (global warming). Dengue sickens 50 to 100 million people a year, according to WHO. Although the mortality rate for dengue fever is lower than some other viruses, at 2.5%, the virus can cause an Ebola-like disease called dengue hemorrhagic fever, and that condition has a mortality rate of 20% if left untreated. A vaccine for Dengue was approved in 2019 by the U.S. Food and Drug Administration for use in children between 9-16 years old living in areas where dengue is common according to the CDC (Center for Disease Control) (Centers for Disease Control and Prevention, NCEZID, 2019).

9. Rotavirus

Rotaviruses are the most common cause of diarrheal disease among infants and young children. The virus is transmitted by the fecal-oral route. It infects and damages the cells that line the small intestine and causes gastroenteritis (which is often called "stomach flu". Nausea, vomiting, watery diarrhea and low-grade fever are the characteristic symptoms of rotaviral enteritis. Once a child is infected by the virus, there is an incubation period of about two days before symptoms appear. There are ten species of rotavirus, referred to as A, B, C, D, E, F, G, H, I and J (Suzuki, 2019). Humans are

primarily infected by the species *rotavirus* A. The genome of rotaviruses consists of 11 unique double helix molecules of RNA (dsRNA) which are 18,555 nucleotides in total (Estes and Cohen, 1989). Twelve proteins are encoded by the rotavirus genome that help the virus to infect and reproduce within the host. Although children in the developed world rarely die from rotavirus infection, the disease is a killer in the developing world, where rehydration treatments are not widely available. Two vaccines are now available to protect children from rotavirus. WHO estimated that worldwide, 453,000 children younger than age 5 died from rotavirus infection in 2008. But countries that have introduced the vaccine have reported sharp decline in rotavirus hospitalizations and deaths.

10. Nipah virus

The Nipah virus (NiV) is a type of RNA virus placed in the newly created Henipavirus genus with the closely related Hendra virus and Cedar virus (Barry *et al.,* 2012). The Henipavirus family is pleomorphic, meaning their shape is varied, and traditionally 40 to 600 nm diameter. The core of a virion contains a linear ribonucleprotein (RNP) comprising of negative sense single stranded RNA. Nipah virus was first recognized in 1999 during an outbreak among pig farmers in Malaysia. In May 2018, an outbreak of the disease resulted in 17 deaths in the Indian state of Kerala (Chatterjee, 2018; Nipah Virus Infection, 2018). Nipah virus is a zoonotic virus and the viral transmission happens in humans from animals (such as bats or pigs), or from consuming contaminated foods or directly from human-to-human. The incubation period ranges from 4 to 14 days. Symptoms include fever, headaches, vomiting and sore throat. In severe cases this could be followed by dizziness, drowsiness, altered consciousness, and neurological signs that indicate acute encephalitis.

11. Corona viruses (SARS- CoV and MERS-CoV)

A. SARS- CoV

Among the corona virus infections in recent times **SARS- CoV, MERS-CoV** and **COVID- 19** have been in news due to their sudden appearance, rapid spread and catching us almost unprepared to mitigate the health threats they present. Severe acute respiratory syndrome coronavirus (SARS-CoV or SARS-CoV-1) is a strain of virus that causes severe acute respiratory syndrome (SARS) (Thiel, 2007; Fehr and Perlman, 2015). It infects the lungs. SARS first appeared in 2002 in Guangdong Province, China, and has spread to several countries. The severity of this disease is such that the mortality rate appears to be ~3 to 6%, although a recent report suggests this rate can be as high as 43 to 55% in people older than 60 years. The virus is considered to have come from bats, which then hopped into nocturnal mammals called civets before finally infecting humans. After triggering an outbreak in China, SARS spread to 26 countries around the world, infecting more than 8000 people and killing more than 770 over the course of two years (Little, 2020). It is an enveloped, positive-sense, single-stranded RNA virus which infects the epithelial

cells within the lungs (Macro *et al.,* 2003). The virus enters the host cell by binding to the angiotensin converting enzyme 2 (ACE2) receptor (Li *et al.,* 2005; de Groot *et al.,* 2013). Transmission of SARS-CoV is primarily from person to person. Symptoms of the disease are influenza-like and include fever, malaise, myalgia, headache, diarrhea and often progresses to pneumonia, a severe condition in which the lungs become inflamed and filled with pus. SARS has an estimated mortality rate of 9.6%, and as of yet, has no approved treatment or vaccine. However, no new cases of SARS have been reported since the early 2000s, according to the CDC.

B. **MERS-CoV**

Middle East respiratory syndrome (MERS), also known as camel flu, is a viral respiratory infection caused by the MERS-coronavirus (MERS-CoV) (Wog *et al.,* 2019; Fehr *et al.,* 2015). Typical symptoms include fever, cough, diarrhea and shortness of breath. The disease is typically more severe in those with other health problems and in severe cases kidney failure, disseminated intravascular coagulation (DIC), and pericarditis have also been reported. This virus sparked an outbreak in Saudi Arabia in 2012 and another in South Korea in 2015. The virus belongs to the same family of viruses as SARS-CoV, and likely have originated in bats, as well. MERS-CoV virus is an enveloped positive-sense single-stranded RNA belonging to the genus betacoronavirus which is distinct from SARS coronavirus and the common-cold coronavirus. It enters its host cell by binding to the DPP4 receptor (Fehr *et al*., 2015). The disease infected camels before passing into humans. MERS often progresses to severe pneumonia and has an estimated mortality rate between 30% and 40%, making it the most lethal of the known coronaviruses that jumped from animals to people. As with SARS-CoV, MERS has no approved treatments or vaccine.

12. SARS-CoV-2: the causative agent in the recent pandemic COVID-19

This is the virus that has gripped the world's apprehension towards its journey into future. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the virus strain that causes the respiratory illness coronavirus disease (COVID-19) (Gorbalenya *et al.,* 2020). It is colloquially known as the coronavirus, and was previously referred to by its provisional name 2019 novel coronavirus (2019-nCoV). The disease has first come into focus in the months of November- December, 2019 in Wuhan province in China. Since then the virus has travels throughout the world at an astonishing speed indicating its highly contagious nature. It is so contagious in humans that the World Health Organization (WHO) has designated the ongoing COVID-19 infection as pandemic and as Public Health Emergency of International Concern (WHO Director-General Opening Remarks at media briefing on COVID-19, 2020). As of 26 April 2020, more than 2.91 million cases of COVID-19 have been reported in 185 countries and territories, resulting in more than 203,000 deaths. SARS-CoV-2 is a positive-sense single-stranded RNA virus with its RNA sequence being ~ 30,000 bases in length. This

virus belongs to the same large family of viruses known as SARS-CoV (corona viruses) and of the subgenus *Sarbecovirus* (beta-CoV lineage B) (Mousavizadeh and Ghasemi, 2020; Sah *et al.,* 2020). The virus likely originated in bats, like SARS-CoV, and passed through an intermediate animal most probably a pangolin before infecting people. Intense research established human-to-human transmission of SARS-CoV-2 on 20 January 2020. The primarily mode of transmission between people is through close contact and via respiratory droplets produced from coughing, sneezing, or talking. Another probable cause of infection is indirect contact via contaminated surfaces. When it infects host cells, it replicates its genomic RNA (gRNA) and produces nine smaller RNAs known as subgenomic RNAs (sgRNAs) that are used for synthesizing various proteins- spike protein (S), envelope protein (E), membrane protein (M), nucleocapsid protein (N), and several accessory proteins. The gRNA is packaged by the structural proteins to assemble progeny virions which burst out of the host cells and repeat the cycles of infection (Chen and Guo, 2020). It mainly enters human cells by binding to the receptor angiotensin converting enzyme 2 (ACE2) (Lan *et al.,* 2020; Verdecchiaa *et al.*, 2020). Further, there is some indication of human-to-animal transmission of SARS-CoV-2, including examples in felids. Researchers have sequenced the viral genome from many infected persons and with sufficient number of sequenced genomes have constructed a phylogenetic tree of the mutation history of a family of viruses. A phylogenetic analysis of those samples showed that these were highly related and as of 27 March 2020, 1,495 SARS-CoV-2 genomes sampled on six continents were publicly available.

On 11 February 2020, the International Committee on Taxonomy of Viruses (ICTV) announced that according to existing rules that compute hierarchical relationships among coronaviruses on the basis of five conserved sequences of nucleic acids, COVID-19 has been placed as a strain of *Severe acute respiratory syndrome-related coronavirus*. Since its appearance, the virus has infected tens of thousands of people in China and thousands of others worldwide (the figure keeps rising). The ongoing outbreak prompted an extensive quarantine in almost all continents, restricting travel to and from affected countries and a worldwide effort to develop diagnostics, treatments and vaccines. The disease COVID-19 has an estimated mortality rate of about 2.3%. COVID-19 affects different people in different ways. People who are older or have underlying health conditions seem to be most at risk. Most infected people will develop mild to moderate symptoms. Common symptoms include fever and tiredness, dry cough and shortness of breath. Some people may experience aches and pains, nasal congestion, runny nose, sore throat and diarrhea. The disease can progress to pneumonia in severe cases. On average it takes 5–6 days from when someone is infected with the virus for symptoms to show, however it can take up to 14 days.

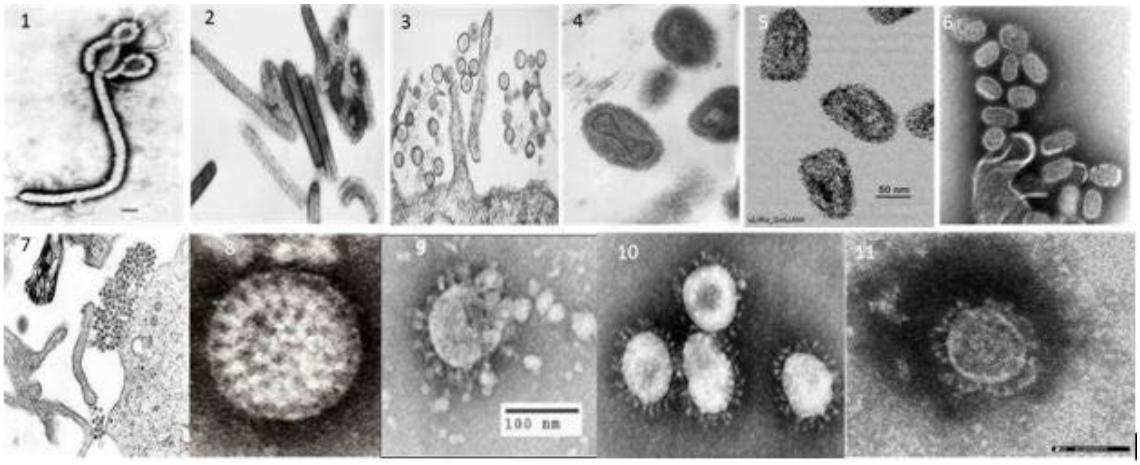

Figure 1: Electron microscopic pictures of different viruses- 1. Ebola 2. Marburg 3. Hantavirus 4. Small pox virus 5. Rabies virus 6. Influenza virus 7. Dengue virus 8. Rotavirus 9. SARS- CoV 10. MERS- CoV 11. SARS- CoV 2. (Micrographs courtesy: Wikipedia).

Mortality of any viral disease is calculated by dividing the number of patients who died by the number who have been infected. Mortality rates tend to range higher early on in an outbreak, because the denominator is falsely low. Without accurate diagnostic testing, the number of patients infected includes only those with obvious symptoms. This seems to be the case with COVID-19. Since symptoms alone make for a sketchy denominator, public health officials rely on laboratory verification of infection, but historically, viruses have been difficult to detect. Because they are hard to grow in a lab, the next best step is to look for antibodies against the virus. Unfortunately that can be inaccurate too as it often misses early infections because the body has yet to mount an antibody response.

In case of Covid-19 uncertainty over whether it is the virus itself or the response of a person's immune system that ultimately overwhelms a patient's organs, is making it difficult for doctors to determine the best way to treat people who are critically ill. There are reports suggesting that the immune system plays a part in the deterioration and death of people infected with the new coronavirus, and this has spurred a push for treatments such as steroids that rein in that immune response. Some of the earliest analyses of people with the coronavirus suggested that it might not be the virus alone that ravages the lungs and kills; rather, an overactive immune response might also contribute (COVID-19: Navigating Uncertainities Together, 2020). High blood levels of proteins called cytokines, which can ramp up immune responses have been reported. They include signaling protein called interleukin-6 (IL-6) which is a call to arms components of the immune system, including macrophages. Macrophage cells fuel inflammation and can damage normal lung cells. The release of cytokines known as a "cytokine storm", also occurs with other viruses. Thus, collateral damage from the immune response may aggravate the illness. There are no specific medicines available to treat COVID-19 as yet, desperate search for a vaccine and hundreds of studies are being carried out (Ledford, 2020). COVID-19

vaccine expected to come out in September may in fact be the most fast-tracked vaccine ever created in all history. For those interested they may look up - What Drugs May Fight Coronavirus COVID-19? Drug Trials, Treatments, Vaccines; reviewed by Charles Patrick Davis,(https://www.onhealth.com/content/1/what_drugs_fight_covid-19_treatments_vaccine_trial).

Conclusion

In conclusion, one can say that we have come a long way in combating various infectious agents. Through scientific pursuit we have learned a lot in terms of understanding the biology of viruses and other pathogens. The more we know the better equipped we are in coming up with therapeutic measures. However, in the virus we have a formidable enemy. Its simple structure probably aids in changing its form frequently which continually poses challenges in developing drugs and vaccines for different strains of the virus. In the end one can only conclude that pandemics of these kinds will continue to appear in the future, how much they kill will depend on the evolution of the strains, how virulent they are, their kill rate, modes of infection, how prepared we are and methods of containment and treatment.

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In vitro seed storage of *Paphiopedilum villosum* Lind., an endangered lady's slipper orchid

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Abstract

Urbanization and commercialization has adversely affected orchid's population. As a result, they are diminishing from the nature very rapidly. Terrestrial orchid, Paphiopedilum villosum (lady's slipper orchid) is one such with horticultural importance. Apart from being listed as endangered in IUCN red data list, it finds a place in Appendix I of CITES in the global platform. In vitro storage with complete regeneration protocol plays an important role for conservation of endangered species. Seed storage is the best option for conservation, however this can be accomplished only after standardizing the correct age of the seed, different temperatures for storage, optimal media for regeneration, etc. There are number of reports suggesting the feasibility of immature orchid seeds for storage. However, storage studies on mature dehisced orchid seeds are few. Therefore, in the present study, we attempted to standardize a protocol for storage of mature dehisced seeds of P. villosum at three different temperatures (0˚C, 25˚C and -196˚C) followed by post storage germination and regeneration studies. We report the feasibility of storing P.villosum seeds in LN (-196˚C), BG1 as the optimum germination medium for the seeds and MS medium for conversion into seedlings. MS medium incorporated with 20 µM BAP+5 µM IAA proved better for growth and development of the seedlings and hence can be used for multiple subcultures till the seedlings are ready to transfer.

Keywords: Endangered orchids, Mature seeds, Liquid Nitrogen, Storage studies

Introduction

The heterogeneous nature of seeds makes suitable for conserving genetic diversity of plant populations in nature. Orchid seeds too exhibit this nature in conjunction with other features like minute seed size and ample availability per capsule. Terrestrial orchids unlike their epiphytic counterparts, are difficult to germinate *in vitro* and fail to establish in soil on a large scale (Batty *et al.*, 2001comprising orchid (Caladenia arenicola; Swarts and Dixon, 2009; Zeng *et al.,* 2013). Besides other factors, prerequisite determination of correct age of the capsule is necessary for maximum *in vitro* germination of orchid seeds. Practically immature undehised capsules age of 4-6 months old are reported to have

high *in vitro* germination rate (Balilashaki *et al.,* 2015; Zhang *et al.,* 2013). On contrary, mature seeds of older capsules results in lower seed germination, due to dormancy owing to many factors of which lignification and cutinisation plays an important role (Yamazaki and Miyoshi, 2006). At the same time, mature orchid seeds may have a greater potential for propagation and storage as orchids have fully developed testa and lower water content and dormancy can be broken (Miyoshi and Mii, 1998; Hu *et al.,* 2013; Fu *et al.,* 2016).

Temperature plays an important role for seed storage. Different temperatures are reported to have potential for prolonged seed storage in different species. Ideally, seed storage in Liquid Nitrogen (-196^{0}C) is an efficient *ex situ* strategy to safeguard the species, but unfortunately, orchid seeds conservation is hampered by poor storage conditions and regeneration protocols that needs to be standardized (Long *et al.,* 2010; Merritt *et al.,* 2014a; Zeng *et al.,* 2015). The advantages of liquid nitrogen storage are: storage for an indefinite period, genetic stability of the individuals, reduced infrastructure, can have independent energy and the stored genetic material does not require manipulation (Cerna *et al.,* 2018).

Therefore, we attempted to store *Paphiopedilum villosum* mature seeds at different temperatures followed by viability test as well as *in vitro* germination after the storage to confirm its feasibility for *ex situ* conservation. *Ex situ* conservation offers not only safer security backup system for conservation but also allows accessibility for research work evaluation (Chugh *et al.,* 2009).

Materials and methods

Seed collection: Dehisced seeds of *P. villosum* were collected from desiccated and dehisced capsules in the polythene bags after 240 days of pollination (DAP). Approximately, 200 mg mature seeds were placed per 2 ml sterile cryovials (polypropylene, Tarsons Pvt. Ltd. Kolkata, India.) followed by fixation in cryocane (Tarsons Pvt. Ltd., Kolkata, India).

Storage of seeds: These cryovials were labelled and stored at different temperatures namely, 0˚C, 25˚C and LN (-196˚C). All seeds were stored for a total of 360 days at different temperature conditions.

Sterilization: Under sterile conditions, after every 30 days of storage, one vial each of stored seeds in 0˚C, 25˚C were sterilized with 3% NaOCl for 30 min followed by rinsing in sterile water. For the seeds stored in -196 ˚C, rewarming was done by dipping the cryovials in distilled water at 45°C for 2 min followed by the same procedure for sterilization.

Viability testing and *in vitro* germination: Simultaneously, after every storage interval, seeds from each storage conditions were divided into two equal proportional quantities. One part was subjected to viability test using 1%TTC (Vujanovic *et al.,* 2000) where, the seeds were soaked 24 h in 1% TTC solution. This was followed by slide observation where seeds showing some degree of pink or red colour were considered viable and scored

accordingly under the microscope. For each treatment, 6 replicates were maintained and the experiment was repeated thrice. The other part of the stored seeds after sterilization were inoculated on different media BG1, ½ MS (Murashige and Skoog), MS and BM terrestrial medium (BM) to assess the optimized growth and development of the seedlings at different stages.

For recording the 1% TTC viability test, the seeds were randomly removed and dispersed in a drop of water on a glass slide and observed under a light microscope. The percentage of seed germination was calculated using the formula:

$$\text{Viability \%} = \frac{\text{No. of pink or red seeds considered viable}}{\text{Total no. of seeds observed}} \text{ x } 100$$

Seeds were considered to have germinated upon emergence of the embryo from the testa (Kumaria and Tandon, 1991). Germination percentage was recorded 60 days after inoculation (DAI). For recording the germination percentage, the seeds were randomly removed and dispersed in a drop of water on a glass slide and observed under a light microscope.

The percentage of seed germination was calculated using the formula:

$$\text{Germination \%} = \frac{\text{No. of seeds showing emergences of the embryo from testa}}{\text{Total no. of seeds observed}}$$

Culture conditions and Statistical analysis: pH of the medium was adjusted to 5.8 using 1N NaOH prior to autoclaving at 15 psi, 121ºC for 15 min. All the cultures were incubated at 25±2ºC and 16 h photoperiod at 50 µM $m^{-2}s^{-1}$ light intensity. Ten replicates were maintained and the experiment was repeated thrice. Statistical analysis was done by analysis of variance (ANOVA) at $p \geq 0.05$ and means compared with Turkey's test using one-way ANOVA (PC version) Origin 8.0 NORTHAMPTON.

Results and discussion

Germination percentage ranging from 78.9 ± 2.6 - 81.5 ± 1.5 was recorded in mature seeds of *P. villosum* stored at -196 °C with similar viability percentage i.e., 78.7 ± 2.4% - 80.1 ± 1.9%, (pink colouration) over 360 days' storage (Figure 3b). The results showed consistency without any significant variation in both germination and viability percentage of mature seeds stored at -196 °C irrespective of different storage time suggesting the feasibility of mature seed storage upto and beyond 360 days. Immature seeds have the zygotic embryo which is not fully developed and the testa might not be lignified, allowing it to be permeable to water and nutrient. But in case of mature seeds, a fully formed testa and low water content may show superior potential for storage as reported earlier (Miyoshi and Mii, 1998; Zhang *et al.,* 2015; Fu *et al.,* 2016). Seeds or shoot tips have been successfully stored in liquid nitrogen (LN) providing protection with the ability for revival and plant regeneration when needed in future. Cryopreservation or LN storage has been used for seed and shoot-tip conservation, providing long-term storage (Engelmann, 2004; Li and Pritchard, 2009; Pritchard and Nadarajan, 2009; Reed

et al., 2011). Poor response was observed in seeds stored at 25 °C, both using TTC test as well as *in vitro* germination suggesting that at higher temperature the seeds are subjected to excessive dehydration stress leading to imbibition injury inducing rapid rehydration in free water as reported by Hirano *et al.* (2011).) Depending on the duration method and temperature adopted, drying and long-term storage may lead to considerable reduction in germination or to eventual death of the seeds. Storage of orchid seeds at higher temperature and humidity can result in reduced seed vigour, low germination and reduced seedling survival (Bewley and Black 1994; Begnami and Cortelazzo 1996). These conditions are believed to affect protein metabolism (Bewley and Black, 1994) and cause a reduction in seed biochemical activity (Bailly *et al.,* 1996; Begnami and Cortelazzo, 1996). Whereas except for 30 days storage at 25°C, there is significant difference in both germination and viability percentage of mature seeds of *P. villosum* stored at both 25°C and 0°C favouring the method of *in vitro* germination to viability test as post storage survival. In orchid seeds, the TTC test has been successfully used for estimation of survival rate (Singh 1981; Van Waes and Debergh 1986a,b) and shown to correlate well with germination percentages after long-term storage (Shoushtari *et al.,* 1994). In contrast, TTC staining could result in overestimation of seed viability when seeds were subjected to prolonged exposure to sodium hypochlorite, which caused high TTC stainability of the embryo because of release of dehydrogenase from damaged embryos (Lauzer *et al.*, 1994).

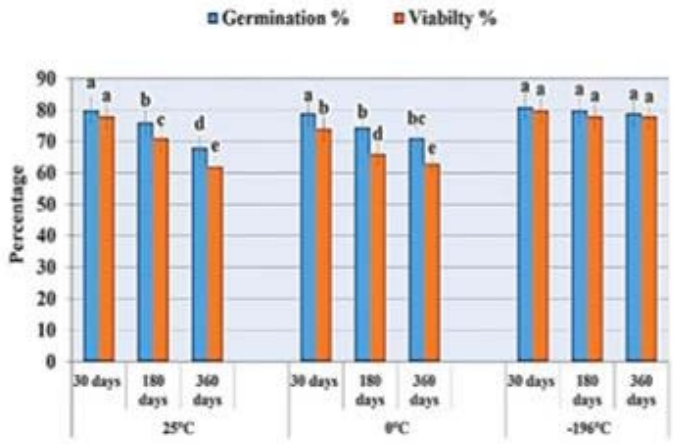


Figure 1: Effect of storage of exposed seed (>240 DAP) on germination recorded after 30 DAI on BG1 medium and 1% TTC viability test of *P. villosum*. Bars with different letters signify statistically different means according to Tukey's test ($p \geq 0.05$)

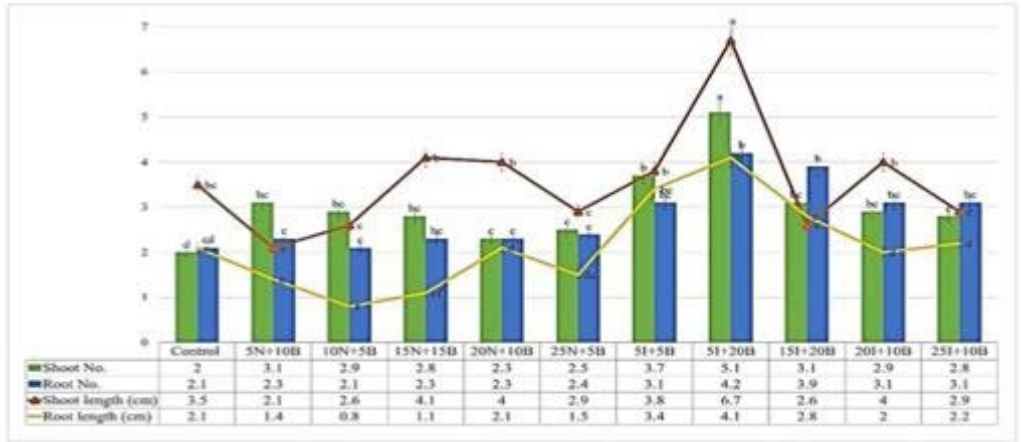
In the present study, reduction in germination as well as viability percentage of the mature seeds in both the temperatures was recorded with increase in storage time (Figure 1).

Table 1: Effect of media on different developmental stages of protocorms (% conversion) of *P. villosum* recorded after 30 DAI post storage in LN.

Media	Stages				Response
	I	II	III	IV	
BG1	**29.6±1.5a**	6.0±0.6b	6.2±0.2c	2.6±1.5d	54.8±2.6
1/2 MS	17.6±1.5 c	24±0.6a	21.6±0.5a	9.2±0.2d	63.3 ±2.6b
MS	9.6±1.5d	12±0.6b	**33.6±0.5a**	**10.2±0.2c**	**70.8±3.6a**
BM	13.6±1.5c	18±0.6b	22.6±0.5a	6.1±0.3d	65.9±2.5b

Means followed by the same letter are not significantly different according to Tukey test (P≥0.005). Values are mean of ± SE of three experiments with ten replicates/ experiment. ANOVA test high significant at 5% level. Stages of development of protocorms (I) Protocorm with pointed apex (II) Leaf initiation (III) and Root initiation (IV).

Maximum seed germination post cryopreservation was recorded in BG1(Figure 1) medium when compared to other media *viz,* MS, ½ MS and BM media tested (Figures not shown for other media). Composition of the medium plays a crucial role in influencing in-vitro seed germination. Morphologically, protocorm colour in modified BG1 medium was light green to whitish as seen in Figure 3c. There are several reports on certain orchid seeds requiring higher salt content medium for germination (Dohling *et al.,* 2008; Paul *et al.,* 2011). On analysing BG1 medium it was found that it had low salts contents in its composition thereby making it more suitable for the seeds of *P. Villosum* seeds to germinate. This is also supported by reports (Pierik *et al.,* 1988; Nikabadi *et al.,* 2014; Zeng *et al.,* 2015) that suggest in few selected orchids the seeds require even less than half of both micro and macronutrients for initiating germination in seeds. Another key factor in the composition of BG1 which varied was in its source of carbohydrates as glucose. Glucose being the simplest form of carbohydrates may have adhered to the easy assimilation by the seeds to germination (Nikabadi *et al.,* 2014). The beneficial effects of glucose for early germination has been reported by Traore and Guiltinan (2006) and Long *et al.* (2010). Germinated seeds were subcultured in different media *viz,* BG1, ½ MS, MS and BM media for conversion into protocorms and seedlings followed by subculture in optimum media incorporated with different growth regulators for further growth and development of the seedlings. Development of different stages (I and II) of protocorm and stages (III and IV) for seedling development was assessed. It was observed that maximum number of protocorms was retained in BG1 medium perhaps the best seed germination being on the same medium, however conversion into seedlings was favoured in MS (Table 1).



	Control	5N+10B	10N+5B	15N+15B	20N+10B	25N+5B	5I+5B	5I+20B	15I+20B	20I+10B	25I+10B
Shoot No.	2	3.1	2.9	2.8	2.3	2.5	3.7	5.1	3.1	2.9	2.8
Root No.	2.1	2.3	2.1	2.3	2.3	2.4	3.1	4.2	3.9	3.1	3.1
Shoot length (cm)	3.5	2.1	2.6	4.1	4	2.9	3.8	6.7	2.6	4	2.9
Root length (cm)	2.1	1.4	0.8	1.1	2.1	1.5	3.4	4.1	2.8	2	2.2

Figure 2: Seedling growth of *P. villosum* in MS medium incorporated NAA (N) + BAP (B) and IAA (I) + BAP in different concentration µM of plant growth regulators. Data recorded after 60 DAI. Mean followed by the same letter are not significantly different according to Tukeys test (P≥0.005)

Protocorm development into seedling is a slow sequential process as was also reported by Robinson *et al.* (2009). The overall conversion percentage till root stage was significantly different in MS medium from that of other media tried (Table 1). Therefore, MS medium was regarded optimal medium for seedling growth. This also shows that the carbohydrate requirement of the protocorm shifts from glucose to sucrose. This implies that the nutrient requirement of the protocorm varies during the different stages of development of shooting and rooting as also suggested in *Cypripedium* (Bae *et al.,* 2010). Further, results of overall seedlings of *P. villosum* showed that, the best growth and development was recorded in MS medium supplemented with 20 µM BAP+5 µM IAA, with maximum average number of shoots (4.5 cm), shoot length (6.5 cm), average root number (4.0 cm) and root length (4.1 cm) recorded after 60 DAI (Figure 2). This shows that the nutrient requirement for different stages of development varies (Nadarajan *et al.,* 2011; Zeng *et al.,* 2013). In the present study, incorporation of BAP and IAA was found stimulatory for seedling growth of *P. villosum* which is in similar to earlier reports (Long *et al.,* 2010; Zeng *et al.,* 2012; Chen *et al.,* 2015) in which BAP and NAA in combination was effectively used for in-vitro propagation of several orchids. It was observed that with further increase in concentration of both the cytokinins and auxins, seedling growth of *P. villosum* was inhibited. Nagaraju *et al.* (2003) had also reported that higher concentrations of cytokinins and auxins do show inhibitory effect on growth of orchids. The concentration of exogenous cytokinins and auxins and its synergistic effects in combinations under balanced condition, varies between species to species (Hossain, 2008; Hossain *et al.,* 2010; Long *et al.,* 2010; Roy *et al.,* 2011; Zeng *et al.,* 2012). The optimized medium with growth regulator was further used for subculturing at interval of 35 days.

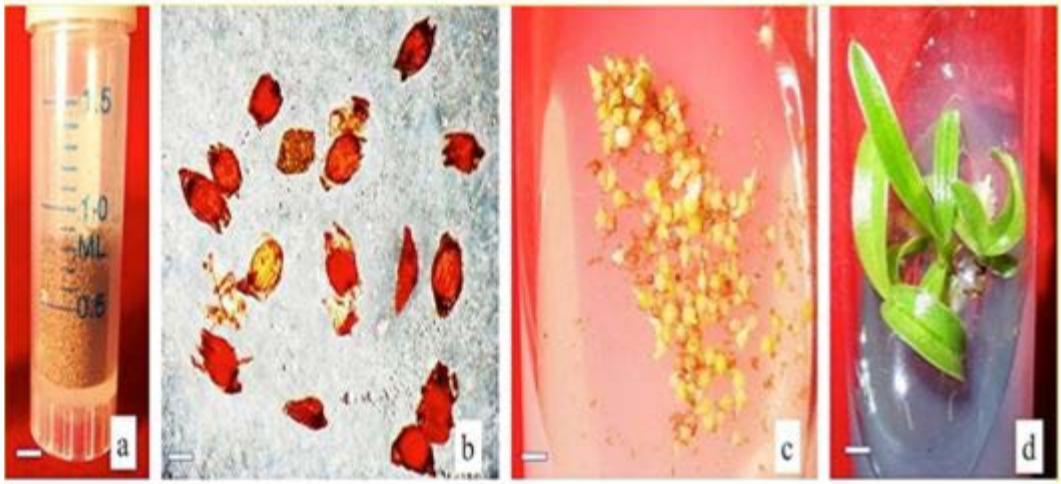


Figure 3: Seed storage (a) Seeds >240DAP of *P. villosum* stored in cryovials; (b) 1% TTC tested staining mature seeds after storage in -196°C liquid nitrogen for 180 days; (c) germination in BG1 medium; (d) seedling growth in MS medium supplemented with 5 µM BAP +10µM IAA.

Thus, in the present study on *P. villosum*, we report on seed storage method recommending in LN (-196 °C) to be most effective in not only storing and retaining the seed viability but also this process helps in protecting the seeds against pathogen attack as has also been reported in other orchid species (Popova *et al.,* 2016; Diengdoh *et al.,* 2017; Schofield *et al.,* 2018).

We have also reported the complete protocol till seedling development of post LN storage of *P.villosum* seeds. Hence the same protocol can be applied for many such threatened species of *Paphiopedilum* as well as other orchid species with some modification.

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Colorimetric detection of Pb^{2+} ions using PVP-capped silver nanoparticles

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Abstract

Colloidal silver nanoparticles (AgNPs) have been synthesized using polyvinylpyrrolidone (PVP) as a capping agent. The particles were characterized using UV-visible absorption spectroscopy and transmission electron microscopy (TEM). The particles were 4-12 nm in diameter. The surface plasmon resonance (SPR) band of the AgNPs at 400 nm was quenched in the presence of 1 μM Pb^{2+} ions at pH 9.6. Additionally, a new absorption band was formed around 600 nm. The colour of the colloidal solution was found to change from yellow to green. The addition of metal ions such as Al^{3+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Ni^{2+}, Sm^{3+} and Zn^{2+} did not alter the SPR band of the AgNPs significantly. The limit of detection (LOD) of Pb^{2+} ions by the colorimetric method was found to be 14.4 nM. The mechanism of the aggregation of the AgNPs in the presence of the Pb^{2+} ions has been discussed.

Keywords: Colorimetric; Colloidal; Nanoparticles; Aggregation; Sensing

Introduction

Lead is categorized as a Class B metal that falls under non-essential trace elements which are highly toxic (Nagajyoti *et al.,* 2010). The contamination by heavy metal ions, particularly Pb^{2+}, poses a serious threat to human health and to the environment. Lead poisoning is known to causes renal malfunction and inhibits brain development particularly in children causing various neurotoxic effects. As lead is nondegradable, it would be persistent in the environment and can produce toxic effects in plants and animals (Chai *et al.,* 2010). It is also known that even long exposure to very low levels of lead can cause neurological, reproductive, cardiovascular, and developmental disorders (Kim *et al.,* 2012). The source of lead in urban areas is mainly the combustion of tetraethyl lead in gasoline, local pollutants from mines and lead based paint pigments (Huheey *et al.,* 2013). Various methods for the detection of Pb^{2+} ions in drinking water or river water have been reported. These include atomic absorption spectrophotometry (Chen *et al.,* 2005, Siraj *et al.,* 2013, Bertenshaw *et al.,* 1981) , voltammetry (Dai *et al.,* 2017) , plasma optimal emission spectrometry (Bispo *et al.,* 2005) , flotation spectrophotometric (Shiri *et al.,* 2011) and fluorescence spectroscopy (Ali *et al.,* 2007; Fu *et al.,* 2012; Li *et al.,* 2013; Singh *et al.,* 2019).

Although several techniques has been employed for the determination of Pb^{2+} ions, the colorimetric methods based on the aggregation of gold (Liu *et al.,* 2003; Liu *et al.,* 2004; Peng *et al.,* 2014; Ratnarathorn *et al.,* 2015) and silver (Qi *et al.,* 2012; Kumar *et al.,* 2014) nanoparticles have attracted considerable attention due to their cost efficiency, rapidity and simplicity. The strategy for designing bifunctionalized gold nanoparticles that can be used for detection of lead have also reported (Zhu *et al.,* 2012). They used two peptide ligands, glutathione and pentapeptide for the selective sensing of lead under physiological conditions. The colorimetric detection of lead using gold nanoparticles is in quite good reported numbers. However, silver nanoparticles based colorimetric methods are fewer.

Herein, we have presented a simple method for the synthesis of the silver nanoparticles using sodium borohydride ($NaBH_4$) as a reducing agent and polyvinylpyrrolidone (PVP) as the capping agent. The colorimetric studies were carried out in the presence of several metal ions such as Al^{3+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Sm^{3+}, Zn^{2+} and Pb^{2+} to ascertain the selectivity of the probe.

Materials and methods

Materials

Aluminium sulphate, calcium chloride, cobalt chloride, cadmium chloride, copper chloride, magnesium sulphate, manganese chloride, lead nitrate, samarium nitrate, mercuric chloride, nickel chloride, polyvinylpyrrolidone, silver nitrate and zinc chloride were obtained from Himedia. Sodium borohydride was obtained from Sigma Aldrich. All other chemicals were of analytical grade. The water used for preparing the solutions was purified through distillation.

Instrumentation

UV-visible absorption spectra were obtained using PerkinElmer 25 spectrophotometer. TEM measurements were carried out using a JEOL 100 CX transmission electron microscope operating at 100 kV.

Synthesis of the PVP-capped AgNPs

200 ml distilled water was taken in a conical flask. Subsequently, 0.2 ml of 0.1 M $AgNO_3$ solution was added and the solution was stirred for 10 minutes. Then 0.02 g of solid $NaBH_4$ was added at room temperature to yield yellow colloidal solution. The solution was stirred and allowed to stand for 10 minutes for the completion of the nucleation process. It was followed by the addition of 1 ml of 1×10^{-3} M PVP solution. The solution was then stirred for 10 minutes and stored in the dark. The pH of the colloidal solution was 9.6.

Results and discussion

Characterization of the PVP-capped AgNPs

The UV-visible absorption spectrum of the PVP-capped AgNPs has been displayed in Figure 1.

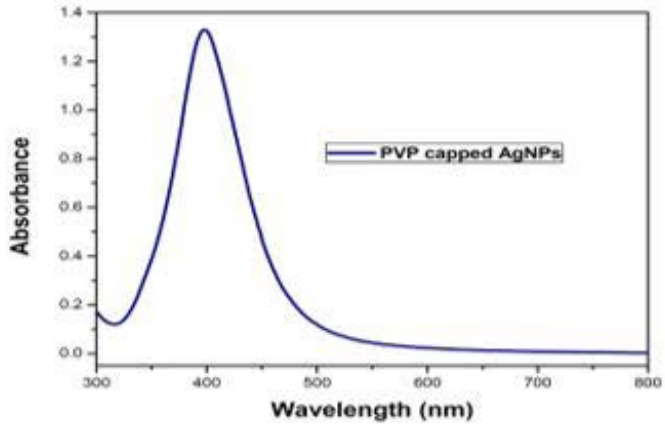

Figure 1. UV-visible absorption spectrum of the PVP-capped AgNPs.

The SPR band of silver was positioned at 400 nm. The intensity and position of the band remained unchanged for a period of two weeks. The morphology of the synthesized AgNPs was determined using the TEM measurements. Figure 2 shows the TEM images of the colloidal PVP- capped AgNPs.

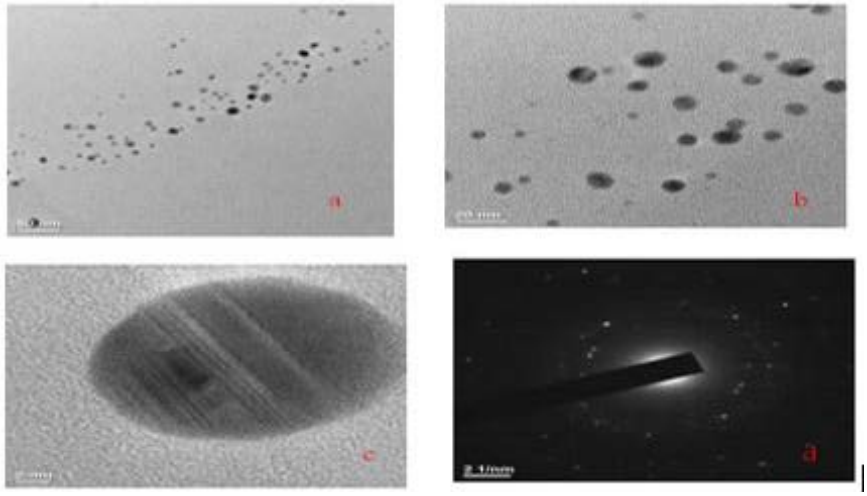

Figure 2 (a) & (b): TEM images of the PVP-capped AgNPs. **(c)** High resolution TEM image of the AgNPs **(d)** SAED pattern of the AgNPs.

The particles were nearly spherical in shape with the diameter ranging from 4 to 12 nm. The high resolution TEM image of the AgNPs has been shown in Figure 2(c). The selected area electron diffraction (SAED) pattern gives information on the crystalline nature of the material (Diamai *et al.,* 2019). The concentric rings in images confirm the

crystalline nature of the synthesized AgNPs.

Effect of the addition of various metals ions on the SPR band of the PVP capped AgNPs

It is well known that the metals ions can induce aggregation of the silver or gold nanoparticles (Saha *et al.,* 2012). The aggregation of the NPs can be confirmed by the change in the colour of the silver NPs from yellow to green. We investigated the effect of the different metal ions such as Al^{3+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}. Mn^{2+}, Ni^{2+}, Pb^{2+}, Sm^{3+} and Zn^{2+} on the SPR band of the AgNPs. The absorption spectra of the PVP-capped AgNPs in the presence of 1 µM concentration of various metal ions at pH 9.6 have been displayed in Figure 3.

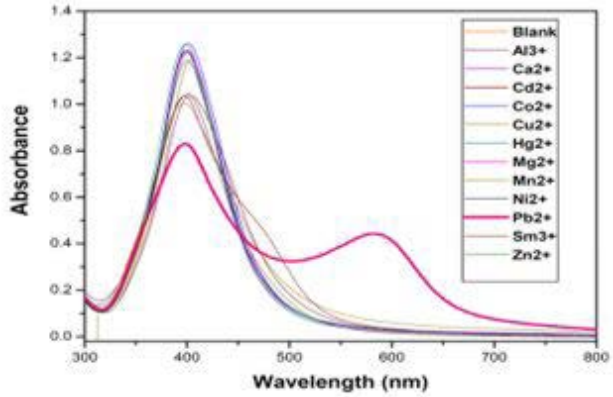

Figure 3. UV-visible spectra of the AgNPs upon the addition of 1 µM concentrations various metal ions at pH 9.6.

It is evident from Figure 3 that the addition of Pb^{2+} ions to the AgNPs resulted in quenching of the 400 nm band and the formation of a new peak around 600 nm. The addition of the other metal ions quenched the SPR band of silver, although not to the same extent as Pb^{2+}. Significantly, no longer wavelength absorption band was observed upon addition of the other metal ions. The colour of the AgNPs turned green upon addition of the Pb^{2+} ions (Figure 4). However, there was no change in the colour upon addition of the other metal ions. Therefore, the PVP-capped AgNPs can be used as a colorimetric probe for the selective detection of Pb^{2+} ions in aqueous medium.

Figure 4. Photograph of the AgNPs in the absence and presence of 1 μM concentrations of the various metal ions.

Selectivity and sensitivity of the colorimetric probe for the Pb^{2+} ions

The plot of the ratio of the absorption intensity response (A_{600}/A_{400}) of the probe for the Pb^{2+} ions against other metal ions at 1 μM have been shown in Figure 5.

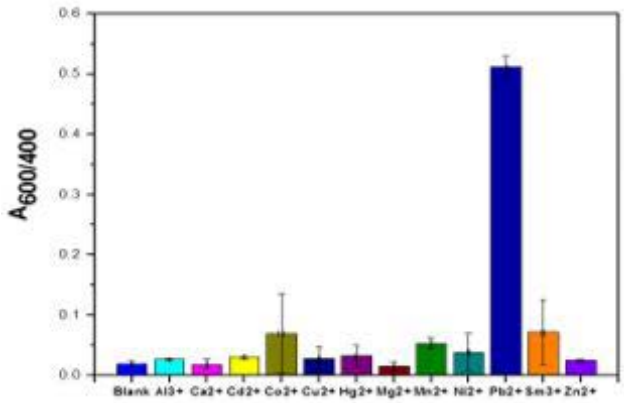

Figure 5. Plot of the response of the PVP-capped AgNPs (A_{600}/A_{400}) in the presence of 1 μM concentration of the various metal ions at pH 9.6.

As can be observed from the figure, the response of the probe was the maximum for the Pb^{2+} ions. The response indicates that Pb^{2+} ions can cause a maximum red shift of the SPR of the probe compared to the other competing metal ions. Thus, aggregation of the AgNPs can be strongly and selectively induced by the Pb^{2+} ions which indicates that the probe is selective for the Pb^{2+} ions. The aggregation of the AgNPs induced by Pb^{2+} ions was confirmed from the TEM measurements as shown in Figure 6. The limit of detection (LOD) was calculated as per the formula 3σ/S, where σ is the standard deviation of the blank and S corresponds to the slope of the calibration curve. The LOD was found to be 14.4 nM.

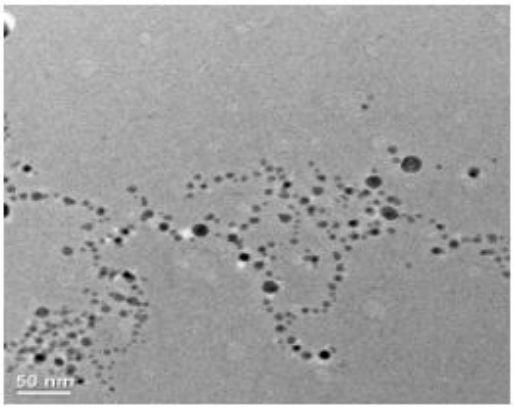

Figure 6. TEM image of the PVP-capped AgNPs upon the addition of 1 μM Pb^{2+} ions.

Mechanism for the aggregation of the PVP-capped AgNPs in the presence of Pb^{2+} ions

The selectivity of the probe towards the Pb^{2+} ions can be explained on the basis of the unique character of this metal ion. The Pb^{2+} ions have highly flexible bond length and geometry with a maximum coordination number up to 12 (Yoosaf *et al.,* 2007). Due to these properties, the Pb^{2+} ions have a high tendency to coordinate with the polymer PVP to form assembly of the AgNPs. Pb^{2+} ion is a borderline acid which can bind to different group of ligands. Thus, when the Pb^{2+} ions are added to the PVP-capped AgNPs, the metal ions coordinate with the N and O group of the PVP as displayed in the inset of Figure 7. Consequently, an assembly of the AgNPs was formed. The other metal ions interact very less or not at all with the ligand due to rigid coordination geometry of these ions. Hence upon the addition of these metal ions, there was little or no change on the absorption spectrum of the AgNPs.

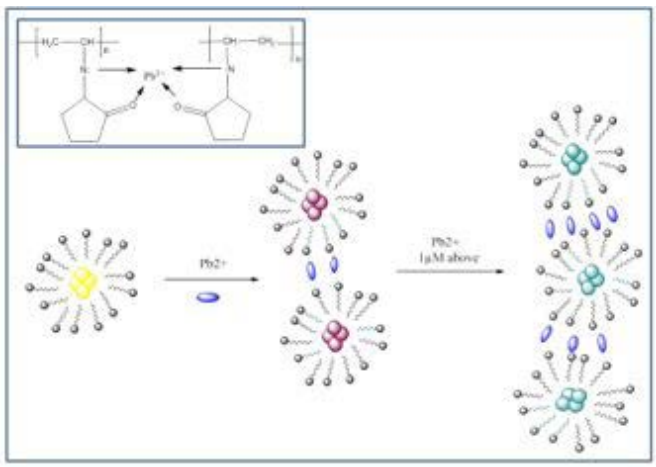

Figure 7. Pictorial representation of the aggregation of the PVP-capped AgNPs in the presence of the Pb^{2+} ions.

Conclusions

The PVP-capped AgNPs have been demonstrated to be a colorimetric probe for the selective detection of the Pb^{2+} ions in the aqueous medium. The LOD of the probe was calculated to be 14.4 nM. The aggregation of the AgNPs in the presence of the Pb^{2+} ions was due to the preferred coordination of Pb^{2+} with the N and O ligand groups of PVP. The present method has potential for the sensing of Pb^{2+} ions in water bodies such as lakes as streams.

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In-vitro comparative studies of *Apium graveolens* L. extracts for antioxidant and anti-inflammatory activity

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Abstract

The purpose of the present study was to find out the secondary metabolites and to evaluate and compare the antioxidant and anti-inflammatory activity of various extracts (aqueous, methanol, chloroform and acetic acid) of Apium graveolens L. Preliminary phytochemical screening revealed the presence of the major class of phytochemicals. The TPC and the TFC were found to be highest in the aqueous extract (61.3 ± 0.8 mg GAE /g) and TFC (47.7 ± 0.4 mg QE /g) with acetic acid extract showing the lowest TPC (2.6 ± 0.3 mg GAE /g) and TFC (13.0 ± 1.1 mg QE/g), respectively. The same trend was found in the case of TAC, where aqueous extract exhibited the highest TAC (9.5 ± 0.06 AAE mg/g) and antioxidant activity with IC_{50}= 0.87 and 0.13 mg/ml for DPPH and NO scavenging activity, respectively. Extracts also showed in vitro anti-inflammatory activity by inhibiting the heat-induced protein denaturation with the IC_{50} values of 0.58, 0.69, 1.06, and 2.82 mg/ml for methanol, aqueous, chloroform and acetic acid extracts, respectively. The results of our study revealed that aqueous extract is the most effective solvent to proceed for the in vivo studies against oxidative stress and inflammation-associated diseases.

Keywords: *Apium graveolens* L., Flavonoid, Antioxidant activity, Anti-inflammatory activity.

Introduction

Since time immemorial, people over the world have been using the medicinal plants for relief of symptoms of diseases (Maqsood *et al.,* 2010). Even today, despite the great advances observed in modern medicine, medicinal plants are being used due to their easy access and low cost. Furthermore, the World Health Organization (WHO) encourages the use of traditional medicines for the treatment of diseases provided they are proven to be efficacious and harmless (WHO, 1985). Epidemiological studies have shown that regular consumption of a plant-based diet containing antioxidants reduces the risk of many oxidative stress-related diseases (Arabshahi-Delouee and Urooj, 2007). Thus, in recent years, interest in natural antioxidants, especially of plant origin, has increased manifolds (Jayaprakash and Rao, 2000). Natural antioxidants either in the form of raw extracts or their chemical constituents are very effective to protect organisms from

damage caused by free radical-induced oxidative stress (Zengin *et al.,* 2011). Presently, the probable toxicity of synthetic antioxidants such as butylated hydroxyl anisole or butylated hydroxyl aniline has been condemned (Zhang *et al.,* 2009). Therefore, a considerable interest in the development of natural antioxidants from plants has gained much interest in recent years, especially in the field of biomedical and nutritional areas.

The therapeutic properties of medicinal plants are mainly due to the presence of phytochemical compounds (Kumar *et al.,* 2009). These are naturally occurring compounds which include primary compounds such as chlorophyll, proteins, and common sugars and secondary compounds such as alkaloids, glycosides, quinones, saponins, terpenoids, flavonoids, tannins and phenolic compound (Zheng and Wang, 2001; Mallikaharajuna *et al.,* 2007; Gracelin *et al.,* 2012). They are also antioxidant compounds that possess anti-inflammatory, anti-tumor, anti-mutagenic, anti-carcinogenic, anti-bacterial, and anti-viral activities (Zheng and Wang, 2001; Sala *et al.,* 2002; Gracelin *et al.,* 2012). One of the most important compounds are polyphenols (E.g. flavonoids, phenolic acids, and transipropanoids). They have been directly associated with antioxidant activity due to their free radical scavenging activities (Nickavar *et al.,* 2007). All these anti-properties of medicinal plants, such as anti-oxidant, anti-cancer, anti-inflammatory, etc. are mainly due to the presence of the biologically active compounds (secondary metabolites) (Zheng and Wang, 2001; Sala *et al.,* 2002; Gracelin *et al.,* 2012). Since these biologically active compounds are present in a very low concentration, the method of extracting them solely dependent on the nature of extracting solvent, due to their chemical characteristics and polarities that may or may not be soluble in a particular solvent. Therefore, the choice of extraction methods and solvents is critical for obtaining these biologically active compounds for phytochemical screening as well as for both qualitative and quantitative studies.

Celery (*Apium graveolens* L.) belonging to the family of Apiaceae, is a medicinal herb used as a food and also in traditional medicine due to its many health benefits (Kooti and Daraei, 2017) and to our best knowledge, this is the first comparative study of this species using different solvent extracts. Therefore, the present study aimed to determine the most effective solvent for extracting phytochemical compounds present in this plant and also to evaluate and compare the antioxidant and anti-inflammatory activity of different solvent extracts.

Materials and methods

Plant material: Fresh leaves of celery (*Apium graveolens* L.) were procured from the local market at Iewduh, Shillong, Meghalaya, India. The leaves were washed thoroughly with tap water and air-dried at room temperature, which was then cut to small pieces and dried in an oven at 40 ºC for 3 days. The dried leaves were then crushed to a fine powder using an electric blender. The powdered sample was stored at 4 ºC in an airtight bottle.

Preparation of extracts: 10 g of powdered plant material was kept in a conical flask

and added 100 ml of a solvent such as water, 80% methanol, 80% acetic acid, and 80% chloroform individually at the ratio of 1:10 (powder/solvent). The mouth of the conical flask was covered with aluminum foil and was subjected to periodically shaking in an electric shaker for 24 h. The extract was filtered by using muslin cloth followed by Whatman # 1 filter paper to obtain the filtrate. The filtrate was then concentrated using a Rotary Evaporator (Model) and was lyophilized to obtain the powdered form of the extracts. All extracts were dissolved in cold saline water containing 0.1% DMSO at the appropriate concentrations and were stored in a refrigerator at 4 ºC for further use.

Chemicals: Standards, such as DPPH, ascorbic acid, gallic acid, tannic acid, and standard anti-inflammatory drug, i.e., Aspirin were purchased from Sigma-Aldrich. Folin-Ciocalteu's reagent was procured from Qualigens. Sodium carbonate, sodium nitrite, aluminum chloride, sodium hydroxide, potassium dihydrogen orthophosphate, sodium hydroxide, potassium ferricyanide, trichloroacetic acid, ferric chloride, and others chemicals used in the study were of analytical grade and purchased from Sigma-Aldrich and HiMedia laboratory, India.

Phytochemical screening

The plant extracts were assessed for the existence of the phytochemicals such as alkaloid (Evans, 2002), phenols (Maze, 1963), flavonoids, terpenoids, cardiac glycosides (Prabhavathi *et al.,* 2016), steroids (Vimalkumar *et al.,* 2014), saponins, tannins and quinones (Deyab *et al.,* 2016).

Quantitative analysis

Estimation of phenols: The amount of Total Phenolic Content (TPC) present in the plant extracts was determined with Folin-Ciocateu (FC) reagent with slight modification (Ainsworth and Gillespie, 2007). Gallic acid was used as a standard for plotting the calibration curves (50-300 µg/ml). The TPC was expressed as mg Gallic Acid Equivalent (GAE)/g dried weight and calculated by the formula, TPC = (C × V)/M where, TPC is total phenolic content (mg/g plant extract in GAE), C is the concentration of gallic acid established from the calibration curve (mg/ml), V is the volume of the extract (ml), M is mass of the extract of the plant (g). All samples were analyzed in triplicates and the results were mean values ± standard deviations.

Estimation of flavonoids: The amount of Total Flavonoid Content (TFC) present in the plant extracts was determined by using a standard method with slight modification (Zhishen et al., 1999). Quercetin was used as a standard for plotting the calibration curves (50-300 µg/ml). The TFC was expressed as mg Quercetin Equivalent (QE)/g dried weight and calculated by the formula, TFC = (C × V)/M where, TFC is total flavonoid content (mg/g plant extract in QE), C is the concentration of Quercetin established from the calibration curve (mg/ml), V is the volume of the extract (ml), M is mass of the extract of the plant (g). All samples were analyzed in triplicates and the results were mean values ± standard deviations.

Estimation of tannins: Total tannins content present in the plant extracts were determined by slightly modified Folin and Ciocalteu method (Ainsworth and Gillespie, 2007). Tannic acid was used as a standard for plotting the calibration curves (10-50 µg/ml). The TTC was expressed as mg Tannic acid Equivalent (TAE)/g dried weight and calculated by the formula, TAE = (C × V)/M where TTC is total Tannin content (mg/g plant extract in TAE), C is the concentration of Tannic acid established from the calibration curve (mg/ml), V is the volume of the extract (ml), M is the mass of the extract of the plant (g). All samples were analyzed in triplicates and the results were mean values ± standard deviations.

Total antioxidant capacity

The total antioxidant capacity (TAC) of the plant extracts was determined by the Phosphomolybdenum method with slight modification (Preito *et al.,* 1999). Ascorbic acid was used as a standard for plotting the calibration curves (10-60 µg/ml). The TAC was expressed as mg Ascorbic Acid Equivalent (AAE)/g dried weight and calculated by the formula, TAC = (C × V)/M where TAC is total Antioxidant capacity (mg/g plant extract in AAE), C is the concentration of Ascorbic acid established from the calibration curve (mg/ml), V is the volume of the extract (ml), M is the mass of the extract of the plant (g). All samples were analyzed in triplicates and the results were mean values ± standard deviations.

***In-vitro* antioxidant activity**

DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay: The ability of the plant extracts to scavenge the DPPH radical was determined by the method with slight modification (Karadag et al., 2009). An aliquot (1 ml) of the plant extracts or standard solution of ascorbic acid (50-300 µg/ml) was added to 2 ml of 1 mM DPPH radical solution prepared in methanol. The reaction mixture was incubated in dark for 30 min and the absorbance was read at 517 nm. A control was prepared by adding 0.5 ml methanol and 0.5 ml saline water containing 0.1 % DMSO to 2 ml of 1 mM DPPH. All samples were analyzed in triplicates and the results were mean values ± standard deviations. The % of DPPH scavenging activity of both samples was calculated using the formula:

$$\% \text{ inhibition} = A_c - A_s / A_c * 100$$

Where, A_c = absorbance of control, A_s = absorbance of sample/standard. The decreasing absorbance indicates a high DPPH scavenging activity.

Total reducing power assay

The total reducing ability of the samples was evaluated (Deore *et al*., 2009) with slight modification. An aliquot (1 ml) of extracts or standard solutions of ascorbic (50-300 µg/ml) were added to 1 ml of phosphate buffer (PB) (0.2 M, pH 6.6) and 1 ml of 1% potassium ferric cyanide [$K_3Fe(CN)_6$]. The mixture was vortex and incubated at 50 °C in a water bath for 30 min and the reaction was terminated by the addition of 1 ml of 10% TCA and the mixture was centrifuged at 3000 rpm for 10 min. 1 ml of the supernatant was transferred into the tube containing 1 ml distilled water and 0.2 ml of 0.1% ferric

chloride ($FeCl_3.6H_2O$). After 5 min, the absorbance was measured at 700 nm against a blank containing 0.5 ml saline containing 0.1 % DMSO and 0.5 ml PB, whereas the control containing all reagent except standard or extracts. All samples were analyzed in triplicates and the results were mean values ± standard deviations. An increase in absorbance indicates their reducing power.

Nitric Oxide scavenging assay

The ability of the plant extracts to scavenge the nitric oxide (NO) was determined (Kumari *et al.,* 2016) with slight modification. An aliquot (1 ml) of extracts or standard solutions of ascorbic (50-300 µg/ml) was added to 2 ml of 10 mM sodium nitroprusside. The mixture was incubated at 25 °C for 2.5 h, 1 ml of the above reaction mixture was taken, followed by mixing with 1 ml of 1% sulfanilamide prepared in 20% glacial acetic acid and after 5 min, 1 ml of 0.1% naphthyl ethylenediamine dichloride prepared in 2% phosphoric acid were added. The absorbance was measured at 546 nm against the blank containing 0.5 ml saline containing 0.1% DMSO and 0.5 ml PBS, whereas the control containing all reagent except standard or extracts. All samples were analyzed in triplicates and the results were mean values ± standard deviations. The % of NO scavenging activity of both samples was calculated using the formula:

$$\% \text{ inhibition} = A_c - A_s / A_c * 100$$

Where, A_c = absorbance of control, A_s = absorbance of sample/standard. The decreasing absorbance indicates a high NO scavenging activity.

In-vitro **anti-inflammatory activity**

Inhibition of protein denaturation: The anti-inflammatory activity of the plant extracts was determined by the inhibition of protein albumin denaturation method with slight modification (Padmanabhan and Jangle, 2012). An aliquot (1 ml) of extracts (100-300 µg/ml) and standard Aspirin (100-200 µg/ml) were added to 0.5 ml of 5% albumin obtained from fresh hen egg and the final volume was raised to 3 ml with PBS (pH 6.4). The reaction mixture was incubated at 37 °C for 20 min and then heated at 80 °C for 5 min. After cooling, the turbidity was measured at 660 nm against the blank containing 0.5 ml saline containing 0.1 % DMSO and 0.5 ml PBS, whereas the control containing all reagent except standard or extracts. All samples were analyzed in triplicates and the results were mean values ± standard deviations. The ability of the plant extracts to inhibit heat-induced protein denaturation were compared with the control and a standard drug aspirin through the formula:

$$\% \text{ inhibition} = A_c - A_s / A_c * 100$$

Where, A_c = absorbance of control, A_s = absorbance of sample/standard. The decreasing absorbance indicates a high inhibition of heat-induced protein denaturation.

Statistical analysis

All experiments were done in triplicates and the results were mean values ± standard deviations. Linear regression analysis was used to calculate IC_{50} for each plant extract. Data were processed with graph pad prism version 8.02 software.

Results

Preliminary phytochemicals screening revealed the presence of all tested parameters in different plant extracts as shown in table 1. The phytochemical screening was performed with aqueous, methanol, chloroform and acetic acid extracts of *Apium graveolens* L. Quinones did not show a positive result for their presence in methanol extract as shown in table 1. The presence or absence of the phytochemicals depends upon the solvent medium used for extraction; therefore, the choice of extraction solvent is critical for phytochemicals screening as well as for both qualitative and quantitative studies.

Table 1: Qualitative analyses of phytochemical substances in different extracts of *Apium graveolens* L.

Sl. No.	Tests	Methanol	Chloroform	Acetic acid	Aqueous
1	Alkaloid	+	+	++	++
2	Flavonoid	++	++	+	++
3	Phenolic	++	+	+	++
4	Saponin	+	+	+	+
5	Tannin	++	+	+	+
6	Glycosides	+	+	+	+
7	Terpenoid	+	+	+	+
8	Quinones	-	++	+	++

++: intensely present, +: Present, - : Absent

The Total Phenolic Content, Total Flavonoid Content, Total Tannin Content and Total Antioxidant Capacity in all extracts was calculated using the linear equation obtained from the standard calibration curve as shown in figure 1, where y is the absorbance and x is the amount of Gallic acid equivalent (GAE), Quercetin equivalent (QE), Tannic acid equivalent (TAE) and Ascorbic acid equivalent (AAE) for TPC, TFC, TTC, and TAC respectively. The TPC (61.3 ± 0.8 mg of TAE/g of extract), TFC (47.7 ± 0.4 mg of QE/g of extract), TTC (12.9 ± 0.7 mg of TAE/g of extract), and TAC (9.5 ± 0.06 mg of AAE/g of extract) was found to be higher in an aqueous extract with acetic acid extract showing the lowest TPC (2.6 ± 0.3 mg GAE /g of extract) and TFC (13.0 ± 1.1mg QE / g of extract), TTC (5.2 ± 0.3 mg TAE/g) and TAC (4.3 ± 0.5 mg of AAE/g of extract), respectively as shown in table 2. The TPC, TTC, and TAC of the four extracts were found to decrease in the order aqueous > methanol > chloroform > acetic acid whereas the TFC was found

to decrease in the order aqueous > chloroform > methanol > acetic acid at a particular concentration, respectively.

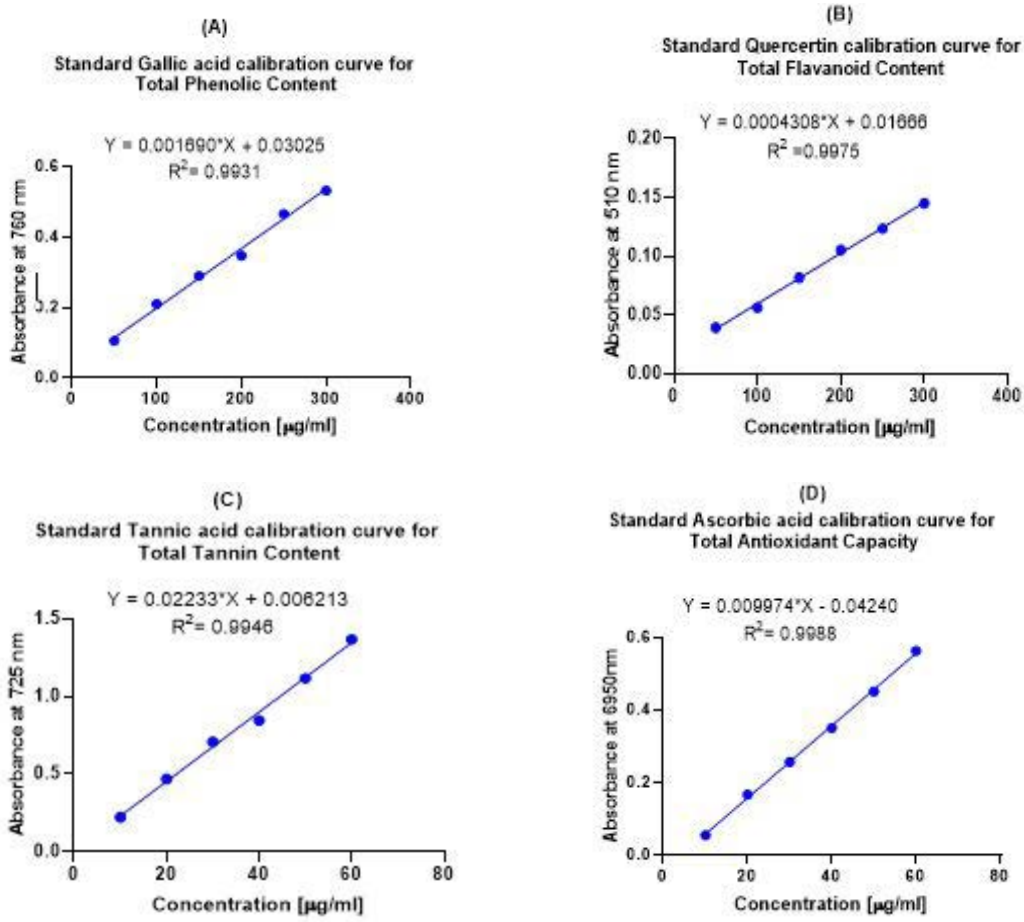

Figure 1: Calibration curve for Gallic acid (A), Quercetin (B), Tannic acid (C) and Ascorbic acid (D) for TPC, TFC, TTC and TAC.

Extracts	**Concentration [mg/ml]**	**Total Phenolic Content (mg GAE/g)**	**Total Flavonoid Content (mg QE/g)**	**Total Tannin Content (mg TAE/g)**	**Total Antioxidant Capacity (mg AAE/G)**
Aqueous	0.5	61.3±0.8	47.7±0.4	12.9±0.7	9.5±0.06
Methanol	0.5	30.9±0.9	20.1±0.7	10.5±0.1	6.1±0.03
Chloroform	0.5	14.1±0.3	24.1±1.5	7.1±0.3	5.4±0.1
Acetic Acid	0.5	2.6±0.3	13.0±1.1	5.2±0.3	4.3±0.5

Table 2: TPC, TFC, TTC, and TAC of different extracts of *Apium graveolens* L. Data represented as Mean ± SD (n = 3).

DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay is based on the principle that DPPH radical in the presence of an antioxidant, accept one or more electron donated by the antioxidant compound and in turn reduces from violet to yellow color which can be quantitatively measured from the change or decrease in absorbance. Here ascorbic acid, a well-known antioxidant was used as a positive control and the calculated % inhibition values of both standard and extracts was plotted against their respective concentration as shown in figure 2(A) and the results were expressed as IC_{50} i.e., the concentration of both samples and standard required to scavenge 50% of the DPPH radical as shown in table 3, which was calculated from the curve of % inhibition plotted against their respective concentration. Low IC_{50} value indicates the strongest scavenging activity. The aqueous extract exhibited the smallest IC_{50} (IC_{50} = 0.87 mg/ml), which indicated that aqueous extract has the highest scavenging activity when compared with methanol (IC_{50} = 1.1 mg/ml), chloroform (IC_{50} = 2.4 mg/ml) and acetic acid (IC_{50} = 5.3 mg/ml) extracts, respectively, although standard ascorbic acid (IC_{50} = 0.023 mg/ml) has the highest scavenging activity in all tested concentration, the extracts still showed good scavenging activities in a dose-dependent manner.

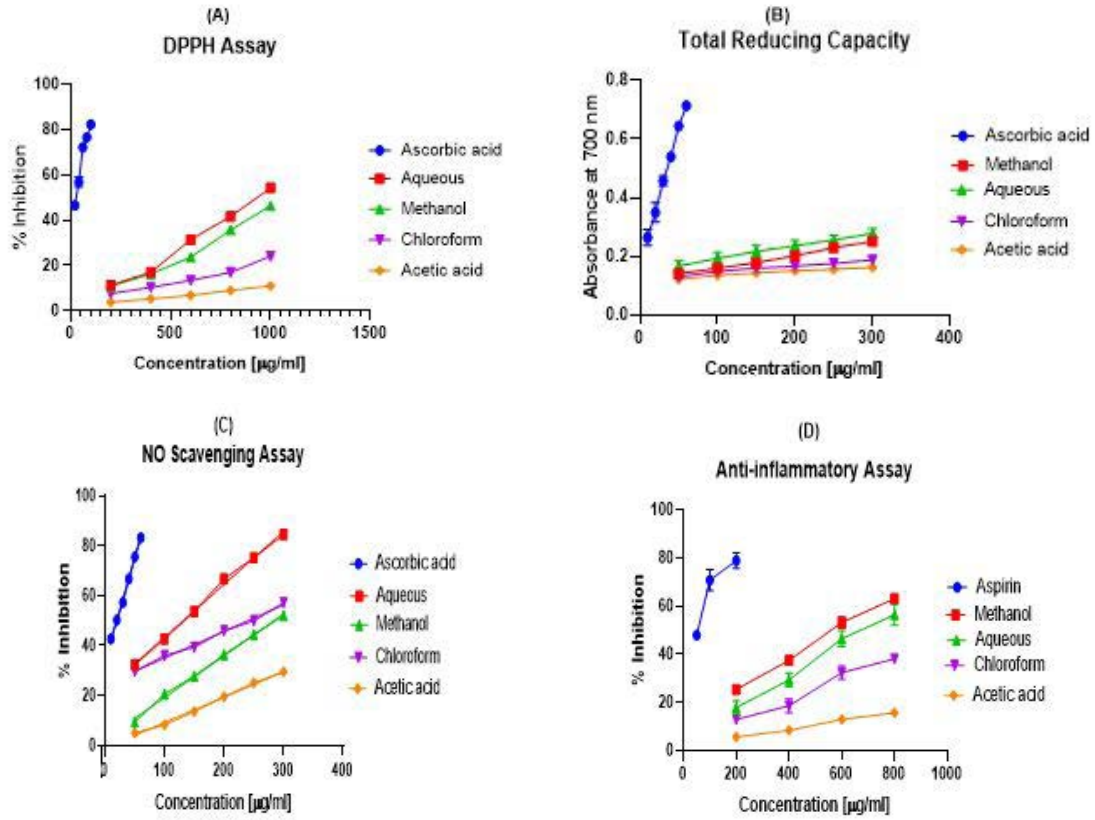

Figure 2: Percentage inhibition of various extracts and standard (Ascorbic acid) on DPPH (A), NO (C), Anti-inflammatory (D), and Absorbance of the total reducing capacity (B).

Table 3: Results of DPPH scavenging activity

Standard/ Extracts	**Concentration [µg/ml] % Inhibition (Mean ± SD)**					**IC_{50} [mg/ml]**
Ascorbic Acid	20 µg	30 µg	40 µg	50 µg	60 µg	0.023
	46.5 ± 0.1	56.9 ± 2.1	72. 1 ± 1.5	76.5 ± 1.3	82.2 ± 0.1	
Aqueous	200 µg	400 µg	600 µg	800 µg	1000 µg	
	11.3 ± 0.05	17.06 ± 0.32	31.6 ± 1.4	42.6 ± 0.79	54.6 ± 0.58	0.87
Methanol	10.9 ± 0.11	16.3 ± 0.4	23.6 ± 0.47	35.7 ± 0.6	46.3 ± 0.76	1.1
Chloroform	7.73 ± 0.4	10.3 ± 1.02	13.4 ± 1.51	17.0 ± 0.75	24.1 ± 1.18	2.4
Acetic Acid	3.9 ± 0.26	5.3 ± 0.05	6.8 ± 0.51	9.0 ± 0.55	11.1 ± 0.32	5.3

The total reducing capacity or the electron-donating ability of the extracts was determined by using Fe^{3+} reduction as an indicator that is based on the chemical reduction of ferricyanide Fe^{3+} complex to Fe^{2+} form in the presence of the extracts or standard and the concentration of ferrous form was determined spectrophotometrically at 700 nm. In this particular assay, the reducing power of both extracts and standard increases with an increase in absorbance as shown in figure 2 (B), and as indicated by their absorbance values as shown in table 4. An increase in absorbance indicates their reducing power. It is obvious that ascorbic acid, a well-known antioxidant compound has the highest reducing power but when compared among extracts, the aqueous extract exhibited the strongest reducing power followed by methanolic, chloroform and acetic acid extracts, respectively.

Table 4: Results of Total Reducing Capacity Assay

Standard/ Extracts	**Concentration [µg/ml]/Absorbance (Mean ± SD)**					
Ascorbic Acid	10 µg	20 µg	30 µg	40 µg	50 µg	60 µg
	0.264 ±0.02	0.349 ±0.03	0.456 ±0.01	0.539 ±0.01	0.644 ±0.01	0.7139 ±0.1
Aqueous	50 µg	100 µg	150 µg	200 µg	250 µg	300 µg
	0.166 ±0.01	0.193 ±0.02	0.2162 ±0.1	0.236 ±0.1	0.255 ±0.01	0.278 ±0.1
Methanol	0.140 ±0.1	0.159 ±0.02	0.178 ±0.2	0.201 ±0.7	0.229 ±0.9	0.250 ±0.1
Chloroform	0.130 ±0.7	0.149 ±0.01	0.159 ±0.09	0.166 ±0.03	0.175 ±0.5	0.187 ±0.1
Acetic Acid	0.122 ±0.3	0.134 ±0.01	0.143 ±0.1	0.150 ±0.4	0.155 ±0.06	0.162 ±0.8

NO scavenging assay is based on the principle that SNP in aqueous solution produces NO, and under aerobic condition, NO react with O_2 to produce a stable product nitrite or nitrate, which can be determined by Griess reagent and the ability of the extracts to scavenge NO is by competing with oxygen for NO, this lead to a reduction in nitrite or nitrate formation, which can be determined spectrophotometrically at 546 nm from the changed or decrease in absorbance. Here ascorbic acid, a well-known antioxidant was used as a positive control and the calculated % inhibition values of both standard and extracts were plotted against their respective concentration as shown in figure 2 (C) and the results were expressed as IC_{50} i.e., the concentration of both samples and standard required to scavenge 50% of NO as shown in table 5, which was calculated from the curve of % inhibition plotted against their respective concentration. Low IC_{50} value indicates the strongest scavenging activity. Aqueous extract exhibited the smallest IC_{50} (IC_{50} = 0.13 mg/ml), which indicated that aqueous extract has the highest scavenging activity followed by chloroform (IC_{50} = 0.24 mg/ml), methanol (IC_{50} = 0.28 mg/ml) and acetic acid (IC_{50} = 0.50 mg/ml) extracts, respectively.

The anti-inflammatory activity of different plant extracts was studied by using inhibition of egg albumin denaturation technique. This assay is based on the principle that when egg albumin induces to heat it denature, and the ability of the plant extracts to

decrease the degree of denaturation was compared with a standard drug aspirin, which can be quantitatively measured from the change or decrease in absorbance. Here aspirin, a well-known anti-inflammatory drug was used as a positive control and the calculated % inhibition values of both standard and extracts was plotted against their respective concentration as shown in figure 2 (D) and the results were expressed as IC_{50} i.e., the concentration of both samples and standard required to inhibit 50% of the denaturation process, as shown in table 6, which was calculated from the curve of % inhibition plotted against their respective concentration. Low IC_{50} value indicates the strongest inhibition activity.

Table 5: The result of Nitric Oxide Scavenging Assay

Standard/ Extracts	Concentration [µg/ml]/ % inhibition (Mean ±SD)						IC_{50} [mg/ml]
Ascorbic Acid	10 µg	20 µg	30 µg	40 µg	50 µg	60 µg	
	42.7 ±2.8	50.3 ±2.8	57.2 ±1 .7	66.7 ±3.1	75.5 ±1.1	83.2 ±4.7	
Aqueous	50 µg	100 µg	150 µg	200 µg	250 µg	300 µg	0.01
	32.3 ±2.2	42.7 ±7.2	53.6 ±6.9	66.7 ±4.6	75.2 ±3.4	84.5 ±3.8	0.13
Chloroform	29.7 ±1 .9	36.0 ±1.6	39.4 ±1 .0	45.9 ±3.9	49.9 ±3.9	57.2 ±2.7	0.24
Methanol	9.4 ±4.2	20.6 ±2.1	27.7 ±1.7	36.3 ±2.6	44.3 ±0.9	52.0 ±0.8	0.28
Acetic Acid	5.0 ±1.9	8.2 ±0.8	13.6 ±1.4	19.5 ±0.5	25.1 ±2.0	29.5 ±1.9	0.50

The methanolic extract exhibited the smallest IC_{50} (IC_{50} = 0.58 mg/ml), which indicated that methanolic extract has the highest inhibition activity when compared with aqueous (IC_{50} = 0.69 mg/ml), chloroform (IC_{50} = 1.08 mg/ml) and acetic acid (IC_{50} = 2.82 mg/ml) extract, respectively, but when compared with aspirin (IC_{50} = 0.04 mg/ml) it is obvious that aspirin a well-known anti-inflammatory drug has the highest inhibition activity in all tested concentration, but the extracts still show good inhibition activities in a dose-dependent manner.

Discussion

The use of medicinal plants to tackle human diseases is as old as mankind. This is because medicinal plants possess great varieties of biologically active compounds or secondary metabolites such as alkaloids, glycosides, quinones, saponins, terpenoids, flavonoids, tannins and phenolic compounds. They are directly responsible for different activities such as antioxidant, anti-inflammatory, anticancer, etc. each through different mechanisms. Phenolics compounds account for most of the antioxidant activity in plants or plant products (Sulaiman *et al.,* 2013). Therefore, the extraction of these biologically active compounds from plant materials is greatly influenced by their solubility in the extraction solvent. In this study, aqueous extracts gave the highest extraction yield of the total extractable compounds, while the acetic acid, being the least polar, gave the

lowest yield. Phytochemicals screening revealed the presence of various phytochemical constituents in the plant extracts (Table.1). Thus, extraction with polar solvents such as aqueous, methanol, acetic acid, and chloroform being the least polar among them all would give a good extraction yield of the biologically active compounds.

Table 6: The result of Protein denaturation Assay.

Standard/ Extracts	Concentration [µg/ml]	%Inhibition (Mean ±SD)	IC_{50} [mg/ml]
Methanol	200	25.2 ±**0.4**	0.58
	400	37.4 ±**1.8**	
	600	53.1 ±**2 .3**	
	800	63.0 ±**1.1**	
Aqueous	200	17.7 ±**2.7**	0.69
	400	29.4 ±**2 .9**	
	600	46.3 ±**3.3**	
	800	56.3 ±**4 .3**	
Chloroform	200	12.7 ±**0.7**	1.06
	400	18.4 ±**2.9**	
	600	32.3 ±**2 .7**	
	800	38.0 ±**1.6**	
Acetic Acid	200	5.5 ±**0 .8**	2.82
	400	8.2 ±**1.2**	
	600	12.8 ±**1.8**	
	800	15.5 ±**0.4**	
Aspirin	50	47.8 ±**1.5**	0.04
	100	67.4 ±**2 .3**	
	200	78.8 ±**3 .2**	

The TPC, TTC, TAC in different extracts were showing that aqueous extract has highest with a descending order of aqueous > methanol > chloroform > acetic acid, and the TFC of different extracts shows that aqueous extract has the highest flavonoid content with a descending order of aqueous > chloroform > methanol > acetic acid. Thus, in all cases aqueous was a better solvent for extraction of polyphenol compounds.

In our present study, several *in vitro* model systems have been used for assessing the free radical scavenging activity. One of the most common and rapid methods is the DPPH free radical scavenging activity. The DPPH radical scavenging activity of different plant extracts of *Apium graveolens* L. is denoted in figure 4. All the extracts showed different levels of DPPH radical scavenging activity and were found to decrease in the order aqueous > methanol > chloroform > acetic acid in a dose-dependent manner. In this assay, the aqueous extract exhibited strongest DPPH free radical scavenging activity

compared to other extracts. Similarly, we also assayed the reducing power of the plant extracts, and in this particular assay, it was observed that higher the absorbance, the stronger is the antioxidant activity; thus, the reducing power of the extracts also increases with the increase in concentration. When compared to other extracts, the aqueous extract exhibited the highest reducing power ability. The reducing power ability of the four extracts was found to decrease in the order aqueous > methanol > chloroform > acetic acid. Both cases follow the same trend as in the case of TPC, TTC, and TAC, indicating that the free radical scavenging activity and the reducing power of *Apium graveolens* L. extracts is highly related to the presence of phenolic compounds which caused the reduction of DPPH radical and Fe^{3+}/ ferricyanide complex.

NO is an important signaling molecule involved in various normal physiological processes (Parul *et al.,* 2013). At low concentration, NO do not harm macromolecules such as DNA, proteins or lipids but excess production of NO can lead to several diseases (Ialenti *et al.,* 1993). Excess NO reacts with O_2 to produce an unstable intermediate (NO_2, N_2O_4, and N_3O_4) that are highly toxic to the cell (Parul *et al.,* 2013). Therefore, antioxidant compounds from plants have gained much interests due to their ability to tackle free radicals in the biological systems. The nitric oxide radical scavenging activity of different plant extracts of *Apium graveolens* L. was presented in figure 4. All the extracts effectively reduced the generation of nitric oxide from sodium nitroprusside. The aqueous extract showed highest nitric oxide radical inhibition compared to other extracts and was found to decrease in the order aqueous > chloroform > methanol > acetic acid, which follows the same trend as in the case of the TFC, indicating that flavonoids might be directly contributing toward the NO scavenging activity which further support the earlier report (Parul *et al.,* 2013). Thus, in all the antioxidant assays, the results correlated between phenolics, flavonoids, and antioxidant activity.

Besides oxidative stress, inflammation is also a major cause of many chronic diseases (Godhandaraman and Ramalingam, 2016). It is considered as a normal protective response and as a part of a host defense system associated with pain and involves occurrences such as the increase in vascular permeability, increase of protein denaturation and membrane alterations (Umapathy *et al.,* 2010). If inflammation is left untreated, it leads to the onset of many chronic diseases (Godhandaraman and Ramalingam, 2016). The most common synthetic drugs used in the present are known as NSAIDs such as aspirin, diclofenac but these compounds are associated with many unwanted side effect (Amir *et al.,* 2010). Since protein denaturation leads to inflammatory and arthritis diseases (Williams *et al.,* 2008), thus, the prevention of protein denaturation may help in inflammatory conditions. Hence, a simple and viable protein denaturation assay was selected to determine the anti-inflammatory activity of *Apium graveolens* L. extracts. All extracts and standard drug aspirin exhibited dose-dependent percentage inhibition of heat-induced protein denaturation in fresh egg albumin and was found to decrease in the order aspirin > methanol > aqueous > chloroform > acetic acid, When compared among extracts, the methanolic extract showed higher anti-inflammatory activity at increasing

concentration. It may due to the presence of active principles of phytocompounds such as tannins, phenols, flavonoids, and related polyphenols that might be responsible for this anti-inflammatory activity. Hence, *Apium graveolens* L. might be used as a putative anti-inflammatory agent.

Conclusion

The results of our study revealed that *Apium graveolens* L. extracts possess various bioactive phytochemical compounds such as alkaloids, flavonoids, phenols, tannins, etc. Extraction solvents have an effect on extraction yield and the total extractable compounds from *Apium graveolens* L. Most of the antioxidant compounds were present in the aqueous extract and it showed the highest activity in most of the assays. Therefore, water appears to be the best extraction solvent for the extraction of various phytochemical compounds from *Apium graveolens* L. The plant extracts also showed remarkable inhibiting activity against heat-induced protein denaturation. Therefore, we can conclude that *Apium graveolens* L. is a potential candidate for a natural source of antioxidants and might be used as an anti-inflammatory agent. However, further detailed investigations are needed to ascertain the specific mechanism and phytochemicals compounds responsible for its anti-inflammatory activities. Nevertheless, consumption of *Apium graveolens* L. can be beneficial in preventing oxidative stress and inflammation-associated diseases.

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Photic and nonphotic cues in regulation of seasonal reproduction in birds

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Abstract

The present review documents the recent advances on the roles of photic and non-photic cues in regulation of seasonal reproduction and associated functions in birds. Annual variation in day length is the most consistent and reliable cue in timing life history stages including reproduction, molt and migration in most birds. Other environmental factors such as temperature, rainfall, availability of food and social interactions etc. are essential supplementary factors used to supplement and/or modify the timing of reproduction. Increasing day lengths of spring and summer trigger hypothalamus-pituitary-gonadal (HPG) axis to secrete gonadotropins, which cause gonadal growth and development and induce endocrine gonads to secrete sex steroids that mediate cellular processes and control development of accessory sex structure, secondary sexual characters like bill and plumage colour and reproductive behavior. Photoperiod also plays an important role in the regulation of fat deposition, feathers molt and migratory immune responses. Apart from its duration, the other photic characters like intensity and wavelength together with some non-photic cues play crucial roles in overall timing of avian reproductive cycles.

Keywords: Photoperiod, Avian, Reproduction, Molt, Seasonal.

1. Introduction

Seasonality involves initiation, termination and reinitiation of physiological processes. It is an important adaptation for survival and is exhibited by many wild species. It ensures the occurrence of seasonal events at the most appropriate time of the year when the environmental factors are most conducive. Birds exploit favorable season by reproducing and avoid or mitigate unfavorable season by migrating or becoming refractory. In nature, they are exposed to changes in different environmental factors that influence their biological functions. Most critical among these functions is the timing of reproduction, a scheme that causes reproductive activity to occur during the period that assures minimum stress on the adults and maximum probability for survival of young and parents (Dixit and Singh 2011, 2020). Most birds reproduce seasonally in order to coincide the birth of young ones when food and shelter are optimally present. They also exhibit seasonality in some other physiological and behavioural functions including migration, molt, bill and plumage coloration and hormone levels, etc. (Dawson, 2007;

Dixit and Singh, 2011). The precise temporal regulation of reproduction is achieved through intricate physiological processes that sense variations in environmental condition, integrate them with internal information and regulate the reproductive state accordingly (Hau *et al.,* 2008). Thus, reproduction being an important part of life cycle greatly depends on environment (Immelman, 1971).

Successful individuals should be fully prepared for appropriate seasonal activity when that season arrives. Thus, fitness in a seasonal environment is all about the timing. There are optimum times to start and stop reproduction, molt and migration which are important life history events in annual cycles of most birds. As all the above events in life cycle of an avian species require preparation, therefore, fitness in a seasonal environment depends not only on the occurrence of seasonal events at the appropriate time in a year but also on the ability of the birds to anticipate and prepare for the favourable seasons in advance of their arrival. Thus, like most other wild vertebrates, birds also show a distinct temporal organization in their reproductive activity (Dawson *et al.,* 2001). The time and duration of the favourable season selected for reproduction differ among different climatic regions and different ecological groups of birds. Many environmental factors help birds in timing of seasonal reproduction and related events in their life history. The predictability of these factors is crucial as the birds need to make necessary physiological preparations before the onset of each life history stage (Dawson, 2008). It is important that the environmental factor selected by bird for control of its annual reproductive cycle must have reliable predictive value. The amplitude and predictability of environmental factors differ in different habitats of birds. These factors also differ in the geographical ranges occupied by them during their life span causing differences in timing and sequence of stages in their life-cycle. Various environmental factors have been classified into two categories by Baker (1938): the proximate and ultimate factors. The proximate factors help birds to select the most appropriate time window in the year for a seasonal event and the ultimate factors help decide the timing of actual seasonal event within this time window. The proximate factors provide the information when the breeding season should be scheduled in nature. This keeps both the sexes physiologically ready to avail any opportunity in the environment for reproduction. Thus, the role of proximate factor is very important with respect to timing of reproduction. The day length plays the role of proximate factor, while food, temperature, rainfall, etc. act possibly as ultimate factors or supplemental cues in control of annual reproductive cycle in majority of birds (Dixit and Singh, 2011). Some ultimate factors including nesting conditions, climate change, competition, predator pressure, etc. influence reproduction under special circumstances (Wingfield *et al.,* 2003). Many birds from both temperate as well as tropical and subtropical regions including India, therefore, use annual cycle of changes in photoperiod as calendar to time their reproductive and associated physiological and behavioural functions (Kumar *et al.,* 2004). The role of day length has been well recognized in the birds that breed at mid and high latitudes (Dawson *et al.,* 2001; Hau *et al.,* 2008). However, because of small annual variations in day length at lower latitudes, it is generally considered less suitable (Hau, 2001) and other nonphotic indicators such as temperature, rainfall, social factors

have been given much importance (Perfito *et al.,* 2008). However, despite little annual variation in tropical and subtropical regions, many avian species have been reported to be photoperiodic (Dixit *et al.,* 2017).

2. Photic cues in control of avian reproduction

The endogenous oscillators involved in control of reproduction are sensitive to certain environmental cues (Daan and Aschoff, 2001). Light is a primary environmental signal that can affect the period of circadian oscillation in birds and thus entrain its physiology and behaviour. Because the light environment varies both in terms of intensity and spectrum, it is logical to expect that endogenous clocks regulating daily and seasonal responses will be sensitive to both the light intensity and spectrum, besides its duration. Birds experience incremental shifts in the length, spectrum and intensity of day-to-day light in nature. Light exhibits its influence on reproduction of most birds by all or any combination of above three characteristics viz. duration, intensity and wavelength (Kumar, 2002).

2.1 Photoperiod

Of all the environmental factors to which the birds are exposed to photoperiod is the most reliable and consistent environmental cue in controlling avian reproduction (Dawson *et al.,* 2001; Dixit and Singh, 2020). Daily and seasonal changes in illumination on earth surface is due to earth's rotation on its axis and its revolution round the sun, respectively. The day length away from equator changes with season and the light intensity at any given time changes with weather even at the equator. The annual variation in day length at a given latitude remains fairly constant for several years. This constancy makes day length a potent environmental factor for birds to rely on in timing physiological preparations for reproduction and related events. Day length not only changes with the season but it also changes with the latitude. Therefore, birds show latitude and season dependent photoperiodic adaptations in their reproductive responses (Dixit and Singh, 2014). Photoperiod is known as the initial predictive cue for initiating reproductive development in most birds (Wingfield, 1983). Following Rowan's (1925) groundbreaking study on Slate-colored Juncos (*Junco hyemalis*), several investigations evidenced the importance of day length as a major source of environmental information in programming seasonal reproduction (Dawson and Sharp, 2007). Day length is predictable and provides reliable environmental information in regulation of reproductive cycles of the birds inhabiting both mid and high latitudes (Hau *et al*., 2008). Studies on sexual cycles of some Indian birds reveal that they correspond to the seasonal changes in day length (Thapliyal and Tewary, 1964). Various investigations on birds that breed in spring/summer months at high latitudes and overwinter in India including blackheaded bunting, redheaded bunting and rosefinch and also on Indian resident species like common myna, yellowthroated sparrow, tree sparrow, house sparrow, and baya weaver reveal that they are responsive to day length. These birds can be placed into following categories based on their responses

to artificial photoperiods: (i) those that exhibit gonadal development only under ultrashort day lengths (0.25 to 6 h) but fail to respond to the customary long and short days (8 to 24 h) for example Spotted Munia (Thapliyal *et al.,*1975; Chandola *et al.,* 1976) (ii) gonadal development occurs under long as well as short (8 to 24 h) days, e.g. Blackheaded Munia (Thapliyal and Saxena, 1964; Pandha and Thapliyal, 1969; Chandola *et al.,* 1973) (iv) short days (9L/15D) can stimulate gonadal development, however, pituitary function can be induced by long days as well, e.g. Lal Munia (Tewary, 1967) and (iv) long days (15L/9D) induce gonadal development while short days (below 9 h light/ day) fail to do so. Long daily photoperiods induce gonadal growth followed by regression and development of photorefractoriness in photoperiodic birds while short daily photoperiods fail to induce above events (Dixit and Singh, 2011). Thus, the seasonal responses in photoperiodic birds cycle between photosensitivity and photorefractoriness. The initiation of gonadal growth is a long day phenomenon while the termination of photorefractoriness and consequent recovery of photosensitivity is a short day phenomenon. Thus, both long and short days are important for photoperiodic regulation of annual reproductive cycles in them, although the birds use them for different purposes. The critical day length for a photoperiodic response is species specific and is a consequence of adaptation for breeding at specific time at particular latitude (Tewary and Dixit, 1986). The threshold photoperiods for initiation of gonadal growth lie between 10 and 11 h in tree sparrow (Dixit and Singh, 2011); 11–12 h in blackheaded bunting (Mishra and Tewary, 1999), weaver bird (Singh and Chandola, 1981), yellowbreasted bunting (Dixit et al., 2014) and between 12 and 13 h in rosefinch (Tewary and Dixit, 1983), redheaded bunting (Prasad, 1983), and yellowthroated sparrow (Tewary and Dixit, 1986). The rates of gonadal growth in these birds also change with changing photoperiods. The photosensitivity and initiation of reproductive events remain associated with the increase in gonadal growth and functions such as increase in sex steroids: testosterone and estradiol 17-β in male and female birds, respectively. Gonadal growth and development, in photoperiodic birds, are accompanied by increase in synthesis and release of hypothalamic gonadotropin releasing hormone (GnRH), pituitary gonadotropins (Leutenizing hormone, LH and follicle stimulating hormone, FSH) and gonadal steroids (testosterone and estradiol 17-β) and decrease in synthesis and release of hypothalamic gonadotropin inhibitory hormone (GnIH) and reverse is true during gonadal regression and photorefractoriness (Dixit and Singh, 2013; Dixit and Byrsat, 2018). Most photoperiodic birds cease to respond to the light stimulus upon long-term exposure to long day length (Bentley *et al.,* 1998). This total loss of sensitivity to long days is known as absolute photorefractoriness (Dawson and Sharp, 2007). It has adaptive significance as it restricts reproduction to best suited portion of the year, provides sufficient time for replenishment of energy stores for post reproductive events like molt and preparation for migration (Dixit and Singh, 2012). Gonads involute and become quiescent in photorefractory birds as the negative feedback mechanism operate to decrease the synthesis and release of GnRH from hypothalamus thereby suppressing pituitary gonadotropin and gonadal sex hormones secretions causing gonadal involution (Chandola *et al.,* 2004; Dixit and Singh, 2012). Restoration of responsiveness

to increasing day lengths (photosensitive) of spring and summer does not occur until photorefractory birds experience decreasing day lengths of autumn and winter in nature or short day lengths in laboratory conditions (Dixit and Singh, 2012). However, there are some birds that do not show phorefractoriness until they experience decreasing day lengths in nature like Japanese quail, *Coturnix coturnix japonica* (Robinson and Follett, 1982) and Indian weaver bird, *Ploceus philippinus* (Chakravorty and Saklani, 1985). This is known as relative photorefractoriness.

Apart from reproduction, birds show distinct seasonality in several other morphological, physiological and behavioural functions related to reproduction such as feathers molt, body fattening, bill and plumage coloration, hormone levels, song production etc. (Dawson *et al.,* 2001). Feather loss can impede flight efficiency (Swaddle *et al.,* 1999), hence good-quality feathers are important for survival. Feathers need to be replaced every year as they wear out. Therefore, feathers are regularly removed and replenished by new feathers through a high energy consuming process called molt. Most passerine birds molt seasonally and once or twice (pre and post nuptial molt) in a year. Reproduction and molt are generally phased at different times in a year as both are energy intensive phenomena (Dixit and Singh, 2011). Molt generally coincides with gonadal regression. However, it is not decreasing gonadal steroids but increase in prolactin during gonadal regression that perhaps regulates molt (Dawson, 2005). It is still not clear whether photoperiod has a direct impact on the molt or molt is a consequence of photoperiodic induction of gonadal regression and its physiological interaction with molt (Dawson, 2008). Photoperiod controls different physiological activities linked to migratory phenomena such as *hyperphagia*, increase in body mass as a result of fat build-up (Zugdisposition), and migratory restlessness or *zugunruhe* (Robart *et al.,* 2018). Photoperiodic induction of body fattening is important for bird migration as the stored fat is a rich source of energy and sustains long migratory flights (King, 1968). However, photoperiodic control of fattening is minimal or nonexistent in non-migratory birds as they have uniform availability of food across the year and any accumulation of fat in them may hamper their flight activities (Dixit and Singh, 2011). Photoperiodically induced secretions of sex-hormones control various developmental and behavioral responses related to breeding cycles of birds such as appearance of secondary sexual characters like changes in bill and plumage color, development of accessary sex structures and a display of reproductive behaviors like establishment of territory, song production, mate selection, nest building etc. (Dixit and Singh, 2011). Immune system is very important in fight against various infections and pathogens. It is reported that photoperiod and temperature take part in regulating the lymphoid organs like thymus, spleen and other parameters of the immune system in mammals (Demas *et al.,* 2003) and these factors have also been speculated to play such roles in birds. Study on lymphoid organ and responses to the mitogen Con A in jungle bush quail (*Perdicula asiatica*) indicates that minor variations in daylength may disturb the immune system by effecting existing levels of melatonin and sex hormones (Verma *et al.,* 2017). The Long days in summer months cause high secretion of sex hormones that in turn are responsible for reproductive activity but decrease the

immune status as steroids inhibit immunity (Singh and Haldar, 2005). Short days improve immune function in a state of energy-compromising conditions (Nelson and Drazen, 1999). This is done by repression of sex steroids and prolactin by the melatonin hormone. Thus, the neuroendocrine and immune system can be correlated to seasonal variations in day length.

2.2 Light Intensity

One of the critical components of light with a major impact on the photoperiodic seasonal responses of birds is the light intensity. It plays significant influence on photoperiodic seasonal responses of birds. Various intensities of light have been reported to influence reproductive activity in house sparrow (Menaker *et al.,* 1970), white-crowned sparrow (Farner, 1959), domestic duck (Benoit, 1964), domestic turkey (Nestor and Brown, 1972) and Japanese quail (Follett and Millette, 1982). Further, studies involving different combinations of light intensity and period suggest that both play significant roles in initiation and development of gonads in birds (Budki *et al.,* 2009). Furthermore, photoperiod-induced seasonal responses tend to have light intensity thresholds (Kumar and Rani, 1996). Light intensity could be a potent zeitgeber in affecting seasonal responses as it changes across the year in both tropical and temperate zones. It has been assigned a greater role in control of reproduction in the birds inhabiting lower latitudes. Although equatorial species have the ability to respond to photoperiod, they are unlikely to detect slight changes in day length prevailing at these latitudes (Moore *et al.,* 2005). Therefore, it is believed that these species utilize seasonal variations in other light parameters such as light intensity (Gwinner and Scheuerlein, 1998). There are reports suggesting that the equatorial birds can monitor seasonal variation in photic information by measuring light intensity (Hau et al., 2008).

2.3 Light Wavelength

The visible spectrum of light consists of seven colors of the rainbow (VIBGYOR) having a wavelength of 400-700 nm, starting from violet and ending to red. Violet and blue colour correspond to short wavelengths, green and yellow to mid-wavelengths and red to long wavelengths. The white light is mixture of the colours present in the visible spectrum while black is total absence of light. The spectral composition of daylight has been found to affect the circadian and seasonal photoperiodic responses in some birds (Rani *et al.,* 2002). Some investigations examining the effects of varying spectral composition of light have revealed the role of light wavelengths in photoperiodic responses of birds (Malik *et al.,* 2014; Yadav *et al.,* 2015). The effects of light wavelengths on egg laying have also been investigated to improve performance in turkeys (Hulet *et al.,* 1992), laying chickens and broilers (Halevy *et al.,* 2006; Zhang *et al.,* 2014). The importance of wavelength of light in control of photoperiodic responses in a long day species was recognized by studies of Oishi and Lauber (1973) and Foster *et al.* (1985) on Japanese quail. Also, the role of wavelength of light in regulation of both circadian and seasonal responses have been shown in studies on black- and red-headed bunting (Misra *et al.,* 2004; Malik *et al.,*

2004, 2014; Rani *et al.,* 2005).

3. Nonphotic cues in control of avian reproduction

The birds of same latitude breed at different times in different habitats, and in the same habitat between different years (Caro *et al.,* 2005). This suggests that apart from day length, other factors such as food availability, social factors, and ambient temperature can fine tune the initial, general reproductive response to photoperiod (Wingfield *et al.,* 1991). The non-photic cues such as temperature, rainfall, food abundance and social stimuli etc. may modulate the exact time of breeding within the time window fixed by photoperiod or may affect the timing of photoperiodic window for reproductive activities. The above factors provide only short term predictive information and can modify and/supplement the photoperiod induced responses (Scott *et al.,* 2007). Photoperiod as a proximate factor has been studied extensively for initiating seasonal reproduction in birds, while the use of other non-photic environmental factors like temperature (Parmesan, 2007; Wingfield *et al.,* 1992), rainfall (Sekercioğlu *et al.,* 2012; Hau, 2001), humidity (Hau *et al.,* 2004), food availability (Both and Visser, 2001) etc., in the regulation of reproductive function and in rare cases associated events is less well understood. Various studies recognize these cues as ultimate factors which help to decide the timing when the actual seasonal event would take place during the time window and supplement as well as modify the period of reproduction in birds. Variation in any one or more of these environmental factors may affect the timing of reproduction in birds. Therefore, it is essential to understand the role of non-photic cues in avian reproduction and its associated events.

3.1. Temperature

Temperature is one of various environmental factors that can influence the process and timing of avian reproduction directly by changing patterns and behaviour or indirectly by interfering with photoperiod and changing its effect (Parmesan, 2007; Dawson and Visser, 2010). The effect of ambient temperature on seasonal reproduction in birds differs among species and within the population of same species. Thus, the issue of whether temperature influences the timing of reproduction remains unanswered (Dixit *et al.,* 2018). There is evidence in some, but not in all species of birds, that the time of egg-laying varies with spring temperature (Torti and Dunn, 2005). It is less clear whether this is a consequence of a direct effect of temperature on photoperiodically induced gonadal maturation. Although attempts have been made to assess the role of temperature on photoperiodic induction of gonadal growth, the results, showing positive or negative effects, were inconclusive (Wingfield *et al.,* 2003; Spencer and Bryant, 2002).

The breeding seasons of many birds have been found to shift in response to rise in global temperature (Walther *et al.,* 2002; Parmesan, 2006). Those birds that adapt to the changing temperature survive and reproduce successfully while those exhibiting rigid life-history stages may perish (Visser *et al.,* 1998; Coppack and Pulido, 2004). There may be shift in breeding schedules of many tropical birds in response to change in spring

temperature (Dixit *et al.,* 2018). There may be advancement of the breeding season in these birds or they may produce lesser offspring due to decreased reproductive rate resulting in population decline (Wormworth and Sekercioğlu, 2011). There are studies suggesting that the birds' egg-laying dates are partly decided by the past ambient temperatures (Visser *et al.,* 2003; Salvante *et al.,* 2007). This is important as the bird's fitness is related to its ability to match reproduction with the short period of temperature-dependent arthropod abundance (Visser *et al.,* 2006). Visser *et al*. (1998) reported that the egg-laying date did not advance in the Dutch population of great tits (*Parus major*) over a period of 23 year period though the selection for early laying was intensified. The seasonal increase in temperature has been reported to fine-tune laying dates in great tits, while they remain unaffected by mean temperature and daily temperature variation (Schaper *et al.,* 2012). The effects of temperature on reproductive activities have been studied in some other avian species and it was found that high temperature fail to induce gonadal maturation in starlings (*Sturnus vulgaris*) and white-crowned sparrows (*Zonotrichia leucophrys gambelli*); however, low temperature delays the development of photorefractoriness in starlings (Dawson, 2005). The above findings, showing both positive and negative effects of temperature on gonadal development, are inconclusive (Spencer and Bryant, 2002; Wingfield *et al.,* 2003). The studies involving causal effects of temperature on seasonal timing of reproduction and related events are crucial in resolving mystery of the biological consequences of climate change (Caro *et al.,* 2013). The day length and temperature cycles are inseparable in nature. Therefore, it is reasonable to believe that the timing and duration of seasonal responses are possibly regulated by day length as well as temperature rather than by day length alone. Furthermore, temperature should also be considered as a predictor of future environmental conditions, in addition to its role as an environmental cue that constrains homeostasis through energetic challenges to the birds (Caro *et al.,* 2013).

3.2. Rainfall

Rainfall is another critically important environmental factor modifying the timing of avian reproduction both directly and indirectly. Inland water birds such as ducks are highly dependent on precipitation to withstand their wetland habitats while tropical species often have fixed breeding seasons related to predictable periods of rainfall (Wikelski *et al.,* 2000). Thus, any alteration in pattern of rainfall has major consequences for these species. Many birds inhabiting Himalayan regions and arid zones start breeding immediately after the first summer rainfall, the timing of which is unpredictable. Nest building in some species starts immediately with the onset of rain (Immelmann, 1973), which shows that the reproductive system by now was fairly mature to respond so quickly to the changes in the surrounding. Zebra finch is an opportunist breeder (Bentley *et al.,* 2000). This species although being a seasonal breeder in predictable habitats breeds opportunistically in arid zones where rainfall pattern is irregular. The gonadal growth is controlled by day length in Rufous-winged sparrow (*Aimophila carpalis*), but the exact timing of breeding occurs in response to rainfall within the breeding window (Deviche

et al., 2006). A similar response is exhibited by other species such as canaries (*Serinus canarius*) which is photoperiodic (Storey and Nicholls, 1976) but breeds in response to rainfall (Leitner *et al.,* 2003). Thus, rainfall acting as a supplementary cue ultimately fine tune the onset of breeding (Wikelski *et al.,* 2000). Many researchers are uncertain of the primary physiological mechanism i.e. the nature of the cue associated with rainfall and also how it interrelates with reproductive physiology. The cue may not be the rainfall itself, but any combination of factors associated with rainfall or consequences of rainfall i.e. the arrival of rain brings about changes in vegetation leading to an improved food supply, the environment is cleaned from dust particles allowing more ultra violet light to reach on earth, the landscape changes from brown to green etc.

3.3. Food Availability

Availability of food, upon which young ones depend, is an important ultimate factor that controls the timing of breeding (Lack, 1968). Food can also act as a proximate factor (Perrins, 1970) because females require sufficient food for the production of eggs. Reproduction in birds can be affected by providing supplement food (Davies and Deviche, 2014). Some studies have shown that egg laying dates in some birds advances if provided with supplemental food and this advancement is normally within the range of few days (Reynolds *et al.,* 2003). In starlings, the egg laying days can be advanced by 5 days on providing supplemental food (Kallander and Karlsson, 1993) and food restriction delays egg laying in captive birds (Meijer and Langer, 1995). In spotted antbirds (O'Brien and Hau, 2005) and wild canaries (*Serinus canaria*) (Voigt *et al.,* 2007), the testis development can be enhanced by food in the absence of photostimulation. Some species such as Crossbills (genus *Loxia*) breed opportunistically at any time of the year when there is sufficient amount of food available. However, the gonadal growth and regression, in this species, are regular seasonal events and the opportunism is mainly limited within this primary seasonality (Deviche and Sharp, 2001). Crossbills possess a long breeding window within which breeding can occur but the exact time of breeding within the window depends upon the food availability. Moreover, breeding does not occur even though food availability is maximum outside this breeding window during gonadal regression and molt (Hahn, 1995). However, food restriction has little or no significance on birds in which gonadal maturation is controlled by photoperiod (Meijer, 1991). Ovarian growth advances in tricolored blackbirds (*Agelaius tricolor*) upon providing live grasshoppers but the effect is not seen in the case of males (Payne, 1969). Few researchers are of the opinion that there exists no correlation between testicular growth and appropriate food items or food restriction (Vleck and Priedkalns, 1985). Thus, the food availability has a greater role as ultimate factors in control of avian reproduction. However, it also acts as proximate factor in special circumstances.

3.4 Social cues

Social cues related to reproduction help in communicating information about the reproductive state and availability and quality of potential mates and help in initiating reproductive process by mate selection, territorial establishment, courtship and clutch initiation. However, some social cues retard gonadal development and egg laying by acting negatively. Due to the multimodal sexual signals, the demonstration of social cues in many experiments conducted on gonadal development and lay is more varied and distinguished than for other kinds of non-photic cues. There are different experimental manipulations which include: inclusion and exclusion of potential partner (Perfito *et al.,* 2015), manipulation of partners reproductive state (Watts *et al*., 2016), intragroup manipulation of social status (Brouwer *et al.,* 2009), presentation of recorded song stimuli on behalf of potential mates (Chmura *et al.,* 2017) or a social group (Setiawan *et al.,* 2007), devocalization (Cheng, 1992) and inclusion or hindrance of visual cues (Meijer and Langer, 1995). Experiments conducted on female birds revealed that the presence of a sexually mature partner enhances follicular maturation (Perfito *et al.,* 2015) and shortens the time of clutch initiation (Crino *et al.,* 2017). However, the presence of male fail to affect the ovarian regression (Silverin and Westin, 1995). Further studies throw light on the characteristics of a partner that initiate gonadal development in females. Matthews (1939) and Stevenson *et al*. (2008) reported how gonadal growth can be influenced by the visual stimulus of a male. Gonadal growth and egg-laying can also be advanced by providing additional stimuli such as song tapes (Morton *et al.,* 1985) and the presence of mate (Shields *et al.,* 1989). In canaries it is seen that heterospecific and conspecific songs enhance follicular growth (Bentley *et al.,* 2000) but between the two, conspecific song is more effective stimulus. There occurs enhancement of gonadal growth in female rock doves when males specifically direct courtship song towards her (Friedman, 1977). Brockway (1965) suggested that the male budgerigar's "soft warble" vocalization can act as an effective way to promote egg laying in females. In female canaries, longer-higher quality vocalizations can result in nest building and formation of larger clutches (Kroodsma, 1976).

4. **Conclusion and Perspective**

Reproduction is the part of life cycle with great environmental dependence. Various photic and non-photic environmental factors help birds in precise timing of their seasonal reproduction when resources in the wild are optimally present and chances for survival of offspring are maximized. The proximate factor like day length, acting as a reliable and predictable cue, helps birds to choose the most appropriate time window for seasonal reproduction; however, the non-photic cues such as ambient temperature, rainfall, food availability and social factors etc. acting as ultimate factors help decide when precisely the actual breeding should occur in the photoperiodic window. The predictability of environmental factors keeps the birds physiologically ready before the favorable season arrives. The integration of environmental factors and neuroendocrine

components regulates the physiological, developmental and behavioral responses related to seasonal reproduction in birds. There is paucity of data on sex-dependent and latitudinal variations in environmental control of avian seasonal reproduction opening up new avenues of research in the field. New experiments designed to focus on molecular mechanisms and physiological pathways are needed to reveal the complete story.

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Multi-spectroscopic investigation on the contradictory relevance of metal nanoparticles in pharmacological milieu of protein glycation and cholinergic inhibition

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Abstract

While unique physico-chemical behaviour of engineered nanoparticles have brought about a large-scale revolution in many fields like medicine, industry, agriculture and technology, it may result in certain toxicity due to unsolicited interactions with diverse biological sections and cellular processes. In this contribution, we report on the inhibitory effect of colloidal gold nanoparticles (AuNPs) towards the formation of Advanced Glycated End (AGE) products, which proves advantageous in the treatment of diabetes mellitus (DM). On the other hand, AuNPs boost the activity of acetylcholinesterase (AChE), the key enzyme which forms the basis of the cholinergic hypothesis for Alzheimer's disease (AD). Since higher activity of AChE increases the severity of AD, this activity-enhancing effect can be considered as nanotoxicity in this context. These seemingly contradictory facets of AuNP interaction with the biological entities associated with two deadly modern-day diseases like DM and AD, are explored in detail.

Key words: Metal nanoparticle; AGE product fluorescence; cholinergic hypothesis; diabetes mellitus; Alzheimer's disease

1. Introduction

The biomolecules responsible for smooth functioning of countless metabolic functions in the body are of nanoscale dimensions. Therefore nanoparticles (NPs), which have gained wide recognition in the past decade for their various applications, make for an interesting system to study nano-bio interactions (Wang *et al.,* 2019; Darr *et al.,* 2017) . The midas touch of nanotechnology has left very few aspects of human life unaffected, and many reports show that NPs exert considerable influence on bodily functions of absorption, distribution, metabolism and excretion (ADME) of life-saving drugs (Yang *et al.,* 2010). They also affect the drug carrying affinity of serum proteins, as well the activity of other biomolecules (Abbasi *et al.,* 2012).

NPs have a plethora of potential innovative applications in many avenues of biomedicine, catalysis, fuel cell, magnetic data storage, agriculture, solar-cell etc (Han *et*

al., 2019). Extensive research has been carried out on a variety of NPs like gold, silver, iron oxide, silica, and carbon-based materials, for diagnostic and therapeutic purposes. NPs loaded with drugs show drug release at precise rates and doses at target sites in the body for specific durations, resulting in accurate transport. This enhances the therapeutic effect of the drug and reduces the toxicity as well as the side effects (Paul and Sharma, 2020). Due to their suitable dimensionality, NPs also possess the ability to cross the Blood Brain Barrier (BBB) (Posadas *et al.,* 2016) further enhancing their usefulness in drug discovery and drug targeting.

Gold nanoparticles (AuNPs) have wide application as biomarkers due to their tunable optical property and are widely used for photo-thermal therapy of cancer cells (Jain *et al.*, 2012). Magnetic NPs, commonly iron oxide nanoparticles, have also been recently used as an alternative for cancer therapy (Saeed *et al.*, 2018). In addition to these, the activity of AuNPs along with their biocompatibility has made them preferable for ophthalmological implications. On a different note, it is well known that oxidative stress plays a significant role in the etiology of several diabetic complications (Giacco and Brownlee, 2010; Samadi *et al.,* 2019). The ability of AuNPs to inhibit the lipid from peroxidation, and thereby preventing the generation of reactive oxygen species (ROS), has restored the imbalances in the antioxidants and liver enzymes which are responsible for the cell dysfunction and destruction in the diabetic control group at hyperglycemic conditions. Thus, AuNPs are also regarded as potent antioxidants (Barathmanikanth *et al.,* 2010).

Typically, smaller NPs favour native-like protein structure, resulting in higher intrinsic enzyme activity of the protein-NP bioconjugate (Kozlowski *et al.,* 2018). The mechanism behind the impact on protein structure is generally explained by a simple model involving the available surface area for varied sizes of NPs. The large surface area of contact for the adsorbed proteins, provided by the relatively bigger sized NPs, results in stronger interactions between proteins and nanoparticles (Saptarshi *et al.,* 2013). This larger degree of interaction leads to greater perturbation in the protein structure.

Serum albumins are proteins which constitute nearly 60% of the blood plasma and play a primary role in the transport of enzymes, minerals as well as drugs in the body. Thus, these carrier proteins provide the most relevant bio-mimicking prototype for analysing the mechanism of drug interactions (Wong and Ho, 2018). It would therefore be interesting to determine the augmented drug carrying capacity of serum proteins quantitatively in presence of colloidal metal NPs. However, incorrect usage of NPs targeting the efficient drug delivery in the body leads to toxic effects. Therefore, an accurate and judicious standardization is required to achieve a nanomaterial showing different properties like enhanced diagnosis, as well as monitoring and treatment of human diseases. It is pertinent to mention here that the use of NPs not only depends on its size and shape, but on a combination of different cellular uptake mechanisms such as varying level of the target receptor, membrane fluidity, and cell cycle, to name a few (Stark, 2011).

Fluorescence-based studies on drug interaction with NP-protein bioconjugate have attracted massive attention in recent years. In these studies, fluorescence quenching is commonly employed to understand the thermodynamic properties of drug binding, number of binding sites etc. Colloidal metal NPs of gold and silver (AuNP and AgNP, respectively) have been shown to increase drug transport capacity and improve drug-protein binding in specific cases, a few reports of which have recently been published by our laboratory (Sonu *et al*., 2019; Sonu and Mitra, 2019). Furthermore, previous experiments on FDA-approved anti-Alzheimer drugs working as AChE inhibitors have revealed that the inhibition potency of the drugs differ considerably in the presence of human serum albumin (HSA), compared to aqueous buffer. HSA significantly reduces the inhibition potency of a series of well-known drugs. This modulatory behaviour of the serum albumin has established it as an important pharmacological medium for studying AChE inhibition efficacies (Islam *et al*., 2016). On the other hand, kinetic enzyme assays are widely employed to mimic relevant biological media to study the effects of NPs on the activity of various enzymes of interest.

In this contribution, we present some of our recent findings on the inhibitory effect of metal NPs on the formation of Advanced Glycated End (AGE) products and their boosting effect on acetylcholinesterase (AChE) enzyme activity. Detailed investigation reveals that the effect of AuNP on glycation is somewhat different from that of an anti-diabetic drug, chlorpropamide (CPM). The effect of AuNP and HSA on the inhibition effect of the FDA-approved AD drug Donepezil (DON) was observed to be analogous, albeit with different mechanisms and degrees of interaction. Before elaborating on the details of the experimental results, we give a brief introduction of the AGE product formation and AChE enzyme activity with reference to two deadly and commonly incurred ailments in modern day human health, namely diabetes mellitus (DM) and Alzheimer's disease (AD). This is followed by a succinct description of the experimental protocols. Finally, we analyse the modulating effect of NPs on these two bio-chemical pathways from a therapeutic point of view.

2. Diabetes Mellitus and formation of Advanced Glycated End (AGE) Product

Hyperglycemia, which refers to a spike in the blood glucose level, is the foremost symptom expressed in diabetic patients (Giacco and Brownlee, 2010). In prolonged hyperglycemia, the aldehyde functional group of the reducing sugar molecules binds to the amino group of the exposed lysine and arginine residues of the protein, leading to the formation of a Schiff base, and thereafter a rearrangement product known as Amadori product (Baynes *et al.,* 1989). Following several steps, combinedly known as Maillard reaction, the formation of advanced glycation end (AGE) products occurs. This entire process is termed as *glycation*. Accumulation of AGE products for an extended time causes many diabetic complications, cataract formation as well as neurodegenerative disorders like Alzheimer's and Parkinson's disease etc (Li *et al.,* 2012; Vicente Miranda *et al.,* 2016).

It is difficult to alter the level of glycated albumins by haemoglobin metabolism. Therefore, the glycated albumins can be used as a short-term measure to gauge glycemic control in diabetic patients, which facilitates its usage as a medical marker for diabetes (Roohk and Zaidi, 2008). Glycation brings about modifications in the structure of serum albumins which in turn alters the enzymatic activity, binding affinity, transportation and distribution of drugs in the body (Anguizola *et al.,* 2013). Many drugs, like metformin and aminoguanidine etc., have been synthesized with promising inhibitory effects towards the glycation of serum albumin (Abbas *et al.*, 2016; Arasteh *et al.*, 2014). However, certain side effects have posed hurdles to their unhindered advancement as a viable therapeutic avenue.

In this regard, the compatibility of NPs with biological systems, as discussed earlier, minimizes the problems related to finding inhibitors of glycation. This is because they can easily pass through the cell membranes and possess a desirably high surface to volume ratio due to which cells, proteins, and biological membranes can be adsorbed easily on the NP surface. Recently ZnO, gold (Au) and selenium (Se) nanoparticles have shown the capacity to inhibit glycation of albumins by D-ribose and glucose (Seneviratne *et al.,* 2012; Liu *et al.,* 2014). In this report, the efficacy of AuNPs (which possess low toxicity and easy bioavailability) in inhibiting the glycation of bovine serum albumin (BSA) induced by L-arabinose, has been quantified and compared with anti-diabetic drug chlorpropamide (CPM) as a reference model system.

3. Cholinergic hypothesis on Alzheimer's disease

Acetylcholinesterase (AChE) is the enzyme involved in terminating impulse transmission through rapid hydrolysis of the neurotransmitter acetylcholine (ACh). Decline in ACh levels has been established as a key element in the pathogenesis of Alzheimer's Disease (AD) (Terry and Buccafusco, 2003). Most of the commercially available drugs currently employed in the treatment of AD are based on this theory, typically known as the *cholinergic hypothesis* (Nordberg, 1992). The modus operandi of the drugs is to increase the concentration of ACh in the synaptic cleft by inhibiting the AChE activity. This subsequently boosts cholinergic neurotransmission in the brain, and thus, AChE inhibition has been established as the most feasible route in the treatment of AD. A direct corollary of this hypothesis is that any substance which reduces the activity of AChE would be considered advantageous in the purview of AD. On the other hand, any entity causing an increase in the activity would be considered toxic in the same regard.

The primary direction in designing and development of AD drugs lies in the search for potent inhibitors of AChE activity, as this leads to a spike in the production of acetylcholine and, consequently, improves neurotransmission. With improved, environmentally benign, and efficient synthetic methodologies in combination with in-silico molecular modeling protocols, the search for novel AChE inhibitors has gained momentum lately. A considerable amount of work in this regard has also been reported by our laboratory (Baruah *et al*., 2019a, 2019b; Rohman *et al*., 2019). In this contribution,

the modulatory AChE inhibition activity of DON in buffer medium and physiologically relevant HSA matrix has been explored in detail. Further, the effect of AuNP on the enzymatic activity of AChE is also reported. The pharmacological consequence, as well as the mode of action in both the cases are discussed in an exhaustive manner.

4. Materials & Methods

All reagents/chemicals used in this study were of highest quality available and purchased from reputed vendors like Sigma-Aldrich, Qualigens, Merck etc. AuNPs (of size 14±2 nm) were synthesized through standard reduction procedure described elsewhere. (Sonu & Mitra, 2019). BSA was glycated by reacting with 0.4 M of L-arabinose wherein the concentration of the working protein solution was kept fixed at 5 μM. A fixed amount of 0.13 nM and 0.68 nM solution of AuNPs was added during the process of glycation to check if AuNPs exerted any influence in the glycation process. The glycated BSA (gBSAara) was incubated for 90 hrs at 37 °C. Excitation at 335 nm is used to monitor protein modifications induced by the monosaccharide's pentosidine type compound; whereas, excitation at 370 nm is used to investigate the alteration in protein due to the formation of different AGE products (Cervantes-Laurean *et al*., 2006). The inhibition of the glycation by AuNPs was examined by observing the AGE's intensity after every 10 hrs.

Absorption measurements were recorded on a double beam, ultraviolet-visible (UV-Vis) spectrophotometer (PerkinElmer Lambda25). Steady state fluorescence spectra and quantum yield measurements were carried out in Quanta Master (QM-40) steady state apparatus obtained from Photon Technology International (PTI). All the fluorescence spectra were corrected for the spectral sensitivity of the photomultiplier tube (PMT).

The acetylcholinesterase enzymatic activity in absence and presence of various inhibitor drugs was measured spectrophometrically in a high-throughput technique following the method originally developed by Ellman and co-workers in 1961 (ELLMAN *et al*., 1961), the details of which have been described elsewhere (Baruah, Basumatary, *et al*., 2019; Baruah, Rohman, *et al*., 2019).

Analysis of enzymatic hydrolysis data using Michalis – Menten (MM) model both in presence and absence of the inhibitor results in the estimation of initial rate (V_0), MM constant (K_m) and maximum hydrolysis rate (V_{max}). The substrate concentration was varied from 50 μM to 2.0 mM. To study the inhibition potency of various inhibitors in all the studied media the substrate concentration was kept fixed at the saturated reaction condition of AChE catalysis reaction (~1.5 mM) and, in some cases, at around the K_m value of the enzyme (0.15 mM) (Khandkar *et al*., 1995); whereas, the inhibitors concentrations were varied from very low till the saturation inhibition condition. In both the non-enzymatic as well as enzymatic reaction conditions, the [DTNB] were kept fixed at ~317 μM; whereas, the enzyme concentrations were ~0.079 units/ml in all the enzymatic reactions on-enzymatic blank was subtracted for each kinetic measurement

and all components were incubated for 5 min on ice cold water bath followed by total run time of 50 min inside the plate reader at the experimental temperature to follow the kinetics.

The well-established Hill protocol (Copeland, 2005) was utilized to study the effectiveness of the various inhibitor drugs towards the AChE enzymatic activity which yielded the values of the decrease in initial velocity in the presence of certain inhibitor concentration (ΔV), the maximum decrease in initial velocity (ΔV_{max}), the concentration of the inhibitor that gives half-maximal initial velocity, $K_{0.5}$ (which is pharmacologically equivalent to IC_{50}), and the Hill coefficient, n_H.

5. Results and Discussion

5.1. Effect of AuNP on the glycation of BSA: Comparing with model anti-diabetic drug

5.1.1. Monitoring AGE product fluorescence. BSA was glycated by incubating the protein with 0.4 M of L-arabinose solution for a period of 48 hours and the AGE'S fluorescence was examined after every 10 hours. It is to be noted here that the concentration of L-arabinose used in this study is substantially higher than that present in the physiological condition (Ning & Segal, 2000) This is to ensure the formation of sufficient and experimentally detectable glycated product in a reasonable time frame. Optimization of the experimental conditions, however, indicates that it is apparently possible to measure the glycation even after 24 hours, which gives adequate signal to noise ratio to quantitatively examine the AGE fluorescence, since the nature of fluorescence spectral profile remains the same even after longer incubation with only an increase in the fluorescence emission intensity.

To comprehend the extent as well as the effect of glycation and formation of AGE products, fluorescence spectrum of the incubated BSA solution was obtained by excitation at 335 nm and 370 nm (Figure 1). Blank data of the buffer and the sugar solution, taken under similar experimental conditions, were subtracted from each spectrum. Further, the solutions of model anti-diabetic drug chlorpropamide (CPM) with varying concentrations (ca. 0.08 and 0.16 mM) were checked to verify if CPM possesses any fluorescence intensity. No significant fluorescence was observed at either of these wavelengths. Excitation of the glycated protein ($gBSA_{ara}$) at 335 nm results in the AGE's fluorescence spectrum at around 415 nm, which is close to the intrinsic emission of the pentosidine molecule. Addition of 0.16 mM concentration of the antidiabetic drug CPM enhances the AGE fluorescence intensity by more than twice in case of $gBSA_{ara}$ (Figure 1a, inset). On the other hand, excitation at 370 nm results in the AGE's fluorescence intensity around 440 nm, which corresponds to the characteristic peak of crossline and vesperlysine. Even with 0.08 mM of CPM added to the glycated protein solutions, an appreciable increase in the AGE fluorescence is observed (Figure 1b, inset).

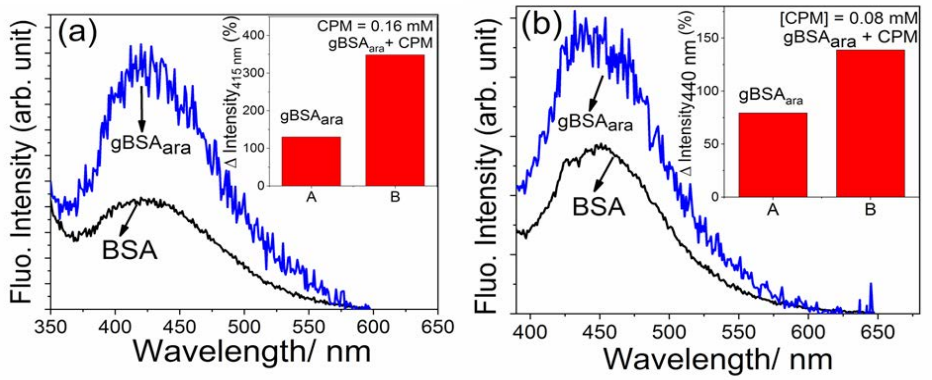

Figure 1: Fluorescence spectra of BSA and L-arabinose induced glycated BSA (gBSAara) excited at 335 nm (a) and 370 nm (b), respectively. Inset shows the bar diagram of AGE's intensity generated (in percentage) relative to that of native BSA when excited at 335 nm and 370 nm respectively.

In presence of colloidal AuNP solution, a sharp diminution is observed in the fluorescence intensity of glycated BSA. The intensity further decreases on increasing the concentrations of AuNP. AGE's fluorescence intensity of gBSAara is reduced by 55% when 0.13 nM of AuNP was added, indicating an inhibition in the development of the AGE's related compounds in presence of AuNP. However, the emission intensity decreases by a significant 80% at [AuNP] = 0.68 nM (Figure 2a). Interestingly, the inhibitory efficacy exerted by AuNPs is observed to decrease over a prolonged time. For example, incubation of 34 hours leads to the decrease in glycated product fluorescence merely by 20% or 40% under the similar concentration of AuNP (Figure 2b). When the excitation wavelength is set at 370 nm, similar effects are also observed as in the case of 335 nm excitation. There is a 49% decrease in the AGE's intensity of gBSAara when incubated with 0.16 nM solution of AuNP, in comparison with 84% decrease at [AuNP] = 0.68 nM. This points towards a concentration and time dependent effect of the AuNPs in the effective inhibition of glycated BSA (Inset, Figure 2a).

These studies show a highly interesting consequence that while CPM increases the extent of protein glycation, AuNPs decrease it significantly. Interestingly, the increase in glycation of serum protein by anti-diabetic drug CPM directly contradicts its therapeutic effectiveness in the purview of diabetes. The clarification perhaps lies in the fact that the modus operandi of CPM is to boost the production of insulin by the beta-pancreas and not to act as an anti-glycating agent (Miller & Moses, 1970). Our studies prove that the presence of an excessive amount of CPM in blood serum either due to protracted usage or drug overdose surges the likelihood of protein glycation and might prove hazardous in the long run.

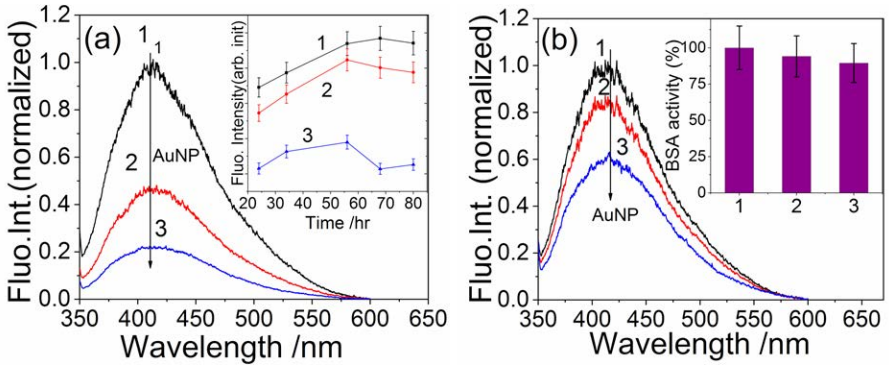

Figure 2. AGE's fluorescence spectra of gBSara (λ_{exc} = 335 nm) after 24 hr (a) and 34 hr (b) incubation with [AuNP] /nM = 0 (1), 0.13 (2) and 0.68 (3). Inset shows the variation in AGE intensity with time in different AuNP concentration (a) and consistent BSA activity at different concentrations of AuNP (b).

Nevertheless, from a pharmacological point of view, AuNP mediated delivery formulation of CPM can be hypothesized to achieve the benefit of both these two pathways. It is expected that this combined formulation would act simultaneously in improving the insulin production through CPM, while inhibiting the AGE product formation through AuNP. However, the possible concerns may be the effect of AuNP on the drug carrying capacity of the serum protein and retention of enzymatic activity of albumin protein – NP bioconjugate.

5.1.2. Interaction of CPM with native protein and protein – AuNP bioconjugate. The binding efficiency of CPM with protein and protein-AuNP conjugate is determined by monitoring the intrinsic tryptophan fluorescence of BSA on varying concentrations of the drug. In both the cases, the fluorescence spectra centred at 342 nm is quenched regularly with an increase in the concentration of the drug without any shift of the emission maxima. Detailed steady state and time-resolved fluorescence analysis along with molecular docking calculation and other complementary spectroscopic investigations confirm that the fluorescence quenching is due to the ground state complex formation between the protein and the drug primarily through an "entropy driven" pathway (Singh & Mitra, 2020). The Stern-Volmer quenching constant (K_{SV} /10^3 M^{-1}) varies in the range of 3.21 ~ 5.85 and 6.76 ~ 14.26 for the interaction of CPM with BSA and BSA – AuNP conjugate, respectively within the temperature range of 298 ~ 318 K. Interestingly, the association constant (K_a) of the protein – drug complex formation remains almost similar for CPM interaction with native BSA ($3.48\pm0.24\cdot10^3$ M^{-1}) and BSA – AuNP bioconjugate ($3.50\pm0.43\cdot10^3$ M^{-1}) at 298 K. This apparently insignificant change in binding efficiency of CPM with BSA and its nanoconjugate is consistent with negligible change in protein secondary structure as confirmed from far-UV circular dichroism (CD) absorption measurement at 208 nm and by monitoring the position of amide I peak in infra-red (IR) spectra. The experimental results confirm that nanoparticle (AuNP) formulation for the delivery of CPM would enhance its efficacy toward the treatment of DM through

inhibiting AGE product formation without compromising the drug carrying ability of serum protein.

5.1.3. Effect of AuNP on the enzymatic activity of BSA. In contrast to many of its counterpart plasma proteins, serum albumin does not contain a carbohydrate moiety on its surface which gives it greater flexibility in performing its numerous biological functions (Bteich, 2019). Albumin is not just a passive but an active participant in several pharmaco- or toxicokinetic processes (SUDLOW *et al*., 1976) The interaction of serum albumins with esters have gained considerable interest in recent times (Wu *et al*., 2016) In numerous experiments, esterase or pseudo-esterase activity of albumin was demonstrated with respect to α- and β-naphthyl acetate or p-nitrophenylacetate (Sakurai et al., 2004). In this study, the enzymatic activity of BSA has been checked spectrophotometrically in presence of p-nitrophenyl acetate (pNA), by monitoring the quantity of pNA released at 410 nm following the protocol mentioned elsewhere (Østdal & Andersen, 1996). The intrinsic absorbance of AuNP was subtracted for obtaining the corrected optical density. The activity of native BSA is taken to be 100%. The interaction of BSA with the two working concentrations of AuNP in the present experiment (i.e. 0.13 and 0.68 nM) results in only about 6.2±0.3 and 10.8±0.2% loss in enzymatic activity relative to native BSA (Figure 2b, inset). This result implies that addition of AuNP, even at the highest working concentration in this study, does not significantly alter the enzymatic activity of serum protein and therefore, gives a viable option in the field of nanomedicine.

5.2. Effect of AuNP on the AChE activity: Comparing with FDA approved AD drug

5.2.1. Quantification of AChE inhibition. Inhibition of AChE activity is quantified in the presence of FDA-approved reversible inhibitor donepezil hydrochloride monohydrate (DON) following modified Ellman method along with kinetic scheme based on MM mechanism. The kinetic parameters for enzyme hydrolysis ([AChE] = 0.079 u/ml) in 0.1 M phosphate buffer medium of pH = 8 are listed in table 1. The results indicate that while the magnitude of MM constant (K_m) remains constant, the inhibition is associated with the reduction of the V_{max} value, suggesting a non-competitive type of inhibition mechanism to be operative in this case. This is a special type of the mixed inhibition mechanism and involves both the competitive [binding of inhibitor (I) with the enzyme (E) to give EI complex in lieu of the substrate (S) to give ES] and uncompetitive (inhibitor binds with ES to give IES complex) inhibition pathways. This mechanism is further ensured from the equivalence of measured α (=1+[EI]/[I]) and α′ (=1+[IES]/[ES]) values. The calculated IC50 value for the enzyme hydrolysis data is found to be 68.3±3.5 nM.

Table 1. Kinetic data of enzyme hydrolysis and the effect of different concentrations of DON on various kinetic parameters in aqueous buffer and in presence of 250 μM HSA.

[DON] /nM	K_m /μM	V_{max} /nMs^{-1}	α	α′
Aqueous buffer of pH =8 (IC_{50} =68 ± 4 nM)				
0	120 ± 20	815 ± 46	1.0 ± 0.3	1.0 ± 0.1
5	100 ± 10	565 ± 29	1.2 ± 0.3	1.4 ± 0.1
10	120 ± 20	390 ± 26	2.1 ± 0.5	2.1 ± 0.2
250 μM HSA matrix (IC_{50} =159 ± 8 nM)				
0	120 ± 20	872 ± 72	1.0 ± 0.3	1.0 ± 0.1
5	150 ± 20	616 ± 28	1.8 ± 0.4	1.4 ± 0.1
10	140 ± 30	606 ± 35	1.7 ± 0.5	1.4 ± 0.1

Considering the severity of the increased number of affected patients and alarming death statistics in AD, there is a lot of research on the development of new drugs with enhanced therapeutic effectiveness at present. Out of the only five chemical species cleared by FDA towards the treatment of AD till date, pharmaco-kinetic parameters confirm the efficiency of DON to be among the best. Therefore, it is often the usual practice to compare the effectiveness of a newly synthesized drug in buffer solution with that of DON before declaring it a potent pro-drug and subsequent animal trial. Interestingly however, while the ADME of DON is well-known, the effect of HSA on the kinetics of AChE inhibition is not studied in detail. Considering this, an in-vitro investigation is done to monitor the AChE inhibition under the condition of fixed HSA concentration of ca. 250 μM, which is close to the reported level of physiological abundance of serum albumins. The experimental results (table 1) reveal that while the mechanism of AChE inhibition remains the same even in HSA medium, the inhibitory potential decreases significantly with calculated IC_{50} = 158.8±7.9 nm. The extent of this modulation, parametrized by the relative change in IC_{50} value, is compared with similar experimental results for several other AD drugs and is found to be strongly correlated with the sequestration ability of HSA toward the inhibitor drug (Islam *et al*., 2016). While our results point toward a significant question mark on "potential" AChE inhibitors in buffer reported randomly in current literature, it also confirms that drug activity as AChE inhibitor can be strongly modulated depending on the experimental medium.

5.2.2. Effect of AuNPs on enzymatic activity of AChE. Numerous reports showing a considerable effect of NPs on biochemical activity of different enzymes propelled us to check the influence of AuNPs on the enzymatic activity and kinetics of AChE. It is to be noted here that all the nanoparticle experiments are conducted in aqueous buffer medium in these preliminary experiments, since the presence of serum protein (HSA) would render additional complications in standardizing the enzymatic reaction due to the formation of protein-corona. Nevertheless, analysis of the kinetic parameters and the MM plots obtained from the enzyme hydrolysis data (Figure 3) revealed that in case of AuNP, only V_{max} shows a sharp increase. An initial value of 801 nM s^{-1} for unadulterated AChE rises to 1052 nM s^{-1} in presence of 10 nM AuNP. However, the values of K_m for AChE and 10 nM AuNP-treated AChE are 170 ±13 μM and 183 ± 11 μM , implying a practically constant value. Since V_{max} is the rate of reaction when the enzyme is saturated with substrate i.e. the maximum achievable reaction velocity, it can be inferred that AuNPs increase the activity of AChE by enhancing the rate of enzymatic reaction. However, since the magnitude of K_m (which is an inverse measure of the enzyme affinity to the substrate) remains unchanged, it is clear that AuNPs do not cause any alteration in the enzyme-substrate affinity.

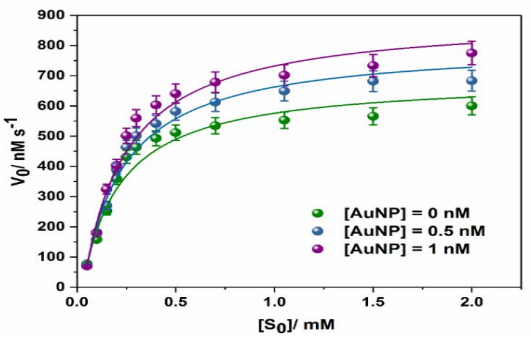

Figure 3. Hydrolysis curve (scattered points) for AChE activity and its inhibition in presence of different concentrations of AuNP in phosphate buffer solution of pH = 8.0. The solid line represents non-linear regression of the experimental data points. [AChE] = 0.079 u/ml.

Comparison of the calculated kinetic parameters indicates that AuNPs act as enzyme activators for AChE, possibly through immobilization on enzyme surface. Typically, enzyme immobilization refers to the optimization of the operational performance of an enzyme by adsorbing it on a matrix of choice. NPs act as very efficient support materials for enzyme immobilization, because of their extreme bio-compatibility. Enzyme bound NPs have been found to show Brownian movement, when dispersed in aqueous solutions, showing the enzymatic activities to be comparatively better than that of the unbound enzyme (Lee & Au-Duong, 2018; Breger *et al*., 2019) Various reviews on immobilization of enzymes on different types of nanoparticles (for example, metal and metal-oxide nanoparticles, magnetic nanoparticles, porous and polymeric nanoparticles etc.) have been reported (Gupta *et al*., 2011; Ansari & Husain, 2012; Verma *et al*., 2013).

In this case too, the activity of AChE is seen to increase in the presence of AuNPs, leading to the conclusion that the enzyme adsorbs itself efficiently on the AuNP surface, leading to a boost in performance.

However, the boosting of AChE activity renders an event of possible toxicity in the purview of Alzheimer's disease (AD) in relation to the cholinergic hypothesis. This is because the higher activity of AChE results in more acetylcholine (ACh) break-down into acetate and choline, a reaction which AChsE catalyses very efficiently. Since a decline in ACh level has been a primary pathological cause of AD, AuNPs (which act as AChE activity boosters) would pose danger in the purview of this deadly disease. Therefore, the unperturbed usage of NPs in neurological functions is restricted despite its advantage of easy BBB permeability due to its small size.

Considered in a wider perspective, the present investigation implies that AuNPs not only put someone who is exposed to suspended NPs through air and/or industrial pollution at risk of incurring AD, but also poses a significant threat towards the effective treatment of the AD-affected population. Proper healthcare formalities might need to be prescribed for patients undergoing treatment for dementia; otherwise, excess use of cholinergic drugs (to achieve the desired result of overcoming the influence of nanotoxicity) may result in drug overdose and additional side effects. This indicates that tweaking the formulations of metallic NPs for usage in medicinal fields, as well as countering the neurologically toxic effects of NPs in people exposed to these for prolonged periods, needs to be approached judiciously

6. Concluding remarks

Nanoparticles have been proven to be extremely valuable in biomedical applications with respect to chemical sensing, bacteria annihilation, biological imaging, drug delivery, and cancer treatment. However, despite all the positive build-up that they have garnered over the years, their effects are not unequivocally beneficial. In this study, we have explored two margins of AuNP with contradictory relevance in the field of medicine.

AuNPs act as potent inhibitors in the glycation of BSA by L-arabinose, demonstrating their usefulness in the treatment of diabetic patients. The anti-glycation properties of AuNPs, followed through their drug binding technique, could be a useful tool in the field of nanomedicine. This result is extremely important because an anti-diabetic drug, CPM, was found to enhance protein glycation, exhibiting a behaviour contrary to what is expected of it. Hence, this might open new avenues, and showcase the necessity of thoroughly examining the pathology of DM, the mechanism of glycation, and their relationship with each other. On the other hand, AuNPs exert a boosting influence on the enzyme kinetics of AChE, implying their toxicity in relation to AD.

Since NP mediated drug delivery is gaining popularity in pharmacology, our

current observations provide new insights, and demand justification of their usage. It further indicates that the haste in using NPs as biological carriers should be accompanied by caution and a thorough understanding of its probable influences on different biological media. Only with exhaustive and strategic approaches can NPs be utilized to their full potential to yield fruitful results in the pharmacological milieu.

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First record of new 'invasion' in northeast India by *Centaurea cyanus* L. (Asteraceae)

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Abstract

The region of northeast India boasts sizeable flora of non-native species which has arrived during last few centuries. Whilst several of these species have escaped in wild and naturalized, others have mingled in the flora and remain oblivious. Some species have gained dominance and replaced native flora and these are considered "invasive". This article reports the first record of an herbaceous plant, Centaurea cyanus L. of Asteraceae family from northeast India. The plant was found in the summer of 2020 in the campus of North-Eastern Hill University, Shillong while it was competing with several other non-native species on poor substratum. An extensive search in the floras of the region revealed that the plant has not been reported previously. It is probable that the plant has arrived here either as a companion with the supplies of packaged seeds of garden flowers procured by the residents from various sources or through some other carrier from nearby villages where it has made presence. Only time will answer if the plant remains oblivious or turns invasive.

Keywords: *Centaurea cyanus*, cornflower, invasive species, medicinal plants.

Introduction

The flora of northeastern region of India has been invaded by a number of plants during the nineteenth and twentieth centuries. The invading species predominantly belong to Asteraceae and are detected only after they have gained dominance in the landscape (Shankar *et al.*, 2011). Many of these species have naturalized and mixed up with the local flora (Anandhapriyan and Shankar, 2018; Sarma *et al.*, 2019). However, several of the invasive species pose a serious threat to the productivity of crops, cause toxicity in the soil and are responsible for health hazards of animals as well as humans (Reddy *et al.*, 2008; Bhatt *et al.*, 2011).

Recently, a beautiful pinkish-flowered herbaceous plant was noticed in the campus of the North-Eastern Hill University, Shillong. Since the plant appeared new, the pictures of the plant were shared with a few taxonomist colleagues. The lead to the generic name was offered by Professor B.K. Datta of Tripura University and with some effort, it was confirmed as *Centaurea cyanus* L. which is popularly known as 'cornflower'. After a reconnaissance of botanical literature, it was concluded that the plant had not been

reported in the floras of the northeastern region (Hooker, 1872-1887; Kanjilal *et al.*, 1934, 1936, 1938, 1940; Balakrishnan, 1981-1983; Deb, 1981-1983; Joseph, 1982; Haridasan, 1985-1987; Hajra and Verma, 1996; Hajra *et al.*, 1996; Chowdhury, 2005; Giri *et al.*, 2008; Chowdhery *et al.*, 2009; Singh *et al.*, 2000, 2002; Boro and Sarma, 2013; Barooah and Ahmed, 2014; Mao *et al.*, 2016, 2017). The ASSAM herbarium houses one sheet of *C. cyanus* collected by N.L. Bor (Collection No. 18317) on 24.03.1938 from Botanical Garden of New Forest (now FRI), Dehradun. Aroused with interest, *in situ* growth of three plants for several weeks was followed, to develop taxonomic diagnosis and illustrations, and review literature on this plant.

Although *Centaurea cyanus* is known to humanity for millennia, its description in the floras is unfinished (Keil, 2012). Notwithstanding, sizeable literature has emerged on its culinary and medicinal properties in last few years (Table 1). The plant is known to have come out from its native range and spread widely in northern hemisphere, but it is not notorious for invasive possessions. Probably, due to its weak competitive ability (Karlsson, 2019), the natural populations of *C. cyanus* have declined drastically in Europe (Anonymous, 2007). The review of literature reveals that the future research on its phytochemistry shall pave the way for proliferation in its utilization for human consumption.

Table 1. Some culinary and therapeutic uses of *C. cyanus* recorded in the literature.

Sl.	Plant part used	Culinary and medicinal uses	Source
1.	Flowers	Aromatic acids and sugars	Swiatek and Zadernowski (1994)
2.	Flower-heads	Anti-inflammatory effect	Garbacki *et al.* (1999)
3.	Flowers	Anthocyanin	Takeda and Tominaga (1999)
4.	Seeds	Indole alkaloids	Sarker *et al.* (2001)
5.	Leaves	Cooked as vegetable	Kültür (2008)
6.	Flowers	Mineral element	Rop *et al.* (2012)
7.	Flowers	Astringent herb used for skin cleansing and eye ailments (concuctivitis and blepharitis)	Al-Snafi (2015)
8.	Ray florets	Infusions, garnish and natural food colorant, antioxidant activity, soothing, and used in ocular inflammation	Fernandes *et al.* (2017)
9.	Dried flowers	Carbohydrates	Pires *et al.* (2019)
10.	Ray florets	Decoration of dishes and desserts	Matyjaszczyk and Śmiechowska (2019)
11.	Flowers	Antioxidant power	Kalemba-Drożdż (2019)
12.	Flower	Antibacterial activity against species like *Listeria monocytogenes* and methicillin-susceptible *S. aureus* (MSSA)	Lockowandt *et al.* (2019)

This study reports the first record of the plant, *Centaurea cyanus* L. of Asteraceae, from northeastern region of India along with taxonomic diagnosis. The principal objective is to draw attention of the people, especially students of plant taxonomy and herbal practitioners for utilization and management of a non-native species in the native landscapes.

Material and methods

This study focuses on *C. cyanus* L. of Asteraceae growing in the campus of the North-Eastern Hill University, Shillong (25°36'56" N latitude and 91°54'21 E longitude) at 1460 m in the State of Meghalaya in northeast India. The plant was growing in the company of other invasive weeds such as *Bidens pilosa* L., *Erigeron annuus* (L.) Pers., *Galinsoga parviflora* Cav., *Persicaria capitata* (Buch.-Ham. ex D.Don) H.Gross, *Tagetes erecta* L. on a thin layer of deposited soil in crevices on road (Figure 1). The flowering is non-synchronous, i.e., inflorescences developing in succession one after another. The flowering continued until last observed in the first week of July, 2020 (Figure 2). Interestingly, *C. cyanus* was competing well with the dense growth of other invasive species. The blooming twigs with flower heads were collected for taxonomic diagnosis and preparation of herbarium specimen. The ray and disc florets were dissected for evaluation. The habitat, habit and plant parts were photographed adequately. The species was confirmed online with the sheets of Kew Herbarium (Figure 3). The herbarium sheet of the collected specimen (*vide* Collection No. US005001, Accession No. 96548) has been deposited in the ASSAM Herbarium of the Botanical Survey of India at Shillong.

Figure 1. Habitat and habit of *C. cyanus* (the plant with pinkish flower in the dense growth) in the North-Eastern Hill University, Shillong recorded in May, 2020.

Figure 2. An *in situ* overhead view of the inflorescence or capitulum of *C. cyanus* showing radiating ray florets and centered disc florets (Picture taken on May 18, 2020).

Figure 3. The herbarium specimens of *C. cyanus*:
a) K000914405 accessed online from Kew Herbarium
b) US005001-01 collected in this study and sheet deposited to ASSAM Herbarium.

Results and discussion

1. Distribution

The native range of *Centaura cyanus* L. (cornflower) is in Central and East Mediterranean region and southern Europe, including Albania, Bulgaria, East Aegean Is., Greece, Italy, Lebanon-Syria, Romania, Sicilia, Turkey, Turkey-in-Europe, Yugoslavia (Beentje, *et al.* 2005, Figure 4). The plant is cultivated in northern hemisphere and has been introduced into United States of America, Canada, Mexico, Europe, Russia, Korea and Siberia (POWO, 2020). In Indian Subcontinent, *C. cyanus* has arrived in Nepal (at 3700 m, Press *et al.*, 2000, Mallick, 2019), and in western regions of India (Tomar, 2017). Although the global distribution by Kew (Figure 4) shows its presence in Pakistan, and the Himalayan region, the e-flora of Pakistan (Anonymous, 2020), but our search in floras of Himalayan region of India shows no documented record. The species is unknown in northeastern region of India and eastward to China and Southeast Asia, although it is reported from Vietnam (Figure 4).

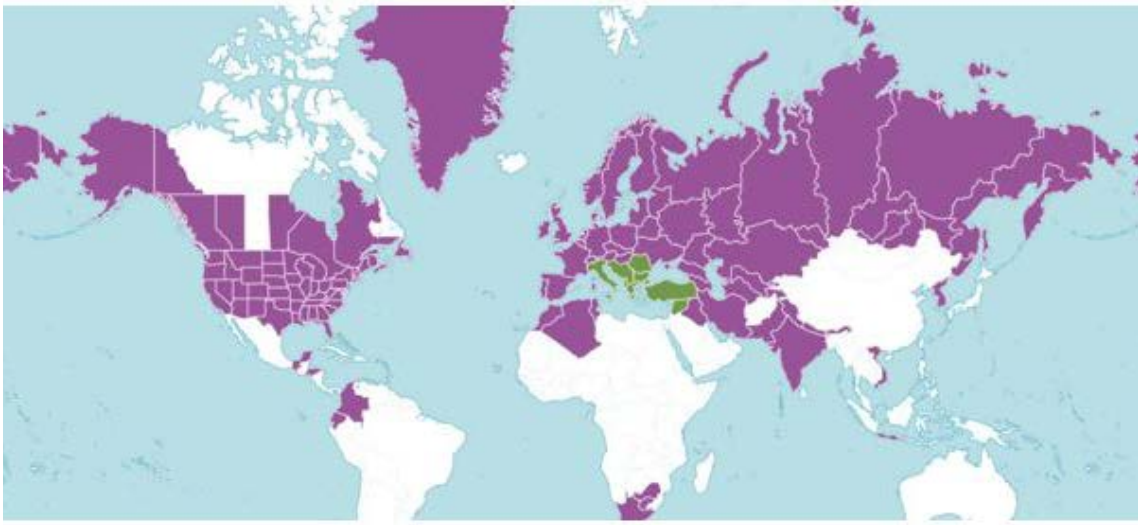

Figure 4. Global distribution of *C. cyanus* L. worked out by the Kew, UK. The regions with green wash show the native range, those with purple wash show the geographies where plant has made presence as non-native, and the uninhabited terrestrial regions are in white (POWO, 2020).

The recent studies in United Kingdom have suggested a decline in much of its range in Europe. For instance, it was once widespread throughout Britain, but is now restricted to small scatter of 'natural' populations mainly in the south and east of England and has now been classified as 'Least Concern' species (Anonymous, 2007). The main causes of decline include the intensification of arable farming, loss of certain crops such as rye and flax in which it was frequent seed contaminate, improved seed cleaning and the introduction of broad-spectrum herbicides, and probably low competitive ability (Karlsson, 2019). This species is listed as 'Priority Species' under the UK Biodiversity Action Plan (Anonymous, 2007).

2. Etymology and synonymy

The species was first published by Linneaus in Sp. Pl. 2: 911 (1753). The generic name *Centaurea* was derived after Greek mythology character, centaur Chiron, who was famous for his knowledge of medicine and was the teacher of Achilles, Asclepius and Hercules. Chiron could heal Achilles after he was wounded with a poisoned arrow (by Herakles), by applying cornflower plants (Al-Snafi, 2015). The species epithet *cyanus* was derived from the vivid colours of the flowers. The common name 'cornflower' comes from the fact that the plant grew wild in the grain fields of southern Europe. In Christian symbolism, cornflower became a symbol of the Queen of Heaven, Mary, and Christ. The cornflower has also been used as a symbol of tenderness, of fidelity, and of reliability.

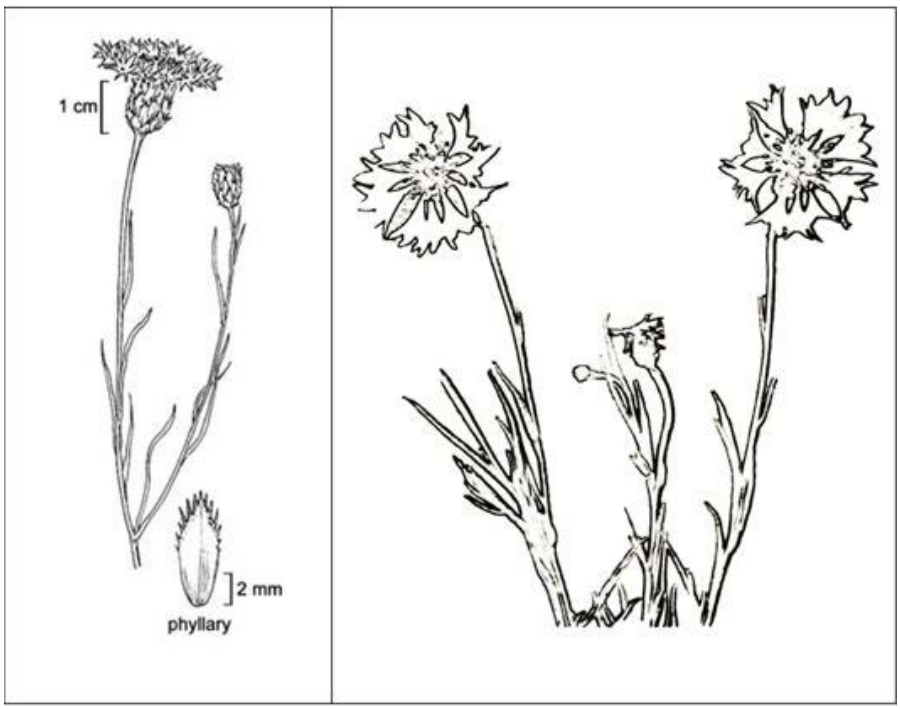

Figure 5. Diagrammatic illustration of the habit of *C. cyanus* as recorded in America by Keil (2012) on the left, and in northeast India in this study on the right.

There are a number of botanical synonyms for cornflower (Barkley, 2006; Bernal *et al.*, 2015; POWO, 2020): *Centaurea concinna* Steud., *Centaurea cyaneum* St.-Lag., *Centaurea cyanocephala* Velen., *Centaurea cyanus* subsp. *coa* Rech.f., *Centaurea cyanus* var. *denudata* Suksd., *Centaurea hortorum* Pau, *Centaurea lanata* Roxb., *Centaurea pulcherrima* Willd., *Centaurea pulcherrima* Wight ex DC., *Centaurea pulchra* DC., *Centaurea rhizocephala* Trautv., *Centaurea segetalis* Salisb., *Centaurea umbrosa* A.Huet ex Reut., *Cyanusa rvensis* Moench, *Cyanus cyanus* Hill, *Cyanus dentato-folius* Gilib., *Cyanus segetum* Hill, *Cyanus vulgaris* Delarbre, *Jacea segetalis* Lam. ex Steud., *Jacea segetum* Lam., *Leucacantha cyanus* Nieuwl. & Lunell, *Setachna cyanus* Dulac.

3. Taxonomic diagnosis

Habitat: agricultural fields, grasslands, woodlands, forests, roadsides, other disturbed sites.

Altitudinal Range: Broad range from about sea level to temperate regions, i.e., 10 to 3700 m. Recorded at 25°36'56" N and 91°54'21 E, 1460 m in the present study.

Habit: Annual herb, 20 cm to 100 cm (Figure 5).

Stem: Weakly erect, often branched distally, up to 3 or 4 mm in diameter, fistular, longitudinally furrowed, pubescent, olive-green, not winged (Figure 6a).

Leaf: Linear-lanceolate with a prominent central vein, densely pubescent on both surfaces, adaxial surface ash-grey and abaxial surface olive-green, lower or basal leaves longer (up to 10 cm) than much-smaller upper ramal and cauline leaves (<3 cm) (Figure 6 h,i). Petiole short, 1-2 mm.

Inflorescence: Each capitulum or head is composed of a ring of radiating ray florets and centrally clustered disc florets. In overhead view (Figure 6b), the diameter of cymiform arrays is 3-4 cm. Capitulum slender-pedunculate with involucre 10-16 mm, campanulate, ovoid or bell-shaped. The hard ovoid involucre of the capitulescence is formed by numerous phyllaries (involucral bracts) arranged in 3-4 whorls (Figure 6c). Each phyllary, 4-6 x 3-4 mm in size, is spear-shaped, olive-green, fringed with brown or deep-purple margins and a tip appendage (Figure 6d). Odour is faint, pleasantly aromatic.

Flower: Flowering in succession (asynchronous) as the plant continues to grow in growth period from May through September. A single plant may produce 20 or more flowers in a season. Florets of both types are pink to fuchsia in colour (Figure 6e,f). Ray florets up to 2 cm in length, sterile, consist of fused petals with 5-8 lobes (Figure 6e). In the disc florets, five petals are fused into a tube with five apical lobes, up to 1 cm in length (Figure 6f). Disc florets are bisexual, containing both fertile anthers and a fertile pistil. Pollen mass white.

Fruit: Cypselae stramineous (straw-colored), 4-5 mm (Figure 6g), linear-oblique (triangular by Rakizadeh *et al.*, 2019), finely short-hairy; pappi of many unequal stiff bristles, 1-3 mm; pappus longer than the seed.

Chromosomes: *2n* = 24 (Beentje *et al.*, 2005)

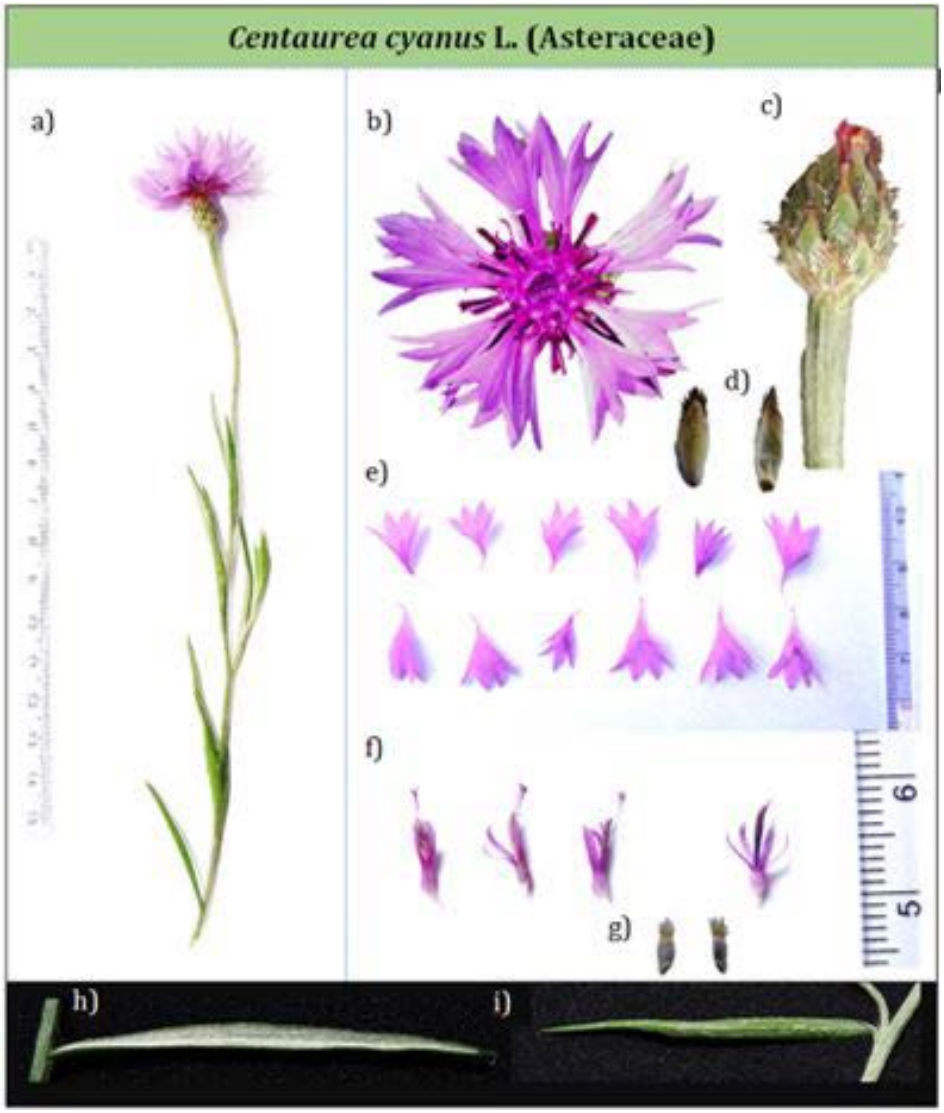

Figure 6. Photographic illustration of *C. cyanus*: a) flowering twig, b) overhead view of the inflorescence or capitulum showing a ring of spreading ray florets and centrally clustered disc florets, c) arrangement of phyllaries, d) abaxial and adaxial sides of a phyllary, e) ray florets retrieved from a single head, f) disc florets showing development of cypselae from the left-most to the right-most flower, g) cypselae with pappi, h) adaxial surface, and i) abaxial surface of narrowly-lanceolate leaf.

Conclusions

The cornflower is an affable herb in Europe and has a long history of cultivation and use for decoration, culinary and medicinal purposes. In America, it has escaped in natural habitats via common route of spread through wildflower mixes. Although native of cooler climate of temperate habitats, cornflower has descended to very low altitudes

in the plains of northern India and also in Nepal. It has not been reported from Bhutan, Eastern Himalaya, northeast India and Myanmar, but seems to be making inroads in these territories as shown by its presence in this study. The habit and morphology of the plant is described from the newer habitat. The precise route of arrival in the recorded habitat remains enigmatic.

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