

**BIOINSPIRED SYNTHESIS, CHARACTERIZATION AND BIOLOGICAL
EVALUATION OF SILVER NANOPARTICLES FROM AQUEOUS
LEAVES EXTRACT OF *HIPPOPHAE RHAMNOIDES* LINN.**



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Doctor of Philosophy in Biotechnology

By

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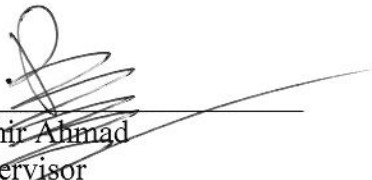
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2013-14

AUTHOR'S DECLARATION

I solemnly declare that the research work presented in this thesis was carried out in accordance with the requirements of the University of Peshawar's regulations for Research Degree Programs. The author has not been submitted this work for any other academic award. The work is original and of author's own data. While work done in collaboration with, or with the assistance of, others, is indicated as such. The views expressed in the thesis, belongs to the authors.

Date 17/01/2014

Signature 

Dedication

Dedicated To My Loving Grandmother
(Mother's mother)

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List of Abbreviations

Hippophae rhamnoides Linn = *H. rhamnoides* L.

PCSIR = Pakistan Council of Scientific and Industrial Research

KPK = Khyber Pakhtunkhwa

AgNPs = Silver nanoparticles

NPs = Nanoparticles

AuNPs = Gold Nanoparticles

SBL = Seabuckthorn leaves

UV-Vis spectroscopy = Ultraviolet-visible spectroscopy

SPR = Surface Plasmon Resonance

FTIR = Fourier transform infrared spectroscopy

SEM = Scanning Electron Microscope

EDX = Energy Dispersive X-ray

TEM = Transmission Electron Microscope

HTEM = High resolution transmission microscopy

XRD = X-ray Diffraction

TG/DTA = Thermogravimetric-differential Thermal Analysis

DMSO = Dimethyl Sulfoxide

ZI = Zone of inhibition

VitC = Vitamin C

Aq = Aqueous

Meth = Methanol

Eth = Ethanol

Eth Act = Ethyl Acetate

Acet = Acetone

Chl = Chloroform

n- Hex = n-Hexane

DPPH = 1,1'-diphenyl- 2-picrylhydrazyl

BHT = *tert*-butyl-1- hydroxytoluene

SDA = Sabouraud Dextrose Agar

MS Basal Medium = Murashige and Skoog Basal Medium

ng/ml = Nanograms per milliliter

hrs = Hours

ml = milliliter

l = litter

C⁺ = Positive control

C⁻ = Negative control

S. aureus = *Staphylococcus aureus*

E. coli = *Escherichia coli*

E. faecalis = *Enterococcus faecalis*

K. pneumoniae = *Klebsiella pneumoniae*

P. aeruginosa = *Pseudomonas aeruginosa*

V. cholerae = *Vibrio cholera*

S. typhi = *Salmonella typhi*

B. cereus = *Bacillus cereus*

B. subtilis = *Bacillus subtilis*

C. freundii = *Citrobacter frondai*

MIC = Minimum Inhibitory Concentration

MBC = Minimum Bactericidal Concentration

MFC = Minimum Fungicidal Concentration

A. niger = *Aspergillus niger*

A. parasiticus = *Aspergillus parasiticus*

A. flavus = *Aspergillus flavus*

A. fumigatus = *Aspergillus fumigatus*

A. oryzae = *Aspergillus oryzae*

F. oxysporum = *Fusarium oxysporum*

R. arrhizus = *Rhizopus arrhizus*

A. alternata = *Alternaria alternata*

C. albicans = *Candida albicans*

S. cerevisiae = *Saccharomyces cerevisiae*

P. digitatum = *Penicillium digitatum*

A. aegypti = *Aedes aegypti*

A. stephensi = *Anopheles stephensi*

Cx. Quinquefasciatus = *Culex Quinquefasciatus*

ROS = Reactive Oxygen Species

GR = Growth regulation

LC = Lethal Concentration

LD = Lethal Dose

GAE = Gallic Acid Equivalent

List of Publications

Publications from this thesis research work:

1. **Bashir Ahmad and Javid Ali.** 2013. Physiochemical, minerals, phytochemical contents, antimicrobial activities evaluation and fourier transform infrared (FTIR) analysis of *Hippophae rhamnoides L.* leaves extracts. African Journal of Pharmacy and Pharmacology.7 (7): 375-388.
2. **Bashir Ahmad and Javid Ali.** 2013. Evaluation of Larvicidal Activity of *Hippophae rhamnoides L.* Leaves Extracts on *Aedes aegypti* and *Anopheles stephensi* (Diptera: Culicidae). Middle-East Journal of Scientific Research 13 (5): 703-709. (ISI Index).
3. **Bashir Ahmad, Javid Ali and Shumaila Bashir.** 2013. Optimization and Effects of Different Reaction Conditions for the Bioinspired Synthesis of Silver Nanoparticles Using *Hippophae Rhamnoides* Linn. Leaves Aqueous Extract. World Applied Sciences Journal 22 (6): 836-843. (ISI Index).

Remaining related research work of this thesis under the process of publication.

ABSTRACT

In this study we have evaluated the physiochemical, minerals contents, phytochemicals and biological activity of *Hippophae rhamnoides* Linn (*H. rhamnoides* L.) leaves. The physiochemical and mineral contents of *H. rhamnoides* L. leaves revealed that fiber was $18.0 \pm 2.64\%$, protein was $10.45 \pm 0.88\%$ and carbohydrate value was found $68.75 \pm 1\%$. Sodium was 30000 ± 1 ppm, calcium 7800 ± 1 ppm and potassium 6200 ± 2 ppm as high concentration. While qualitative phytochemicals investigations showed that tannins, phenols and flavonoids are present in large quantity. The Fourier transform infrared spectroscopy (FTIR) spectra of all extracts revealed, the presence of different functional groups like OH stretching for hydroxyl group, alkanes, alkenes, aromatic rings, carboxylic and amides (aromatic).

The highest antibacterial zone of inhibition was observed in aqueous and methanolic extracts against *S. aureus* and *V. cholerae* with 21 ± 1 mm and lowest Zone of inhibition measured 07 ± 0 mm against *V. cholerae* (ethyl acetate extracts). The antibacterial minimum inhibitory concentration values of extracts were determined ranging between 40 to 120 mg/ml and minimum bactericidal concentration values of the extracts ranged between 50 and 135 mg/ml. The highest antifungal zone of inhibition was calculated against *Alternaria alternata* (18 ± 0 mm) followed by *Aspergillus parasiticus* (17 ± 1 mm) of methanolic extract, while *Alternaria alternata* (07 ± 1 mm) and *Penicillium digitatum* (07 ± 0 mm) was the least found in chloroform and aqueous extract respectively. Further antifungal minimum inhibitory concentration and minimum fungicidal concentration are between 40 – 135 mg/ml and 50 – 180 mg/ml respectively.

The antioxidant activity of *H. rhamnoides* L. leaves extracts were increased in a dose dependent manner. The antioxidant activity (% inhibition) of all the tested extracts were increased in the order i.e. menthol > ethanol > aqueous > acetone > chloroform > ethyl acetate > *n*-hexane. The methanol extract LC₅₀ (µg/ml) value was compatible with vitamin C (standard). The maximum cytotoxic activity of methanol and ethanol extract of *H. rhamnoides* L. were observed having low LD₅₀ (µg/ml) value i.e. 1199.97 and 1206.91 respectively. Low phytotoxic activity was calculated for all tested extracts against *L. minor*.

The crude leaves extract of *H. rhamnoides* L. in different solvents were tested for larval mortality against fourth instars of *Anopheles stephensi* and *Aedes aegypti* viz., vector mosquito species. The ethanol and methanol extract have highest percent mortality at 72 hrs i.e 84±0.82 and 81±1.71 at 2000 ppm respectively against *Aedes aegypti*. For ethanol extract the lowest LC₅₀ (ppm) at 24 hrs, 48 hrs and 72 hrs were 1424.45, 1104.53 and 1035.21 respectively against *Aedes aegypti*. The lowest LC₉₀ (ppm) were 2841.20, 2632.08 and 2643.63 at 24 hrs, 48 hrs and 72 hrs calculated against *Aedes aegypti* of ethyl acetate, acetone and ethanol extract respectively. The highest mortality (%) of *Anopheles stephensi* at 72 hrs (2000 ppm) was 90±1.30 (ethanol) and 89.0±0.50 (methanol). The highest LC₅₀ (ppm) of *Anopheles stephensi* at 24 hrs, 48 hrs and 72 hrs were 1725.80 for chloroform, 1508.97 for acetone and 1361.07 for chloroform. The lowest LC₉₀ (ppm) were 2605.78 (ethanol) at 24 hrs, 2142.63 (ethanol) at 48 hrs and 2042.06 (methanol) at 72 hrs. These results suggest that *H. rhamnoides* L. leaves exhibited noteworthy activity and could be measured as an effective natural larvicidal means against vector mosquitoes.

There is a growing marketable demand for nanoparticles because of its broad utility in diverse fields like energy, chemistry, electronics, catalysis, medicine and food. Metals based nanoparticles are conventionally prepared from physical and chemical methods, but in these methods poisonous and flammable chemicals are used. In the current study, we illustrated a low cost and plant origin technique for the synthesis of silver nanoparticles (AgNPs) from 1 mM silver nitrate solution and aqueous leaves extract of *H. rhamnoides* L. as capping as well as reducing agent. Synthesized AgNPs were characterized using UV-Vis absorption spectrophotometer, FTIR, scanning electron microscope (SEM), energy dispersive X-ray (EDX), X-ray diffraction (XRD) and Thermogravimetric/Differential Thermal Analysis (TGA/DTA) techniques. *H. rhamnoides* L. leaves aqueous extract were used for the biosynthesis of AgNPs. Confirmation of AgNPs was carried out by UV-Visible spectra of the reaction solution containing AgNPs showed maximum absorption at 435 nm. FTIR confirm the presence of OH groups, amide, carbonyl compounds, aldehyde, aliphatic esters and alkynes, in both leaves powder and prepared AgNPs. The SEM analysis showed the particle size ranging from 135- 300 nm with spherical shape. The EDX analysis supported the presence of silver along with other biomolecules residue like C, O, Na and K. These last four elements originated from plant extract which act as stabilizing as well as capping agent for AgNPs. The XRD data confirm the crystalline nature of the prepared NPs. The average crystal size size was 21.909 nm according to Debye-Scherrer's equation. The TGA/DTA thermogram revealed that at first phase the weight loss was due the moisture loss from the surface of AgNPs, and then a sudden weight loss was seen due to the burning of the organic substances present on the surface of AgNPs.

Different experimental variables conditions like reaction pH, silver nitrate concentrations, time, temperature and mixing ratio of the reactants were optimized for the synthesis of AgNPs using aqueous extract of *H. rhamnoides* L. leaves. The formation of yellowish brown color confirmed the synthesis of AgNPs. The optimized condition for the bioinspired synthesis of AgNPs revealed that silver nitrate concentration was 1mM, temperature was 75°C, pH was 7, incubation time was 1 hrs and aqueous extract and silver nitrate ratio was 5:95. These finding revealed that good modification of the bioprocess parameters will improve potential of desired nano-product for particular applications.

Additionally these bioinspired fabricated nanoparticles were found to be very toxic against various pathogenic bacteria and fungi. Further the 1,1'-diphenyl- 2-picrylhydrazyl (DPPH) scavenging activity, cytotoxic, phytotoxic and larvicidal activity against vector mosquitoes were also evaluated. Prepared silver nanoparticles were affective against all tested bacterial strains and showed lowest zone of inhibition 12 ± 0 mm against *E. faecalis* and highest zone of inhibition was found against *E. coli* (19 mm). Minimum inhibitory concentration was low as 150 µg/ml and maximum was 450 µg/ml. Minimum bactericidal concentration were in the range 220 – 1000 µg/ml. It was proved that biosynthesized AgNPs was exerted its antibacterial activity at 1 mg/ml concentration, which was a very low concentration as compared to the all extracts of *H. rhamnoides* L. leaves. Results summarized that synthesized AgNPs were having efficient antibacterial activity against G (-) bacteria as compared with G (+) bacteria. For antifungal activity of AgNPs the highest zone of inhibition 18 ± 0 mm was observed against *A. alternata*, while the lowest zone of inhibition was calculated against *P. digitatum*. The most susceptible

fungi was *A. alternata* showing MIC value 1200 µg/ml, while the same moderate MIC values were 1400 µg/ml observed against *A. paraciticus* and *F. oxysporum*. The highest MIC value 1700 µg/ml was accountable for *P. digitatum*. Similarly the low MFC value 1500 µg/ml was calculated against *A. alternata* and high resistant was noted for *A. niger*, *A. fumigatus* and *P. digitatum*. The antimicrobial activity of the prepared AgNPs documented that it was active at low concentration 2 mg/ml against all the tested human and plant pathogenic fungi. The antioxidant activity (1,1'-diphenyl- 2-picrylhydrazyl) of prepared AgNPs was compatible with the standard (*tert*-butyl-1- hydroxytoluene). The cytotoxic activity of synthesized AgNPs was found maximum mortality 73.33% at 1000 µg/ml, having LD₅₀ value of 145.03 µg/ml. The data showed that the bioinspired synthesized AgNPs was 16.67% and 36.67% GR at 50 µg/ml and 500 µg/ml respectively phytotoxic activity. The mortality of AgNPs against vector mosquitoes *A. aegypti* was 93% at 600 ppm concentration with 72 hrs incubation, similarly against *A. stephensi* mortality was 95% on 72 hrs incubation at 600 ppm concentration.

Chapter-1

Introduction and literature review

Introduction and Review of Literature

Plants are precious gift of Allah to the human beings on this planet. Plants are always known to provide shelter, food, medicine etc to human beings and other living organisms. Medicinal plants hold some pharmacologically active compounds which were used from the ancient times for the cure of different diseases.

The growing urbanization and industrialization are disturbing our surroundings by release of huge quantity of dangerous gases, chemical or substances. Directly or indirectly it leads to create health, social and economical problems. Currently it is necessary to find out concerning the natural secrets there in the natural world and its derivatives, guiding to the synthesis of bioinspired protocol for the advancements development in the preparation methodology of nanoparticles (NPs). It is the character of natural molecules through which it can assume greatly hierarchical and controlled assemblage to prepare them appropriate for the synthesis of environment friendly, low cost, fast and reliable route for metal NPs production. It is also a need of the day to synthesize plants origin products having versatile properties.

All over the worlds medicinal plants are abundantly available and are more focused than ever because they have the ability to produce many benefits to human society, especially to cure different types of diseases. World health organization (WHO) reported that above eighty percent inhabitants of the world depended on conventional medicines for various types of ailment. A number of chemical compounds are present in plants which have possessed the therapeutic properties and on the human body cause a specific physiological action. Phenolic compounds, alkaloids, tannins and flavonoids are the primarily significant bioactive compounds present in plants [1].

The alkaloids have antimicrobial, anticancer, cytotoxic and antimalarial properties while flavonoids possess high antibacterial activity and are more effective in the treatment of inflammation, allergy, cancer, viral infection and hypertension. Tannin has shown high activities against viral and bacterial infection as well as act as strong antioxidant [2]. Based on ethno-pharmacological knowledge the phytochemical research was usually considered a useful methodology in the innovation of novel antimicrobial compounds from higher plants [1].

1.1. *Hippophae rhamnoides* Linn.

Hippophae rhamnoides Linn (*H. rhamnoides* L.) commonly known as Seabuckthorn is a medicinal plant, grown on in large areas of Asia and Europe. Its different components have been long utilized for the cure of many diseases including pain, rheumatoid arthritis and colds. It has possessed many types of active compounds, which have latent applications in human health. The seeds, leaves and fruits hold high amounts of useful substances such as flavonoids, carotenoids, phenolic compounds and vitamins with antibacterial, antifungal and free radical scavenging activities [3]. Iron, magnesium, calcium, sodium, potassium, phosphorus and silver were found in seabuckthorn seeds [4]. The twigs of *H. rhamnoides* methanol and chloroform: methanol extracts have reported [5] the presence of glycoside, terpenoids, steroids, flavonoids, reducing sugar and tannins, while alkaloids, coumarins and saponins were absent. It has been investigated scientifically [6] that *H. rhamnoides* extracts from berries, oil and leaf have been found various pharmacological activities such as anti-inflammation, immune-modulation, radio protective and tissue regeneration.

1.2. Nanotechnology Background

The prefix "nano," derivative from the Greek "nanos" suggestive of "dwarf" and nearly all of the thrilling properties of 'nano' commence to be obvious in systems lesser than 1000 nm, or 1 micrometer (1 μm). Nanoparticles (NPs) are particles with at least one dimension lesser than one micron [7]. Richard Feynman a physicist in 1959 for the first time laid the hypothetical underpinnings of NPs in his dialogue, "There is sufficient space at the bed". Feynman discovered the option for manipulating the material at the base of personage atoms and molecules, the explored the choice of manipulating substance at the level of personage molecules and atoms, forecasting the whole of the Encyclopedia Britannica written on the top of a pin and seeing in future the increasing ability to manage and observed material by the nanoscale. The term "nanotechnology" was first used by Norio Taniguchi, at the Tokyo University, used this term to submit the ability to manage the materials correctly on the nanometer phase in 1974 [8].

Bionanotechnology has emerged as the combination of biotechnology and nanotechnology for increasing environment friendly and biosynthetic technology for production of nanomaterials [9]. Novel and innovative uses of nanomaterials and NPs are increasing at a high pace [10]. Nanoparticles have established substantial concentration in current years due to their utilization in the areas of optoelectronics, material chemistry, biological tagging, environmental pollution control, catalysis, pharmaceutical applications, photonics and drug delivery systems [11]. Nanotechnology makes a well-off involvement of biotechnology and biomedical technology by producing numerous products and devices [12].

1.3. Silver: Prehistorical, Past and Present Status

Caldeans were aware about metallic silver as 4,000 (B.C.E.) before Common Era. Father of history “Herodotus” wrote so as to none of the Persian king, along with Cirrus, would drink water which was not given in silver pots that kept the water clean and fresh for many years. The food and water were protected by the use of silver; as documented by the earliest Greeks, Egyptians, Phoenicians, Romans and many others [13]. Hippocrates documented the function of silver in the avoidance of ailment. Silver vessels were used for the storage of wine in order to avoid spoilage were used by Romans [14]. For more than 100 years the health professionals were familiar about the silver antibacterial and antifungal activity. The basic silver element rarely occurs in nature and is elastic and soft. It shows maximum thermal and electrical conductivity along with less contact resistance among all metals. Silver has been used since the primeval times for manufacturing different materials like jewelry, utensils, monetary currency, explosives, photography, dental alloy etc [15]. Silver is harmless and non toxic (in less concentration) inorganic agent having antibacterial properties and is able to inhibit 650 microorganisms responsible for various types of ailments. It is also an agent to control spoilage [16]. Different silver compounds which were used from ancient times as antimicrobial agents are silver oxide, silver powder, silver chloride, silver nitrate, silver sulfadiazine, silver cadmium powder and silver zeolite [15].

1.4. Biological Approach of NPs Synthesis

Nanomaterials can be synthesized by both the so-called ‘bottom-up’ and ‘top-down’ approaches. The top-down technique involves mechanically grinding of massiveness

metals and following stabilization of the resultant NPs by adding the colloidal capping substances. On the other hand, the bottom-up methods comprise electrochemical methods, reduction of metals and sonodecomposition [17].

The NPs were formed by physical, chemical and biological methods; the last method was used preferably due to its safety and environment friendly nature [18]. The biological methods have some advantages over chemical and physical synthesis of NPs as these are injurious by all means and are mostly flammable, poisonous, have low fabrication rate and not easily disposable due to environmental issues etc. As a result, great attention has been given to search methods for the synthesis of metal NPs from biological system including plants and plants extracts, enzyme and micro-organisms. However the synthesis of NPs from plant parts was of great interest as it was very rapid, eco-friendly, low cost and a single step technique. It has been observed that by using plants, the rate of reduction of metal ions found to be more rapidly than micro-organisms along with stable metal NPs formation. Plant based fabricated NPs can be beneficial on other biological methods through avoiding the complex process of maintaining cell culture [19, 20]. The most important benefit of using plant extracts for silver nanoparticles (AgNPs) synthesis was that they were safe, effortlessly available, harmless and nontoxic in most cases. The plant extracts were contained a wide diversity of phytochemicals that can help in the reduction of Ag ions and were faster as compared to microorganisms in the fabrication [17].

Worldwide generally and in the third world countries particularly, the infectious diseases (bacterial) are responsible for the death of human beings [21]. Bacteria species such as *Pseudomonas*, *Bacillus*, *Staphylococcus* and *Salmonella* are the major

pathogens which are responsible for many life threatening diseases. These pathogens have the ability to live in unsuitable environment due to their poly habitats in the earth [22]. Pathogenic fungi were responsible for many diseases due to superficial and deep inside infections. The numbers of humans infected with pathogenic fungi have increased drastically in all parts of the world. Human systemic infections are caused by *Candida albicans*, *Aspergillus flavus* and *Aspergillus niger*. Candidiasis and aspergillosis are the diseases caused by these fungi [23]. In immunocompromised patients fungal infections are more common due to HIV, cancer chemotherapy, or organ infections. The availability of antifungal drugs is limited and with time resistant strains are also appearing. Therefore, there is an urgent and inevitable need for new antifungals agent [24]. Scientists and pharmaceutical industries are now working for novel antimicrobial compounds. In the current situation, distinctive physical, greater surface area to volume ratio and chemical properties of NPs have emerged as new antimicrobial agents [25].

Antioxidants give protection to human beings from harmful effect of reactive oxygen species (ROS) and related protein damage, DNA strand breaking and lipids peroxidation [26]. Free radical is a molecular level component having one or more unpaired electrons. Reduction in antioxidant quantity or excess synthesis of free radicals causes oxidative stress responsible for diabetes mellitus, aging, neurodegenerative diseases, atherosclerosis and rheumatoid arthritis. Antioxidants work as free radical scavengers, put off and restore reparation which are cause due to ROS. It's reduced the cancer risk and degenerative diseases and develops the immune defense system [27]. Greater part of the world population, especially in third world

countries depends on the folk system of medicine for a number of diseases. Many plant genera are used medicinally, which provide major sources of powerful and potent drugs [28].

Pathogenic vectors responsible for different ailment including dengue, Japanese encephalitis, chickungunya, filariasis and malaria are commonly caused by a number of mosquito species which belong to the *Culex*, *Aedes* and *Anopheles* genera [29]. Mosquito control programmes were established in various countries but slight development in the malaria management and infections can be seen, causing financial and human losses [30]. Due to increase pesticides resistance, unavailability of vaccines and medicines, it was very difficult to control mosquito born diseases [29].

1.5. Bioinspired Synthesis and Characterization of NPs

The plant based synthesis was rather rapid and AgNPs were synthesized [11] from utilization of tuber extract of *Dioscorea bulbifera* in five hours. The yellowish-brown color was the confirmation of the synthesis of AgNPs due to the surface plasmon vibrations excitation at 450 nm (maximum absorbance). Transmission electron microscope (TEM) images observed that most of the NPs were triangles and nanorods having 8–20 nm size. Larger size NPs were also synthesized having a size of 75 nm. Energy Dispersive X-ray (EDX) analysis observed that prominent peaks of silver were found without contamination. At approximately 3 keV the optical absorption peak was observed as (3 1 1), (2 2 0), (2 0 0) and (1 1 1) which showed the confirmed reduction of Ag.

The *Fissidens minutus* [31] aqueous extract treated with 0.5 mM silver nitrate solution at room temperature rapidly produced AgNPs. Absorption spectra of

yellowish brown AgNPs solution indicated peak of 412.8 nm for Surface Plasmon Resonance (SPR). The EDX spectrum observed a strong signal from the silver atoms (62.00 % mass and 17.81% Atomic) in the NPs along with weak signal from carbon and oxygen. These elements were originated from the plant organic constituents.

The extract of seaweed was used for the fabrication of AgNPs [9] by the reduction of Ag^+ into Ag^0 with the solution of 1 mM silver nitrate. The colorless silver nitrate solution changed into dark brownish yellow color representing the synthesis of AgNPs. The biosynthesis of AgNPs was checked by UV-vis absorption spectra at 200 - 600 nm which detected a clear strong band at 430 nm. Fourier transform infrared spectroscopy (FTIR) spectral study showed an array of absorbance bands in 600 cm^{-1} . The spectral bands were interpreted for the detection of different functional groups of compounds (organic) which were adhering to the AgNPs. The FTIR spectrum of seaweed showing peaks (cm^{-1}) at 1039, 1645 and 2917 shows the presence of alcoholic group (C-O), carbonyl (C=O) and hydroxyl (-OH) group respectively. Spherical shaped NPs were observed with 20 to 30 nm in size as suggested by TEM. Three distinct diffraction peaks at 2θ angle were 35.1° , 38.41° and 46° as shown by X-ray diffraction (XRD) analysis.

Utilization of *Argemone maxicana* (weed) was carried out for the synthesis of AgNPs [10]. Absorption spectra of AgNPs biosynthesized in the reaction mixture has absorbance peak at 440 nm and expansion of peak suggested that the polydispersed NPs were formed. FTIR analysis indicated that the biological reductions of Ag^+ to AgNPs were because of the plant extract capping material reduction. The XRD

analysis showed three deep spectra in the entire spectrum in range of 10 - 80 at 2 θ . The average size (20 nm) with hexagonal and cubic shape.

Preparation of AgNPs [32] from leaves extract of *Mollugo nudicaulis* showed a prominent SPR at 520 nm in UV-Vis spectroscopy. The XRD analysis revealed that diffracted intensities at 2 theta angles were recorded from 10° - 70°. The XRD peaks indicated that the particle size was small (9.3 nm). Further, XRD image observed that the end product exhibited diverse nature of AgNPs, while noise and broadening were most likely connected to the different bioactive compounds present in the extracts. The FTIR spectra revealed the occurrence of different functional groups as alkane, alkene, amine, aromatic, ether or ester, secondary alcohol and alkyl halide.

The prepared AgNPs solution [33] has a yellowish-brown color with maximum of 425 nm surface plasmon absorption band, representing sphere-shaped AgNPs. XRD peaks revealed that AgNPs were in pure silver metal powder and sphere-shaped structure. Similarly SEM analysis observed that the synthesized NPs size was 29-68 nm in diameter.

Oscillatoria willei (cyanobacterium) [34] were used for the biological formation of AgNPs, through which silver ions reduce and AgNPs stabilization was due to secreted protein. The AgNO₃ solution was mixed with washed cyanobacteria and the appearance of yellow color after 72 hrs indicated the synthesis of AgNPs. FTIR bands (cm⁻¹) shown at 2924 and 3280 were representing the stretching vibrations of secondary and primary amines respectively. Due to the capping agent SEM images showed that the synthesis of agglomerated AgNPs in the range of 100 – 200 nm.

Green fabrication of AgNPs [35] showed the reddish-yellow solution after mixing of latex aqueous extract and 1 mM AgNO₃ with characteristic SPR absorption band at 425 nm. TEM image indicated 20–30 nm range diameter. The XRD patterns of AgNPs observed number of Bragg reflections with 2θ values of 77.18°, 63.43°, 46.18° and 38.03° sets of pattern planes were recorded which might be indexed to the (3 1 1), (2 2 0), (2 0 0) and (1 1 1) facets of AgNPs. The synthesized AgNPs were crystalline in nature as clearly illustrated by XRD pattern. The EDX analysis gave sharp signal in the Ag area proving AgNPs formation. The FTIR spectra of plant extract exhibited absorption band (cm⁻¹) at 1618 and 1537 corresponding to the stretching vibration of (NH) C=O group and bending vibration of secondary amine of proteins. After reduction of silver nitrate, the decline in band (cm⁻¹) intensity at 1537 signs the participation of the secondary amines in the process of reduction. On the other hand the transfer from 1618 to 1604 of the band (cm⁻¹) was credited to the attachment of (NH) C=O group with NPs, while a member of (NH) C=O group inside the cage of cyclic peptides were involved in stabilization of AgNPs, the transfer of (NH) C=O band was fairly minute.

1.6. Optimization of Different Conditions for NPs Biosynthesis

Flower extract of *Rhododendron dauricum* was utilized [36] for synthesis of AgNPs. Different reaction parameters (concentration of plant extract, substrate concentration, pH, temperature and reaction time) were optimized for syntheses of AgNPs with controlled properties. Maximum synthesis of AgNPs was observed when metal salt concentration was increased from 0.5 – 4.0 mM. Beyond this there was again fall in absorbance; hence 4.0 mM of AgNO₃ was selected for further experiments. Diluted

flower extract containing 2 ml extract in 100 ml water i.e. 0.02 dilution was able to produce the maximum concentration of AgNPs as observed by higher absorbance at 420 nm. Absorbance increased with increase in the temperature from 25 to 45 °C and thereafter decreased at higher temperatures. Effect of pH on the synthesis of AgNPs by *R. dauricum* was tested over a wide pH range (pH 3-13). At acidic pH, larger sizes NPs were formed, whereas, at alkaline pH, smaller size NP formation was observed. Absorbance of silver colloidal solution increased with span of time and maximum absorption was observed after 12 hrs of reaction. Aggregation of NPs after 36 hrs of reaction was observed, which was a sign of instability.

The AgNPs were synthesized [37] from *Cacumen platycladi* extract. It was observed that increasing the temperature at 60 or 30 °C the mean size increased. Flavonoids and reducing sugars were mainly accountable for silver ion reduction. At 90 °C their reduction ability increased; as a result 18.4 ± 4.6 nm of AgNPs was synthesized. Moreover, the explanation of bioreduction means of Ag ions by quantifying the alteration of the bioactive compounds in extract of plant was a clear example to understand the synthesis mechanism of biological preparation of AgNPs.

The AgNPs were prepared from the *Garcinia mangostana* leaf extract [38]. The average size (35 nm) of AgNPs was formed by treating silver ions with leaf extract. The UV–Visible spectra denoted that the prepared AgNPs have absorbance peak was at 438 nm in the reaction media. The TEM evident that there was dissimilarity in particle size ranging from 6 to 57 nm and the average size was 35 nm. The overall optimized reaction condition was: temperature= 75 °C, time= 60 min, concentration

of silver nitrate = 1 mM, pH - neutral while silver nitrate and mangosteen leaf extract concentration = 1:19.

The leaves extract [39] of *Murraya koenigii* were employed for biological preparation of AgNPs. UV-visible spectrophotometry was used for monitoring the rapid reduction of silver (Ag^+) and the peak appeared at 435 nm (λ_{max}) which correspond to the absorbance of AgNPs with formation time 15 minutes. After 90 minutes there was no significant colour change, which was evidence for the completion of reduction reaction. The complete reaction along the rapid rate of reduction showed that the reaction was optimized at the 1:20 leaf broth to silver nitrate solution. From this study, it was found that the increasing broth concentration increases the rate of reduction and decreases the particle size.

Azadirachta indica [40] different leaf extract concentrations were employed to observe the UV-visible spectrum of the prepared AgNPs. At 5% leaves concentration the synthesized AgNPs noted 0.509 absorbance was 417 nm weak SPR spectra. Further rising to 20% concentration, the absorbance value increases to 0.815 as λ_{max} also increase to 444 nm. Additional raising 40% concentration absorbance declined to 0.476, while small alteration in λ_{max} to 441 nm was noted. The recorded difference in the λ_{max} values between 20 and 40% were not so considered. So the optimized *Azadirachta indica* leaf extract was 20%. The leaves extract to silver nitrate blending ratio (1:4) were recorded for λ_{max} . With rising incubation period from 30 min - 4 hrs 15 min, λ_{max} as of 420 to 444 nm was noted with a red shift. Incubation time increased up to 24 hrs, change in λ_{max} value did not occur considerably, even though increased in absorbance values were noted.

1.7. Mechanistic Approach of NPs Synthesis

The phytochemicals present in plants extract was responsible for the synthesis of NPs. The major phytochemicals concerned were carboxylic acids, ketones, terpenoids, amides, flavones and aldehydes. Aqueous soluble biomolecules (quinones, flavones and organic acids) were accountable for the instant reduction of the ions. Studies have proved that xerophytes hold emodin, an anthraquinone that undergoes tautomerization, resulting in the synthesis of the AgNPs. Although the precise mechanism concerned in every plant varies as the biomolecules differs in different plants, the reduction of the ions was the main mechanism involved [17].

Three different ways have been recommended by [41] to reduce silver in the plant extracts. Firstly, the active biomolecules present plant, were accountable for the bioreduction and biosynthesis of AgNPs. Electron or energy was the second biogenic route. It was released through glycolysis to transform nicotinamide adenine dinucleotide to reduce nicotinamide adenine dinucleotide. Finally conversion of silver nitrate for the preparation of NPs. Another method was the release of electron, reduces the silver at the time of formation of ascorbate radicals from ascorbate ions.

The leaves extract of *Ficus benghalensis* [42] contains high level of water soluble antioxidants, poly-phenols (Flavonoids). Kenotic and hydroxyl groups present in these phenolic compounds have the ability to attach with metals and give chelate effect. Flavonoids have proved antioxidant activity, which meant to donate hydrogen atoms or electrons. At the end the extract of leaves has proteins/enzymes and flavonoids which participate in gold nanoparticles (AuNPs) biofabrication. The

proteins/enzymes provide the stability and flavonoids have capacity to reduce the Au^{+3} to Au for the AuNPs biosynthesis.

1.8. Biological Evaluation of NPs

1.8.1. AgNPs as Antibacterial Agent

Toxic effects of NPs on pathogenic microbes open a new application door of nanotechnology in health. Biosynthesis of metallic NPs was a conventional route and the plants utilization has a new strategy for disease control [43]. Though gold, silver, palladium and platinum (Nobel metals), were generally used for the NPs preparation but silver gained importance and popularity due to its potent efficiency as it is used in the vivo systems, medicine and other important biological application [44].

Few years before, there has been a great research for the development of inorganic NPs. Predominantly, silver nano compound has latent benefits as recognized by several industries because of dominant anti activity of silver against fungi, viruses and bacteria, and the little incidence of increases in resistance [45]. Silver nanoparticles have been used for centuries to avoid and take care of different diseases [46]. The antifungal and antibacterial properties of AgNPs were utilized in a variety of customer goods like air filters, detergents, air sanitizer sprayers, food storage containers and toothpastes [2]. The most widely used and recognized applications of silver and AgNPs in the pharmaceutical industries. These contain topical ointments and creams having silver to get rid of infection due to burns and open wounds [47].

The prepared AgNPs [11] were found effective for Gram +ve and Gram -ve bacteria. Silver nanoparticles showed a 3 and 3.6-fold increase with Macrolide (erythromycin) and Beta-lactam (piperacillin) antibiotics respectively, against *Acinetobacter*

baumannii (multidrug-resistant). Vancomycin or chloramphenicol with AgNPs give synergetic affects and observed increase in the diameter zone inhibition (ZI) 4.2-fold and 4.9-fold against *Pseudomonas aeruginosa*. In the same way, streptomycin in combination with AgNPs found a maximum 11.8-fold increase in the diameter of zone of inhibition against *E. coli*, showing well proof of the synergistic effect.

An investigation was carried out by [25] to determine the minimum inhibitory concentration (MIC) of nano colloidal silver towards food-borne pathogens such as *E. coli* O157:H7 (12.43 ppm), *L. monocytogenes* (24.58 ppm), *S. typhi* (23.75 ppm), *V. cholerae* (7.71 ppm), *V. parahaemolyticus* (9.64 ppm), *B. cereus* (10.63 ppm) and *S. aureus* (13.85 ppm) and minimum bactericidal concentration (MBC) of nano colloidal silver towards all the tested food-borne pathogens were ≥ 100 ppm. The results obtained suggested that nano colloidal silver exhibit a good bacteriostatic effect but poor bactericidal effect towards all tested food-borne pathogens. Nano colloidal silver can be a potential antimicrobial agent due to its low cost of production and high effectiveness, which may find wide applications in various food industries to address food safety issues.

The AgNPs was prepared [48] using root, bark and leaves of *Avicenna marina* showing high antibacterial activity. As a whole the synthesis of AgNPs from the leaf extract was found maximum. The prepared NPs (100 μg / disk concentration) shows minimum activity (10.87 ± 1.33 mm ZI) against *S. aureus*, whereas highest ZI (18.40 ± 0.97 mm) against the *E. coli* but the MIC and MBC values ranged from 6.25 and 50.0 $\mu\text{g}/\text{ml}$ among the selected bacterial strains. It was proved that, the *A. marina*

leaf extract can be used for the biosynthesis of AgNPs showing a high potential as antibacterial agent.

The prepared AgNPs [49] were found active against common pathogens. MIC ($\mu\text{g/ml}$) of AgNPs against *Staphylococcus* spp was 53, *E. coli* 27 and *B. subtilis* 54. The MIC of Gentamycin ($\mu\text{g/ml}$) against *Staphylococcus* spp was 1255, *E. coli* 1.2 and *B. subtilis* <1.0. MIC of active iodine ($\mu\text{g/ml}$) was 1562, 1.5 and <1.2 against *Staphylococcus* spp, *E. coli* and *B. subtilis* respectively.

The silver nanocrystals were studied [50] for its antibacterial activity. As the emergence and resistance to multiple antibiotics against microorganism increased, therefore, researchers have made attempt to prepare novel antibiotics. The particles size was less than 100 nm, approximately. This study showed that synthesis of mono-metallic and composite nanocrystals, with oxalate decomposition method was simple and so useful. Also the Ag/ZnO nanocrystals are the great antimicrobial agent against all the strains and just combination of zinc oxide and silver nanocrystals give increase the bactericidal effect.

1.8.2. AgNPs as Antifungal Agent

Acalypha indica leaf extract was applied [51] to prepare AgNPs. Different concentrations of AgNPs were applied to find out the inhibition of phytopathogenic fungi namely *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Macrophomina phaseolina*, *Curvularia lunata*, *Alternaria alternata* and *Botrytis cinerea*. It was found that AgNPs with concentration of 15 mg exhibited high antimicrobial activity against these pathogenic fungal strains. These findings obviously propose that AgNPs may have a significant role to manage plants diseases caused by fungi.

A study demonstrated the comparative antimicrobial activity of silver-silica nanocomposite and conservative compounds, such as silver zeolite and silver nitrate [45]. The silver-silica-containing polystyrene synthesized material showed strong antimicrobial activity. Silver nanocomposite inhibits the growth of *C. albicans* by using 125 µg/ml and 2 mg/ml was the minimal fungicidal concentration. Silver nanocomposite of 2 mg/ml also inhibit the growth of *A. niger* on agar plates.

Spherical AgNPs [24] were synthesized and their antifungal activity was examined on fungal pathogens of the skin. The AgNPs were found effective against Candida species (IC_{80} , 1-7 µg/ml), *Trichophyton mentagrophytes* and clinical isolates. The activity of AgNPs was higher than fluconazole (IC_{80} = 10- 30 µg/ml) and nearly equal to amphotericin B (IC_{80} = 1-5 µg/ml). This study also showed that AgNPs exerts its activity on the mycelia. Therefore, this study specifies that AgNPs showed significant activity against fungi and require further study for clinical use.

The minimum inhibitory concentration (MIC_{50}) of AgNPs, Fluconazole and Amphotericin B [52] on *C. albicans* was 0.5 mg/ml, 8mg/ml and 1mg/ml, respectively. Moreover, MIC_{90} of AgNPs, Fluconazole and Amphotericin B was 2 mg/ml, 16 mg/ml and 4 mg/ml respectively. MIC_{50} of AgNPs, Fluconazole and Amphotericin B on *S. cerevisiae* were 4 mg/ml, 64 mg/ml and 16 mg/ml respectively. MIC_{90} of AgNPs, Fluconazole and Amphotericin B on *S. cerevisiae* were 32 mg/ml, 256 mg/ml and 32 mg/ml respectively. This study showed that fungal cell was inhibited because of pores in its membrane structure. The result of SEM on *C. albicans* and *S. cerevisiae* demonstrated reciprocal relationship among AgNPs and cell membrane, which damage the fungal cell followed by death. This study indicated

that AgNPs has significant antifungal effect as compared to other antifungal agents, therefore, it needs further trails for clinical use.

1.8.3. The Role of NPs as Antioxidant

The inorganic NPs can act against different damages due to free radicals by effective scavenging action [27]. *Prunus armeniaca* (apricot) fruit extract was used for the NPs synthesis as a reducing agent. The antioxidant activity of the NPs was carried out by modified procedure of 2, 20-azinobis, 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and DPPH assay. Dose dependent antioxidant activity was recorded for AgNPs and AuNPs in both DPPH and ABTS in-vitro assay. 50 % scavenging activity for DPPH was 11.27 and 16.18 mg and for ABTS 3.40 and 7.12 mg with AuNPs and AgNPs, respectively [53]. Aqueous leaves extract of *Iresine herbstii* was used [54] for the biosynthesis of AgNPs. It observed potent DPPH assay and cytotoxic effect against HeLa cervical cell lines.

Piper nigrum extract of fruit were utilized as reducing agent for the biosynthesis of AgNPs [55]. The DPPH free radical scavenging evaluation proved strong inhibitory ability of biosynthesized AgNPs when matched with ascorbic acid at high concentrations. Increase in concentration of substrates means increased inhibition percentage of free radicals. The IC_{50} value was 183.24 $\mu\text{g/ml}$. This data proposed that at above 120 $\mu\text{g/ml}$, the fabricated AgNPs might act as effective antioxidants.

The research work was carried out to synthesize and evaluates antioxidant activity of AgNPs from *Cassia auriculata* flower extract [56]. The antioxidant activity of AgNPs increased as the AgNPs and flower extract concentration increased. The antioxidant activity of AgNPs was high as compared to L ascorbic acid and flower extract.

Hydrogen peroxide positive tests confirmed that AgNPs, ascorbic acid and flower extract were free radical scavengers. The probable mechanistic approach of antioxidant activity of AgNPs includes electron donation, scavengers of radicals and reductive ability. These prepared AgNPs were beneficial for pharmaceutical and medical purposes and can be produced industrially.

1.8.4. Cytotoxic Effect of NPs

The cytotoxic effects of biosynthesized AgNPs were tested on brine shrimps showing that 75% of inhibition at 25 µg/ml, while 50 to 100 µg/ml showed total inhibition [57].

The study regarding aquatic stability and impact of titanium dioxide nanoparticles (TiO₂NPs, 10–30 nm) was conducted using *Artemia salina* (*A. salina*) [58]. Acute exposure was conducted on nauplii (larvae) and adults in seawater in a concentration range from 10 to 100 mg/L TiO₂NPs for 24 and 96 hrs. Average TiO₂ content in nauplii ranged from 0.47 to 3.19 and from 1.29 to 4.43 mg/g in 24 and 96 hrs, respectively. Accumulation in adults was higher, ranging from 2.30 to 4.19 and from 4.38 to 6.20 mg/g in 24 and 96 hrs, respectively. Phase contrast microscopy images revealed that *Artemia* was unable to excrete the particles. Thus, the TiO₂ aggregates filled inside the guts. No significant mortality or toxicity occurred within 24 hrs at any dose. Lipid peroxidation levels characterized with malondialdehyde concentrations were not statistically different from those of the controls ($p > 0.05$). These results suggested that suspensions of the TiO₂NPs were nontoxic to *Artemia*, most likely due to the formation of benign TiO₂ aggregates in water. In contrast, both mortality and lipid peroxidation increased in extended exposure to 96 hrs. Highest

mortality occurred in 100 mg/L TiO₂NP suspensions; 18 % for nauplii and 14 % for adults (LC₅₀>100 mg/L). These effects were attributed to the particle loading inside the guts leading to oxidative stress as a result of impaired food uptake for a long period of time.

The cytotoxic activity of biosynthesized AgNPs were studied [59]. The experiment revealed that the cell exposed with various plant extracts and NPs condensed the cell development of vero cell line in a dose dependent mode. Single transformed cell lines were tested against NPs in this study. The results data neither involve the activity of the flower extracts in other cell lines (transformed or primary), nor the anti-proliferative measures in vivo. As observed in a study, silver colloidal solution induced dosage dependent cytotoxic effect on breast cancer MCF-7 cells; the median lethal (LD₅₀) dose was 3.5 ng/ml and the lethal (LD₁₀₀) dose was 14 ng/ml [60].

1.8.5. Phytotoxic Effect of NPs

The growth inhibition effect of AgNPs and TiO₂NP was checked against aquatic plant duckweed (*Lemna paucicostata*) [61]. TiO₂NPs size was 2-3 nm and AgNPs size was 50 nm, which were phytotoxic against Lemna in dissimilar manner and the calculated EC₅₀ were 538.5 ppm and 13.8 ppm respectively. According to these findings it was observed that even in low concentration range (≥ 1 ppm), AgNPs might cause Lemna growth inhibition, while TiO₂NP in the concentration range of ≥ 250 ppm showed negative effect on the development of Lemna. AgNPs, in the concentration of ≥ 100 ppm, completely inhibited the growth of Lemna in the growing media for seven days of incubation period. Similarly, TiO₂NPs at 500 ppm were decreased 51% of the Lemna specific growth rate as compared the control specific growth rate.

This study assesses the biological effects of NPs based on seed germination and root elongation tests [62]. Cucumber, lettuce and radish seeds were incubated with different metal oxide nanoparticles (Co_3O_4 , TiO_2 , CuO , NiO , Fe_2O_3), of which only NiO and CuO showed harmful effect on the behavior of all three seeds. For seed germinations the measured EC_{50} (mg/l) were: lettuce (CuO : 13, NiO : 28), radish (CuO : 398, NiO : 401) and cucumber (CuO : 228, NiO : 175). Progress of root elongation of radish seedling was shown by the application of Co_3O_4 NP solution (5 g/l). Nanoparticles were absorbed in aqueous medium on surface of seed and near the seeds metals ions were released. In the equivalent concentrations free metal ions had lesser phytotoxicity than metal oxide NPs. Small seeds like lettuce were highly susceptible to NPs (NiO and CuO), because phytotoxicity was related to the surface area-to-volume ratio of seeds.

Silver nanoparticles were synthesized from stem bark powder of *Boswellia ovalifoliolata* [63]. These AgNPs were tested to investigate its effect on seed growth and germination. The seeds kept in Murashige and Skoog (MS) basal medium with AgNPs observed 90% germination, but in control seeds 70% germination was recorded. The reason could be that the AgNPs can enter in seed coat and activate the embryo. Seeds sprouting were noted from 7th day beyond in AgNPs tested (treated) test tubes and in control seeds sprouting was found on 15th day. Within 7 to 10 days all the seeds germination was completed, when seed treated with AgNPs. When the seeds were treated with 30 mg/ml of AgNPs on maximum height i.e. 10.6 cm, while the control experiments showed shorter seedling (5 cm) in more than one month. It was observed that treated seeds with AgNPs reach maximum growth in twenty to

twenty five days. The data of these finding may perhaps be useful to get better the percentage of seedling growth and seed germination in seeds, particularly, in inactive seeds. This technique can increase the amplification of plants particularly endemic trees with hard seed coat which are on the verge of extinction.

Germination and growth profile of common wetland plants species (eleven) were investigated [64], when exposed to gum arabic coated silver nanoparticles (GA-AgNPs) and Polyvinylpyrrolidone-coated silver nanoparticles (PVP-AgNPs). Exposure to silver nitrate and GA-AgNPs considerably affected the germination rates of seed for numerous plant species, while no measurable effects on germination were found for PVP-AgNPs exposure. High dosage (40 mg Ag/l) exposures were recorded 25%, 45% and 55% average reduction in leaf growth in the PVP-AgNPs, AgNO₃ and GA-AgNPs treatments, respectively. Commonly growth of root was highly effected to silver exposure as compared to the leaf. It was concluded that GA-AgNPs had greater or equal effects in degree than silver nitrate on growth seedling, proving that the greater phytotoxic effect of AgNPs were not merely because of the ionic silver content. Further species of wetland plant differ in their sensitivity to AgNPs and AgNO₃. The data proposed that wetland plant were affected by increasing release of AgNPs into the surroundings.

1.8.6. Mosquitocidal Potential of NPs

Millions of deaths every year are due to the transmission of serious human diseases. The application of man-made insecticides against vector mosquitoes results in hazards to the environment, developed insect's resistance as well as high production

price. The formations of non-synthetic compounds as insecticide against vector are of great importance in this part [65].

Substances derived from plants have drawn a greater concentration of researchers and about 2000 plant species were formerly identified for its insecticidal activities [66]. Plant derived products has been used in many areas of the world against the species of insects and vectors conventionally by human communities. Many researchers have found that the phytochemicals constituents of plant origin can kill larvae, regulate the insect growth, repel the insects and ovipositional attractants and have preventive actions [67]. Agents acting as repellents hold a major role in saving humans from insect bites. The spreading risk of diseases and humans contact with vectors can only be reduced by effective repellents. The compounds which were repellants should be non toxic, should not have any irritation and should have effects for longer time e.g. amides, imides, esters and other multifunctional compounds [68].

Larvicidal activity of AgNPs synthesized [69] using *Euphorbia hirta* (*E. hirta*) plant leaf extract against malarial vector *Anopheles stephensi* was determined. Range of concentrations of synthesized AgNPs (3.125, 6.25, 12.5, 25, and 50 ppm) and methanolic crude extract (50, 100, 150, 200, and 250 ppm) were used against larvae of *A. stephensi*. The AgNPs synthesized from *E. hirta* was found more toxic against, *A. stephensi* as compared to methanolic crude extract. After 24 hrs of contact of AgNPs with mosquito larva (*A. stephensi*) observed larvicidal activity; the maximum larval activity of AgNPs was observed against the instar larvae (I, II, III and IV) and pupae. The LC_{50} values were (10.14 ppm, 16.82 ppm, 21.51 ppm, and 27.89 ppm) and 34.52 ppm, respectively, and LC_{90} (ppm) of instar larvae (I, II, III and IV) and

pupae values were 31.98, 50.38, 60.09, 69.94 and 79.76, respectively. Methanol extract exhibited the larval toxicity against instar larvae (I, II, III and IV) and pupae with LC_{50} (ppm) values as (121.51, 145.40, 169.11 and 197.40) and 219.15 respectively. Similarly LC_{90} (ppm) were 236.44, 293.75, 331.42, 371.34 and 396.70 found against instar larvae I, II, III and IV and pupae respectively. Nil mortality was found in the distilled water serving as control.

The *Eclipta prostrate* (Asteraceae) aqueous extract was used for the synthesis of AgNPs and were tested for larvicidal activity against *Anopheles subpictus* (malaria vector) and IV instar larvae of *Culex quinquefasciatus* (filariasis vector) [65]. The AgNPs toxicity against larvae was checked at different concentrations for 24 hrs. The highest activity was calculated in AgNPs and aqueous crude extract against *A. subpictus* with LC_{50} (mg/l) values were 5.14 and 27.85 respectively and similarly the LC_{90} (mg/l) values were 25.68 and 71.45 respectively. While LC_{50} (mg/l) values were 4.56 (AgNPs) and 27.49 (extract), LC_{90} (mg/l) values 13.14 (AgNPs) and 70.38 (extract) against the larvae of *C. quinquefasciatus* respectively.

The larvicidal and Pupicidal activity of AgNPs synthesized from stem aqueous extract of *Pedilanthus tithymaloides* (*P. tithymaloides*) was determined against different developmental stages of *A. aegypti* [70]. It was concluded that by increasing the NPs concentrations increased the % mortality rate. The LC_{50} of instar larvae 1st, 2nd, 3rd, 4th and pupa of *A. aegypti* was 0.046, 0.051, 0.046, 0.167 and 0.054% respectively. At 0.25% AgNPs 100% mortality was observed for pupa. While 100% mortality was found for the same concentrations for instar II and III. The mosquitocidal activity of *P. tithymaloides* stem aqueous extract at 1% concentrations

mortality (%) was 36, 32, 26, 16 and 30 against *A. aegypti* instar larvae first, second, third, fourth and pupa, respectively. while the LC₅₀ values were 1.529 (instar I), 1.282 (instar II), 1.450 (instar III), 2.210 (instar IV) and 1.196 (pupa).

The larvicidal activities of AgNPs synthesized from *Rhizophora mucronata* leaves observed that percent mortality increases as the concentrations of NPs increases [71]. At 20.0 mg/l, 10.0 mg/l and 5.0 mg/l concentrations 100% mortality was calculated against *A. aegypti*. While 100% mortality was recorded at 20.0 mg/l and 10.0 mg/l concentrations for *Cx. Quinquefasciatus*. The LC₅₀ (mg/l) and LC₉₀ (mg/l) of AgNPs against *A. aegypti* was 0.891 and 6.291 respectively and the LC₅₀ and LC₉₀ of AgNPs against *Cx. Quinquefasciatus* were 0.585 and 2.615, respectively.

The larvicidal activities of AgNPs against *Aedes albopictus* larvae were obtained and the results showed that these NPs gave outstanding *Aedes albopictus* larval control [72]. High mortality was observed in larvae exposed to AgNPs as compared to the aqueous extract. At 1.0 mg/l of AgNPs 50% viability decreased of larvae to twelve hours incubation. Whereas 5.0 mg/l NPs in three hours hundred percent mortality of the larvae were counted. Larvae gradually killed by NPs at 1.0 mg/l and almost 90% death were observed after 16 hrs incubation.

Larvicidal activity results [73] showed that LC₅₀ of *Vinca rosea* extract (aqueous) was 78.62 mg/ml and LC₉₀ was 184.85 mg/ml against *A. stephensi*. The LC₅₀ and LC₉₀ of aqueous extract against *C. quinquefasciatus* were 55.21 mg/ml and 112.72 mg/ml respectively. The extract showed 100% mortality at 72 hrs in the 50 mg/ml concentration against *A. stephensi* and *C. quinquefasciatus*. The AgNPs LC₅₀ and LC₉₀ at 48 hrs were 12.47 mg/ml and 36.33 mg/ml, LC₅₀ and LC₉₀ at 72 hrs were

168.84 mg/ml and 68.62 mg/ml against *A. stephensi* respectively. Similarly, the LC₅₀ and LC₉₀ at 72 hrs exposure AgNPs against *C. quinquefasciatus* were 43.80 mg/ml and 120.54 mg/ml respectively. Silver nitrate was found no mortality at 10 mg/ml.

Aims and Objectives:

The aims of the current research study were:

1. To evaluate the physiochemicals, minerals, phytochemicals and biological activities of *H. rhamnoides* L. leaves.
2. To utilize the *H. rhamnoides* L. leaves for the bioinspired synthesis of AgNPs.
3. To characterize the bioinspired prepared AgNPs.
4. To optimize different reactions conditions for the biosynthesis of AgNPs.
5. To study the biological activities of bioinspired AgNPs.

Chapter -2

Material and Methods

Materials and Methods

2.1. Collection of *H. rhamnoides* L. Leaves

The fully matured healthy leaves of *H. rhamnoides* L. were collected from Pakistan Council of Scientific and Industrial Research (PCSIR) Skardu Gilgit Baltistan, Pakistan. The leaves were slightly washed to remove any dust, shade dried and powdered with a laboratory mill. The crushed leaves were kept in an air-tight plastic bag till used.

The voucher specimen was deposited in Department of Botany, University of Peshawar, Khyber Pakhtunkhwa- Pakistan, with herbarium number Bot.20006 (PUP).

2.2. Physiochemical Analysis of *H. rhamnoides* L. Dry Leaves Powder

The *H. rhamnoides* L. leaves powder were used for physiochemical analysis i.e. moisture, ash, fat, pH, total soluble solids, acidity, crude fiber, nitrogen, protein, reducing sugar, non-reducing sugar and total sugar [74].

2.2.1. Determination of Moisture

The *H. rhamnoides* L. leaves powder (1 g) was taken in a moisture determination bottle (W1) and kept in an oven for 2 hrs at 105 °C. The leaves powder was then transfer to desiccator to cool and weight, for a second time it was shifted to oven awaiting steady weight followed drying was achieved (W2) and percent moisture was calculated by the formula:

$$\text{Moisture (\%)} = \frac{W1 - W2}{\text{Wt. of Sample}} \times 100$$

2.2.2. Determination of Ash

Clean empty crucible was kept in a muffle furnace for 1 hrs at 600 °C, cooled and afterward weight (W1). In a crucible *H. rhamnoides* L. leaves powder 1 g (W2) was taken and 5 ml of HNO₃ was added to it. It was continuously heated at low flame unless the powder starts to burn. Following charring the powder, shifted in muffle furnace at 650 °C for four hrs and weighed (W3) to estimated percent ash of powder:

$$\text{Ash (\%)} = \frac{(W3) - (W1)}{\text{Wt. of sample}} \times 100$$

2.2.3. Determination of % Fat content

The *H. rhamnoides* L. leaves powder was weighed into formerly weighed filter paper. The filter paper (W1) and the leaves powder were transfer to filter paper (W2) and kept in the extractor thimble. The petroleum ether was poured into the extractor. The condenser was placed and connects were tightened. The assembly was kept on the heating mantle. The heat source was in tune so that petroleum ether boiled softly and refluxed numerous times for 6 hrs until the petroleum ether extracted all the fat content. The sample was then kept in an oven at 52°C and dried to constant weight (W3). The percentage lipid (fat) was calculated:

$$(\%) \text{ Fat} = \frac{(W2) - (W3)}{(W2) - (W1)} \times 100$$

2.2.4. Determination of Crude fiber

A fat and moisture free sample was first digested with diluted sulphuric acid and next with dilute KOH solution. The collected undigested residue following digestion was ignited and after ignition loss in weight was calculated as crude fiber.

2.2.5. Determination of Total, Reducing and Non-reducing Sugar

Weighed five (05) gram *H. rhamnoides* L. leaves powder, transferred to beaker and mixed with hundred ml of hot H₂O. The slurry was well shaken to dissolve the whole soluble constituents and then filtered the slurry with the help of Whatman No.1 filter paper into a volumetric flask (250 ml). In conical flask the resultant solution hundred (100) ml and ten (10) ml diluted hydrochloric acid were taken. Boiled the mixture for five minute. After cooling, the mixture was neutralize to phenolphthalein by means of ten percent sodium hydroxide solution and makes the volume up to the mark of 250 ml volumetric flask. Titrate this solution against Fehling's solution.

$$\text{Total Sugar \%} = \frac{\text{Factor (4.95)} \times \text{dilution (250)} \times 2.5}{\text{Titre} \times \text{wt. of leaves powder} \times 10}$$

$$\text{Reducing Sugar \%} = \frac{\text{Factor (49.5)} \times \text{dilution (250)}}{\text{Titre} \times \text{wt. of leaves powder} \times 10}$$

$$\text{Non Reducing Sugar} = (\text{Total Sugars percentage} - \text{Reducing Sugars percentage}) \times 0.95$$

2.2.6. Crude protein and Nitrogen

The Kjeldahl technique was employed for protein estimation. Transferred 0.5 g leaves powder into micro-Kjeldahl flask. Ten (10) ml concentrated H₂SO₄ and 8 g of digestion mixture (K₂SO₄: CuSO₄) in 8:1. These were put in the suitable outlet of the digestion block heaters in a fume hood. The digestion was carried out for four hours following which in the tube an apparent colorless solution was left. The digest was cautiously shifted into volumetric flask (100 ml), cautiously wash the digestion tube with distilled water and the amount of the flask make up to the sign by means of distilled water. Then five (05) ml digested sample was shift to Kjeldahl equipment

and 5ml of 40% (w/v) NaOH was added. Then steam distilled the mixture and the produce ammonia received into a 50 ml conical flask having ten milliliter of boric acid (2%) beside with mixed solution of indicator. The solution of green colour was afterward titrated against HCl (0.01N) solutions. At the finish spot, the green colour alter to wine colour, which denotes that, whole nitrogen trapped as ammonium borate have been detached as ammonium chloride.

Crude Protein (%) = $6.25 \times X \% N$ (*Corrected factor)

$$\text{Nitrogen (\%)} = \frac{(S-B) \times N \times 0.014 \times D \times 100}{\text{Sample weight} \times V}$$

Where

S = Reading of titrated sample

B = Reading of titration (Blank)

N = HCL normality

D = Sample dilution after digestion

V = Use volume for distillation

0.014 = Nitrogen (Milli equivalent weight)

2.2.7. pH

The pH was measured in a mixture obtained by homogenization of 25 g sample diluted in 300 ml distilled water using pH meter (EcoMet Korea).

2.2.8. Total Soluble Solids (TSS)

The total soluble solid (°Brix) of *H. rhamnoides* L. leaves powder was determined using Digital Refractometer (ATAGO, Japan).

2.2.9. Acidity

Total titratable acidity as percent of citric acid was calculated through sodium hydroxide solution (0.1 N) and phenolphthalein as an indicator. Total acidity was calculated with amalgamation a 25 g sample with distilled water (300 ml) to prepare harmonized slurry. Sample filtered slurry of 10 ml was titrated with 0.10 mol/L NaOH. The total acidity was estimated as % citric acid.

2.2.10. Determination of carbohydrates

Percentage carbohydrate value was calculated by the described method [75].

% Carbohydrate = $100 - (\text{moisture}\% + \text{ash}\% + \text{fat}\% + \text{protein}\%)$.

2.3. Minerals Analysis of *H. rhamnoides* L. Leaves Powder

2.3.1. Wet Digestion

One gram sample was taken in digesting glass tube. 12 ml HNO₃ was mixed with sample and reserved for overnight at room temperature. Subsequent to that HClO₄ (4.0 ml) was added to this fusion and was set for digestion in the fumes hood. The temperature was increased gradually, beginning from 50°C and rising upto 300°C. The process was completed in seventy to eighty minutes as denoted via the white fumes appeared. Cooled down the mixture and the materials of the tubes were shifted to volumetric flasks (100 ml) and the contents volumes were prepared to hundred milliliters with distilled H₂O. The end solution was kept in glass bottles and used for elemental analysis [76].

2.3.2. Procedure

Minerals like sodium and potassium were quantified using Flame Photometer (Jenway PFP7). Metals like Ca, Mg, Fe, Al, Mn, Zn, Si, Ba, Cd, Pb, Cr and Ni, were

calculated using Atomic Absorption Spectrometer (Hitachi Zeeman Japan Z-8000) with air/acetylene flame at 2200 – 2400 K (photo multiplier tube detector), against the standard according to the standard methods [74].

2.4. *H. rhamnoides* L. Leaves Extraction

Fifty grams powder of *H. rhamnoides* L. leaves was extracted in 250 ml of water, ethanol, acetone, methanol, ethyl acetate, chloroform and *n*-hexane for 48 hrs. These extracts were afterward filtered under vacuum in the course of No.1 Whatman filter paper into a Buchner flask. The extracts were concentrated in rotary evaporator and transferred in a sterilized beaker for heating on water bath at 50°C to obtain dried residue. The resultant crude extract was transferred into airtight sample bottles and kept at 4°C until used.

2.5. Phytochemical Screening of *H. rhamnoides* L. Leaves Powder (Qualitative)

Terpenoids, steroids, glycosides, alkaloids, phenols, flavonoids, flavones, tannins, saponins and amino acids were determined qualitatively by the described methods [77, 78].

2.5.1. Terpenoids

The crude extract of each solvent was dissolved in two ml of chloroform and evaporated till dryness. Then 2 ml of H₂SO₄ (concentrated) was supplemented to it and boil for two minutes. A grayish colour indicates the presence of terpenoids.

2.5.2 Steroids

Each extract of 01 ml was dissolved in 10 ml of chloroform and equivalent amount of concentrated H₂SO₄ was poured by edges of the test tube. The higher level become

red and H_2SO_4 level become yellow with green light. This represents the existence of steroids.

2.5.3. Glycosides

The sample was mixed with hydrochloric acid on a water bath for few hours. To the hydrolysate, pyridine (01 ml) and sodium nitroprusside solutions (few drops) was added and after that it was made alkaline with NaOH solution. Change of pink to red color indicates the existence of glycosides.

2.5.4. Alkaloids

Each extract of 5 ml was blended with HCl (2 ml) and 1 ml of Dragendroff's reagent was added. Precipitate of an orange or red color shows the occurrence of alkaloids.

2.5.5. Phenols

Equal volume of the extract was mixed to equal volume of ferric chloride, a deep bluish green solution was taken as a positive test for the presence of phenols.

2.5.6. Flavonoids

Small amount (drops) of dilute NaOH was added to 01 ml of each extract. A deep yellow colour was formed in each extract, which turn into colorless on adding of a few drops of acid (dilute) shows the presence of flavonoids.

2.5.7. Flavones

The extract (03 ml) was blended in test tubes with 4 ml of one percent aluminium chloride in methanol and noted the colour. Development of color (yellow) represents the presence of flavones.

2.5.8. Tannins

To each extract (5 ml) add one drops of lead acetate (1%). Precipitate of yellow color was produced, showing the presence of tannins.

2.5.9. Saponins

Each extract was diluted with twenty ml of distilled water and agitated for 15 minutes in a graduated cylinder. The creation of foam having 1cm layer indicate the existence of saponins.

2.5.10. Amino Acids

A few drops of Ninhydrin reagent was added to 01 ml of the each extract and development of purple colour shows the presence of amino acids.

2.6. FTIR Analysis of *H. rhamnoides* L. Leaves Extracts

Fourier transform infrared (FTIR) technique was used for the recognition of functional groups in all extracts. The IR peaks was obtained using FTIR Prestige -21 Shimadzu Japan. The sample was scanned from 3900 to 500 cm^{-1} and operating at a resolution of 4 cm^{-1} with 10 number of scan.

2.7. Biological Evaluations of *H. rhamnoides* L. Leaves Extracts

2.7.1. Microorganism used in Current Study

Pure bacterial cultures of *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Vibrio cholera*, *Salmonella typhi*, *Bacillus cereus*, *Bacillus subtilis*, *Citrobacter frondai* and pure fungal culture of *Aspergillus niger*, *Aspergillus parasiticus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus oryzae*, *Fusarium oxysporum*, *Rhizopus arrhizus*, *Alternaria alternata*, *Candida albicans* and *Penicillium digitatum* were obtained from Food

Microbiology Laboratory of PCSIR Laboratories Complex Jamrude Road Peshawar Khyber Pakhtunkhwa (KPK), Pakistan. These microbes were maintained on slants of nutrient agar (bacteria) and potato dextrose agar (fungus). These strains were stored in refrigerator and subcultured every week.

2.7.2. Preparation of Bacterial inoculum

Standardization of the bacterial cultures was carried out by described protocol [79]. 1 ml of bacterial culture was pipetted into sterile test tubes containing 1 ml of nutrient broth. After that normal saline was mixed slowly to it therefore as to balance the turbidity to that of 0.5 McFarland standards that corresponded to approximately 1.0×10^8 cells.

2.7.3. Antibacterial Activity of *H. rhamnoides* L. Leaves Extracts

Antibacterial activity was determined using well agar diffusion method [80]. One milliliter (1 ml) of test bacterial strain was inoculated into Petri plate. In each Petri plate melted nutrient agar at 45°C was add and shaken smoothly to make homogeneous amalgamation of the culture and media. Wells were punched in the solidified agar with the help of a sterile 6 mm cork borer. Stock solution was made by dissolving 1000 mg in 5 ml of DMSO. Fifty microliter (50 μ l) of the 200 mg/ml of each extracts was pipetted into each well. Fifty microliter of each of the DMSO and Ciproxin (0.5 mg/ml) solution served as negative and positive control respectively. The plates were incubated at 37°C for 18 hrs. The antibacterial activity was determined by measuring the diameters of zone of inhibition.

2.7.4. Minimum Inhibitory Concentration (MIC) of *H. rhamnoides* L. Leaves Extracts

The broth dilution method was followed to determine the minimum inhibitory concentration. The *H. rhamnoides* L. leaves extracts were diluted to various concentrations ranging from 20-200 mg/ml in nutrient broth. Five hundred microliter (500 µl) of each concentration was added to sterile nutrient broth (2ml) in test tubes. Then 1ml (1×10^8 cfu/ml) of bacterial culture of the respective strain was added to the content of the test tubes and incubated at 37°C for 18 hrs. Ciproxin solution with different concentration ranging from 0.10 – 0.5 mg/ml was used as positive control. The lowest concentration of the testing material that did not allow any visible growth against experimental bacteria was taken as MIC [80].

2.7.5. Minimum Bactericidal Concentration (MBC) of *H. rhamnoides* L. Leaves Extracts

The MBC was determined by pipetting 100µl of culture from each of the broth tubes having no growth and introduced into fresh agar plates. The plates were incubated for 48 hrs and then observed for growth. The concentration of the extracts/control without visible growth was calculated as the MBC [80].

2.7.6. Preparation of Fungal Inoculum

After seven days old slant culture of Sabouraud Dextrose Agar (SDA) fungal spores were rinsed with ten milliliters normal saline solution in two percent Tween 80 with the help of glass beads to facilitate spores dispersion. Standardize the spore suspensions to 10^5 spores /ml [81].

2.7.7. Antifungal activity of *H. rhamnoides* L. Leaves Extracts

Antifungal activities were carried out according to the method described elsewhere [81]. One ml of each standardized spore suspension (10^5 spores/ml) was spread on the surface of the SDA plates. Then, sterile cork borer (6mm in diameter) was used to make well at the centre of each inoculated/cultured plates. *H. rhamnoides* L. leaves extracts of 1000 mg were dissolved in 5 ml of DMSO (200 mg/ml) and 50 μ l of each extract was applied into each respective well. 50 μ l each of pure DMSO and fluconazole (0.5 mg/ml) were used as negative and positive control respectively. The plates were kept in incubator for 1-7 days at ambient temperature and then observed for anti-fungal activities.

2.7.8. MIC of *H. rhamnoides* L. Leaves Extracts

The MIC of the extracts was carried out using broth dilution method [81]. Serial dilutions of each extracts were made in the broth to obtain the dilutions in the range of 20- 200 mg/ml (extracts) and 0.1 – 0.5 mg/ml (fluconazole). 1 ml standardized inoculums (10^5 spores/ml) were inoculated into each broth having different extract concentrations and then incubated for 1-7 days at 30°C to observe turbidity. MIC was calculated on the basis of no turbidity observation of lowest concentration in the test tubes.

2.7.9. Minimum Fungicidal Concentration (MFC) of *H. rhamnoides* L. Leaves Extracts

For MFC determination the contents of the MIC in the serial dilution were then sub-cultured on the media (SDA) and incubated at 30°C for one to seven days and

observed for colony growth. The MFC was the plate with the lowest concentration of extracts and without colony growth.

2. 7.10. Antioxidant Activity of *H. rhamnoides* L. Leaves Extracts

Antioxidant activities of *H. rhamnoides* L. leaves extracts were measured by 1, 1'-diphenyl- 2-picrylhydrazyl (DPPH) scavenging procedure [82]. Different concentrations of each extract (10 - 80 µg/ml) were made in methanolic (95%) and mix this extract solution (1 ml) with 1 ml methanol (95%) solution of DPPH (0.004%) and also with vitamin C (standard) solution separately. DPPH and methanol (95%) with 1:1 ml was used as blank. These solutions were kept in a dark room for 20 min and absorbance was measured at 517 nm using UV-visible spectrophotometer (UV-1700, Shimadzu, Japan). The scavenging inhibition (I %) was calculated by following formula.

$$\text{Inhibition percentage (I \%)} = (A - B)/A \times 100$$

A = Absorbance (Blank), B = Absorbance (Sample).

2. 7.11. Brine Shrimp Cytotoxicity Assay of *H. rhamnoides* L. Leaves Extracts

Cytotoxicity of *H. rhamnoides* L. extracts was determined through brine shrimp (*Artemia salina*) assay [83]. In this method eggs of 50 mg were used to hatch larvae during incubation in artificial seawater for 48-72 hrs. 20 mg of each extract was dissolved in two (02) ml DMSO to make stock solution. From this stock solution transfer 500, 50 and 5 µl to vials corresponding to 1000, 100 and 10 µg/ml respectively and evaporate DMSO overnight. Three vials for each concentration of each extract were made and to each concentration 10 shrimp per vial (30 shrimp per dilution) were transferred before 5 ml/vial addition of sea water. Cytotoxic drug

(Etoposide) served as positive and sea water as negative control. The brine shrimps were observed for its mortality after 24 hrs. The % mortality of brine shrimp was calculated as shown bellow.

$$\% \text{Mortality} = \frac{(\text{Number of died or immobile brine shrimp})}{(\text{Total number of brine shrimps})} \times 100$$

2. 7. 12. In vitro Phytotoxic Assay of *H. rhamnoides* L. Leaves Extracts

H. rhamnoides L. leaves extracts were subjected for its phytotoxic activity against the *Lemna minor* [83]. Each extract (15 mg) was dissolve in 15 ml DMSO, 10, 100 and 1000 µl solution were added to vials to make 5, 50 and 500 ppm solution. Evaporate all solvent overnight. Then add E medium (2 ml) and a single plant to each vial (10 vials/dose), sterile E medium was used as a negative and Paraquat as positive control. Then vials were placed in the growth chamber (27-29°C) for 7 days and were checked on daily basis. After incubation fronds per vials were counted and the growth regulation (in percent) was measured with reference to negative controls.

$$\% \text{Growth Regulation} = 100 - \frac{\text{Number of fronds in sample (test)}}{\text{Number of fronds in control (negative)}} \times 100$$

2. 7.13. Larvicidal Activity

2.7.13.1. Test Larvae

Mosquitoes *Aedes aegypti* (*A. aegypti*) and *Anopheles stephensi* (*A. stephensi*) were reared in the Food Technology Center, PCSIR Laboratories Complex Peshawar KPK Pakistan. The mosquito's vectors cyclic generations were under specified conditions of relative humidity i.e. 80-90% and temperature i.e. 25-29 °C in insectarium. The food used for the growth of larvae consisted of yeast and powdered dog biscuits (1:3).

2.7.13.2. Larvicidal Activity of *H. rhamnoides* L. Leaves Extracts

The larvicidal activity test was carried out by described method of WHO [84]. Twenty-five instar (IV) larvae of *A. stephensi* and *A. aegypti* were transferred to plastic cups (500 ml) having 249 ml (distilled H₂O) and 1 ml of extract from the desired concentration. Four replicates for each concentration were set up. Distilled H₂O having 25 larvae were used as a control. Abbott's formula (1987) [85] was applied to correct the control mortality and (Finney 1971) [86] probit analysis were used to calculate regression equation, LC₉₀, LC₅₀ and Chi-square value (X^2).

$$\% \text{ mortality} = \frac{\% \text{ Mortality (sample)} - \% \text{ Mortality (control)}}{100 - \% \text{ Mortality (control)}} \times 100$$

2.8. Leaves Extraction for AgNPs Biosynthesis

Twenty five gram (25 g) of the leaves powdered of *H. rhamnoides* L. were weighed and kept into 1000-ml conical flask containing 500 ml double distilled water, well mixed and then boiled for 25 min. The extract obtained was filtered through muslin clothe and then filtered through filter paper (Whatman No.1) and the filtrates were received in Erlenmeyer flask (500 ml) and kept at low temperature to use further.

2.8.1. Bioinspired Synthesis of AgNPs

Silver nitrate aqueous solution (1 mM) was prepared and used for the bioinspired synthesis of AgNPs. *H. rhamnoides* L. leaves extract (5 ml) was mixed with 95 ml aqueous solution of 1 mM AgNO₃ and heated on horizontal shaking water bath at 75 °C for 60 min in a dark room. Reduction of AgNO₃ to silver ions was confirmed by change in color from colorless to brown. Furthermore confirmation of AgNPs synthesis was carried out by spectrophotometric determination. The fully reduced

solution was concentrated on rotary evaporator (R-200, Buchi Rotavapor, Switzerland) on 50°C. The concentrated AgNPs were dried in an oven overnight at 50°C and grinded in mortar and pestels.

2.9. Characterization of Bioinspired Synthesis of AgNPs

2.9.1. UV-Vis Spectrophotometer Analysis

The silver ions reduction confirmation was carried out by UV-Visible Spectrophotometer UV-1700 (Shimadzu, Japan) spectrum of the reaction solution after cooling at room temperature. It was measured by mixing 20 ml distilled water with 1ml sample reduced solution (AgNPs).

2.9.2. FTIR Measurements of AgNPs

The IR spectrum was obtained using FTIR Prestige -21 Shimadzu Japan. The AgNPs and *H. rhamnoides* L. leaves powder were scanned from 3900 to 500 cm^{-1} and operating at a resolution of 4 cm^{-1} with 10 number of scan using IR solution software.

2.9.3. Scanning Electron Microscope (SEM) Analysis of AgNPs

Sample was prepared in the form of thin films on a copper grid carbon coated by taking a very little quantity of AgNPs powder in distilled water and then on the SEM grid AgNPs layer were keep for dryness with the help of hot air (50 – 60°C) for 5 min. The sample was then gold coated through sputter coater (SPI, USA) at 30 mA and 120 seconds. Scanning Electron Microscopic (SEM) images was carried out by JSM-5910 (JEOL, Japan) machine.

2.9.4. Energy Dispersive X-Ray (EDX) Analysis

The AgNPs elemental composition study was carried out with EDX coupled with SEM JSM-5910 (JEOL, Japan) with model INCA 200, Oxford Instruments UK. The

dispersed particles were sprinkled onto double sided sticky tape which was mounted on a microscopic stub of aluminum.

2.9.5. X-ray Diffraction (XRD) Analysis

X-Ray Diffractometer measurement of the prepared AgNPs was carried out by model JDX-3532, JEOL Japan. A slight layer of the AgNPs was preparing by sinking a plate of glass in the solution. The diffracted intensities were calculated from 10° to 80° of 2θ angles. Measurement condition was as a current of 30 mA with Cu K α radiation with a wavelength of $1.5418 \text{ \AA}$ in θ - 2θ configurations and voltage of 20 to 40 kV. The scanning was done in the region of 10° to 80° for 2θ at $0.02^\circ/\text{min}$ and 2 second was time constant. The XRD peaks width was used for the calculation of crystalline domain. It was assumed with the aim that they are free commencing non-uniform strains, with the formula of Scherrer.

$$D = 0.94 \lambda / \beta \cos \theta$$

D = crystallite domain size (mean) at right angles to the reflecting planes

β = full width at half maximum (FWHM),

λ = X-ray wavelength

θ = Diffraction angle.

2.9.6. Thermogravimetric/Differential Thermal Analysis (TGA/DTA)

Diamond TG-DTA Perkin Elmer Instrument USA was used to evaluate the thermal crystallization and decomposition temperature of the AgNPs.

2.10. Optimization of Bioinspired Synthesis of AgNPs

To improve the optimization and reproducibility of the trial results of the bioinspired synthesis method as a whole, the factorial Design of Experiments, the “one factor- at-

a time'' method, was used in this research work. Here, the investigational factors are different one at a time with the left over factors constant.

2.10.1. Temperature

The pre mentioned synthesis method of AgNPs was repeated for temperature optimization, where the temperature of the reaction was set at 25, 35, 45, 55, 65, 75 and 85°C. The resulting solutions (AgNPs) absorbance was monitored using UV-Vis spectrophotometer.

2.10.2. pH

The AgNPs synthesis protocol was repeated for optimization of pH where the interaction pH was adjusted at 4, 5, 6, 7 and 8 respectively. For pH adjustment, solution of NaOH (0.1N) and 0.1 N HCl were used. At the end, absorbance of the resultant mixture solutions (AgNPs) was noted by UV-Vis spectrophotometer.

2.10.3. Time

The AgNPs synthesis procedure was repeated to optimize the time required for the reaction completion. For this purposes different reactions periods (10 min, 20 min, 30 min, 40 min, 50 min, 60 min and 70 min) were chosen. At the end the absorbance was observed by UV-Vis spectrophotometer.

2.10.4. Concentration of Silver Nitrate Solution

The AgNPs synthesis approach was repeated for optimization of silver nitrate concentration, where the reaction was monitored using 0.25, 0.5, 0.75, 1 and 2 mM of silver nitrate. At the end the absorbance of the resultant mixture solutions was calculated by UV-Vis spectrophotometer.

2.10.5. Leaf extract concentration and AgNO₃ ratio

The above described method was also repeated for optimization of leaf extract concentration and silver nitrate required for the AgNPs maximum synthesis, where the reaction was monitored by using different ratio of leaf extract and AgNO₃ solution (0.5:99.5, 1:99, 2.5:97.5, 5:95 and 10:90). The absorbance of the resultant mixture solutions was calculated by UV-Vis spectrophotometer.

2. 11. Biological Evaluation of Bioinspired Synthesis of AgNPs

2.11.1. Antibacterial activity of AgNPs

Took 0.01 g of silver nitrate and AgNPs were dissolved into 10ml of sterilized distilled water. 50 µl of sterile distilled water was used as negative and 50 µl Streptomycin (500 µg/ml) as positive control. The rest procedure was adopted as in 2.7.3.

2.11.2. MIC of AgNPs

The AgNO₃ and AgNPs were diluted independently to diverse concentrations ranging from 10 - 1000 µg/ml in distilled water. 500 µl of Streptomycin solutions (10 - 500 µg/ml) was used as positive control. The rest protocol was as in 2.7.4

2.11.3. MBC of AgNPs

The same procedures were applied as mentioned in 2.7.5.

2.11.4. Antifungal Activity of AgNPs

Fifty microliter (50 µl) of the 2 mg/ml each of the test material (silver nitrate and AgNPs) was pipetted into holes bored in the agar plates. Fifty microliter standard drug Bifonazole (2 mg/ ml) and sterile distilled water was use as positive and

negative control respectively. The same methodology was carried out as described in 2.7.7.

2.11.5. MIC of AgNPs

Different concentrations (20- 2000 $\mu\text{g/ml}$) of each tests material were made from the stock solution. The methodology of 2.7.8 was carried then.

2.11.6. MFC of AgNPs

The same experimental procedure as followed in 2.7.9.

2.11.7. Antioxidant Activity of AgNPs

Antioxidant activities of AgNPs were carried out according to the described procedure as mentioned in 2.7.10.

3.11.8. Brine Shrimp Cytotoxicity Assay of AgNPs

Took 20 mg of AgNPs and dissolve in 2 ml distilled water. Further the pre mentioned procedure in section 2.7.11 was followed.

2.11.9. In Vitro Phytotoxicity Assay of AgNPs

Silver nanoparticles were subjected for its phytotoxic activity according to the described protocol of 2.7.12.

2.11.10. Larvicidal Activity of AgNPs

The larvicidal activity test was carried out according to the procedure as described in section 2.7.13.

2.12. Statistical Analysis: The statistical analysis mean, standard deviation($\pm\text{SD}$), EC_{50} , LC_{50} , LC_{90} , LD_{50} , regression equations and Chi-square value (χ^2) values were carried out by using computer program SPSS.

Chapter -3

Results and Discussion

Results and Discussion

H. rhamnoides L. leaves were collected from PCSIR Skardu, dried and grinded into powder. These powders were analyzed for physiochemical, minerals, phytochemicals and biological parameters. The aqueous extract was utilized for the bioinspired synthesis of AgNPs using silver nitrate as precursor. The resultant AgNPs were characterized by UV-visible spectroscopy, FTIR, SEM, EDX, XRD and TGA/DTA techniques. Different parameters were optimized for the enhanced preparation of AgNPs and also biological evaluations were carried out.

3.1. Physiochemical Analysis of *H. rhamnoides* L. Leaves

Table 1 revealed the physicochemical analysis of *H. rhamnoides* L. leaves showing moisture contents $8\pm 0.1\%$, ash $7\pm 0.3\%$, fat $5.8\pm 0.4\%$, pH 4.0, total soluble solid ($^{\circ}\text{Brix}$) 1 ± 0.25 , acidity $0.6\pm 0.17\%$, fibers $18\pm 2.14\%$, total sugar $0.8\pm 0.01\%$, reducing sugar $0.7\pm 0.01\%$ and non-reducing sugar $0.1\pm 0.01\%$. Nitrogen contents $1.82\pm 0.24\%$ were recorded, while protein $10.45\pm 0.88\%$ and carbohydrate $68.75\pm 0.1\%$.

A normal human diet consists of water, vitamins, fats, proteins, carbohydrates and minerals. Human health always depend on balanced minerals contents in the body. Balanced mineral diet play an important role in human health, any disturbance in balance (below or above the limits) results in the abnormalities of human health [87]. Hippophae leaves [88] contained significant amounts of proteins (20.7%), amino acids (0.73% lysine, 0.13% methionine & cystine) and minerals (Ca, Mg and K). Seabuckthorn seed showed [4] that ash, moisture and pH values were high and titratable acidity was low as compared to the current study. Glucose and fructose [88] have the large components present in berry juice from 0.6 to 24.2 g/100 ml.

H. rhamnoides L. [89] berries, pulp and seed moisture content was high as compared leaves (Table 1). The difference of moisture contents was blamed to the weather and origin variation. The chemical analysis of *H. rhamnoides* L. pulp revealed that ash, fat, fiber and protein content were low, while moisture, total acidity and total sugar were high as compared to the current study [90]. The moisture and total sugar content in pulp of *H. rhamnoides* L. were high as compared to the current study [91].

The vitamins, carbohydrate, amino acids, protein and organic acids were found abundantly in fruit of *H. rhamnoides* L. [92]. The protein content [93] in *H. rhamnoides* L. *Subsp. sinensis* was 17.1 and 16.2 g protein 100/g dried leaf. Based on the investigation it was concluded that harvesting of *H. rhamnoides* leaves start from end of July to start of August in view of the fact that protein leaves were highest during this period and in the mid august significant decline was started. *H. rhamnoides* fresh leaves [94] moisture content was high, but when dried at 50°C it was 8.2%, which was a close agreement to our findings. Seabuckthorn berries [95] TSS and acidity were high and pH was low as compared to our study. The nitrogen content in pulp of seabuckthorn was low, but in seed was high as compared to the present study [96]. The whole berries, pulp and seed of *H. rhamnoides* L. [97] reported that ash, acidity and total sugar content were high, while as content was low as compared to the present study. *H. rhamnoides* L. different fruit cultivars were found total sugar and reducing sugar was high as compared to the present study [98]. *H. rhamnoides* L. fruit pulp of different localities [96] were account that moisture, TSS, acidity, reducing sugar, fat and total protein was high, while fiber and carbohydrate were low as compared to the current study.

Table.1. Physiochemical Analysis of *H. rhamnoides* L. Leaves.

S#	Analysis parameters	Results
1	Moisture (%)	8±01
2	Ash (%)	7±0.3
3	Fat (%)	5.8±0.4
4	pH	4±0.2
5	TSS (°Brix)	1±0.25
6	Acidity (%)	0.6±0.17
7	Fiber (%)	18±2.14
8	Total sugar (%)	0.8±0.01
9	Reducing Sugar (%)	0.7±0.01
10	Non-Reducing Sugar (%)	0.1±0.01
11	Nitrogen (%)	1.82±0.24
12	Protein (%)	10.45±0.88
13	Carbohydrates (%)	68.75±01

Average Results of three replicates, ± Standard deviation

3.2. Minerals Analysis of *H. rhamnoides* L. Leaves

Minerals analysis of *H. rhamnoides* L. leaves is shown in Fig.1. Sodium was 30000 ± 01 ppm and the concentration of calcium was determined 7800 ± 01 ppm. Potassium contents were recorded 6200 ± 02 ppm followed by magnesium 1170 ± 01 ppm. The iron quantity 380 ± 02 ppm was noted in the *H. rhamnoides* L. leaves. While the concentration of Mn was observed 80 ± 01 ppm whereas the amount of silicon and aluminium were determined 60 ± 01 ppm and 50 ± 01 ppm respectively. The concentration of chromium was found 10 ± 0 ppm followed by zinc 8 ± 01 ppm. The *H. rhamnoides* L. leaves were also evaluated for barium, lead, cadmium and nickel were found below detection limit.

Seabuckthorn seeds were reported [4] that Ca, Mg, Fe, K and Na quantity were low, while Zn was high as compared to our findings. Variation may originate from the natural content of minerals in the earth, air contamination, soil contamination, geographical position, dissimilar procedure and instrument utilized for analysis. The seeds and fruits of *H. rhamnoides* [89] observed that potassium content was high as compared to the other analyzed minerals. In the pulp it diverse among 10.12 and 14.84 ppm and in seed it vary among 9.33 and 13.42 ppm. The minerals constituent (mg/l) in seabuckthorn pulp reported by [91] that Ca was 176.6, Fe was 30.9, Mg was 22.5, P was 84.2, K was 647.2, Na was 414.9, Zn was 1.4, Cu was 0.7 and Mn was 1.1. It was reported [95] that *H. rhamnoides* berries content of K, Mg and Zn were found high and Ca, Mn and Fe were found low as compared to the current study. *H. rhamnoides* pulp and seed of different localities revealed [96] that Fe, Mg, Zn and Na were low; however K was high as compared to the present investigation.

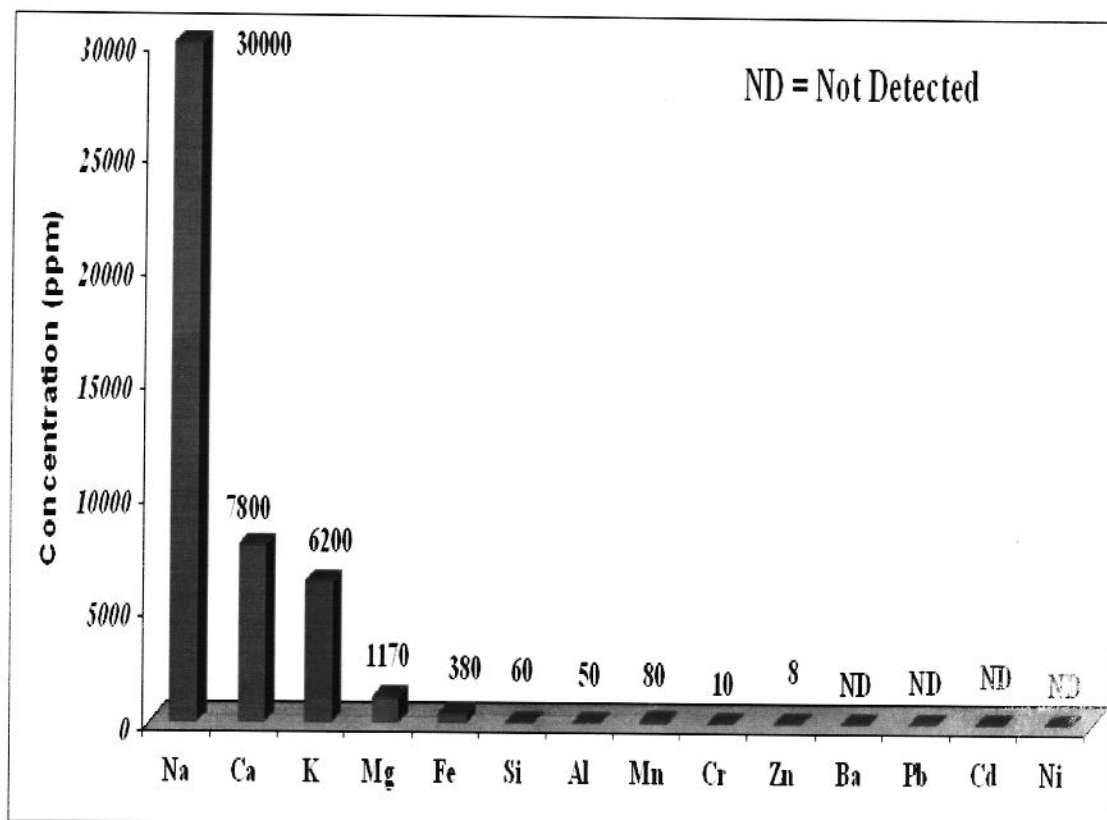


Fig.1. Minerals Analysis of *H. rhamnoides* L. Leaves.

3.3. Extractive Values and Phytochemicals Contents of *H. rhamnoides* L. Leaves Extracts

The *H. rhamnoides* L. leaves extraction values are shown in Fig.2. The % extract value for different solvents was: Aqueous (8.74 ± 01), *n*-hexane (1.50 ± 01), ethanol (8.83 ± 01), chloroform (5.41 ± 01), methanol (9.47 ± 01), ethyl acetate (2.38 ± 01) and acetone (22.95 ± 01).

Table 2 shows that terpenoids was not detected in the aqueous extract of *H. rhamnoides* L. leaves while average contents (++) were detected in the methanolic extract. Ethanolic extract showed small quantity (+) terpenoids and average amount was determined in the ethyl acetate extract. Low amount of terpenoids was found in acetone extract whereas average quantity or terpenoids was recorded in both chloroform and *n*-hexane extracts of *H. rhamnoides* L. leaves. Small amounts of steroids were present in all extracts (aqueous, methanol, ethanol, ethyl acetate, acetone, chloroform and *n*-hexane) of *H. rhamnoides* L. leaves, while glycosides were not detected in aqueous, ethyl acetate, chloroform and *n*-hexane extracts. Glycosides were found in methanolic, ethanolic and acetone extract of *H. rhamnoides* L. with less quantity. The alkaloids were not detected in the aqueous extract of *H. rhamnoides* L. leaves whereas less content of alkaloids were observed in the methanolic, ethanolic, ethyl acetate, acetone, chloroform and *n*-hexane extract of *H. rhamnoides* L. leaves. The phenolic contents were found in large quantity (+++) in the aqueous, methanolic and ethanolic extracts followed by average quantity in ethyl acetate, acetone and chloroform extracts. Phenols contents were not detected in the *n*-hexane extract. The contents of flavonoids were present in all the extracts of *H.*

ramnoides L. leaves except *n*-hexane. High amounts of flavonoids were observed in aqueous, methanolic and ethanolic extracts, while average concentration was recorded in both ethyl acetate and acetone extracts followed by less concentration in chloroform extract. Flavones were absent in the ethyl acetate, acetone, chloroform and *n*-hexane extracts and present in high amount in the methanolic extract, while average amounts were recorded in extracts of ethanol and aqueous. The tannins contents were found maximum in methanol, ethanol and ethyl acetate extracts. While the average amounts were found in aqueous, acetone and chloroform extracts and found positive for less quantity of tannins in the *n*-hexane extract. Saponins were not detected in all the extract of *H. ramnoides* L. leaves including aqueous, methanolic, ethanolic, ethyl acetate, acetone, chloroform and *n*-hexane extracts. Amino acids were not detected in the *n*-hexane extract, while less amount of amino acids were present in the ethyl acetate, acetone and chloroform extracts followed by average contents in aqueous, methanolic and ethanolic extracts.

Hippophae leaves [88] contain folic acid, catechins, esterified sterols, triterpenols and isoprenols, while juice contains 1182 mg/l flavonoids. The flavonoid in seabuckthorn leaves [92] were calculated in the range of 0.310 to 2.100 g/100g, in fruits was found 0.120 to 1.000 g/100 g and dry matter in fruits was 24.6% to 33.8%. The flower, stem, leaves, root and fruits of *H. ramnoides* L. [99] contained flavonoid compounds. Five flavonoids reported [100] in the ethanol extract of *H. ramnoides* L. leaves i.e. catechin (0.04%), rutin (0.30%), quercetin(0.49%), kaempferol (0.37%) and isorhamnetin (0.42%). The study [5] documented the % extractive values of *H. ramnoides* (twigs) as 4.647 and 7.02 for chloroform/methanol (1:1) and methanol

respectively. The methanol and chloroform: methanol extract of *H. rhamnoides* (twigs) revealed the presence of glycoside, terpenoids, steroids, flavonoids, reducing sugars and tannins, while lacking alkaloids and saponins. The methanol extract of leaves, seed and pomace of seabuckthorn [101] were reported to have total phenolic content (mg GAE/g extract) 278.80, 162.56 and 107.01 respectively. Similarly aqueous extract exhibited phenolic content (mg GAE/g extract) 184.89, 109.57 and 87.35 respectively.

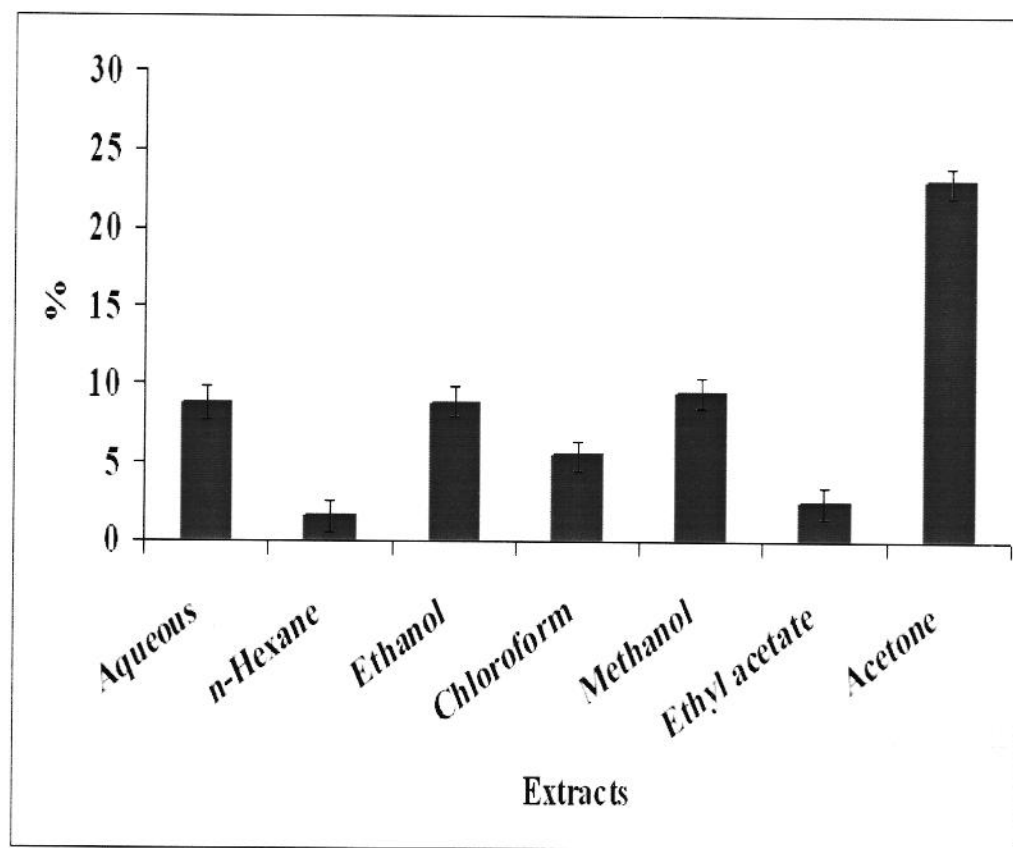


Fig. 2. Extraction values of *H. rhamnoides* L. Leaves Extracts.

Table 2. Phytochemical Analysis of *H. rhamnoides* L. Leaves Extracts.

Chemical constituents	Aqueous	Methanol	Ethanol	Ethyl Acetate	Acetone	Chloroform	<i>n</i> -hexane
Terpenoids	ND	++	+	++	+	++	++
Steroids	+	+	+	+	+	+	+
Glycosides	ND	+	+	ND	+	ND	ND
Alkaloids	ND	+	+	+	+	+	+
Phenols	+++	+++	+++	++	++	++	ND
Flavonoids	+++	+++	+++	++	++	+	ND
Flavones	++	+++	++	ND	ND	ND	ND
Tannins	++	+++	+++	+++	++	++	+
Saponins	ND	ND	ND	ND	ND	ND	ND
Amino Acids	++	++	++	+	+	+	ND

Note: + sign shows detection level of the phytochemicals present in extracts. + = Small quantity, ++ = Average quantity, +++ = Large quantity, ND = Not detected.

3.4. FTIR Analysis of *H. rhamnoides* L. Leaves Extracts

The FTIR spectra analysis was utilized to identify the functional group of the active ingredients on the basis of peak value in the vicinity of infrared radiation. The results of FTIR peak values and functional groups of *H. rhamnoides* L. aqueous extract are presented in (Fig. 3). IR-spectrum shows strong absorption peaks at 3242.34 (broad), 2929.87 (small short) and 1593.20 cm^{-1} (sharp) which correspond to the presence of hydroxyl (OH), alkane (C-H) and aromatic rings (C=O) functional groups respectively. *H. rhamnoides* L. methanol extract (Fig. 4) showed that peaks values 2922.16 cm^{-1} and 2852.72 cm^{-1} are attributed to the existence of functional groups (alkanes), while 1693.50 and 1600.92 cm^{-1} are carboxylic and amides respectively. Ethanol extracts (Fig. 5) shows the broad peaks values (3315.63 cm^{-1}) of hydroxyl group (OH), sharp and strong peak (2924.09 cm^{-1}) of alkane, small peak (2854.65 cm^{-1}) for alkane, small and weak peaks of alkene (1735.93 and 1604.77 cm^{-1}). Ethyl acetate extract peak (cm^{-1}) data are shown in Fig. 6 and confirm the presence of hydroxyl, alkane and carboxyl group. Acetone extract FTIR spectra Fig. 7 peaks values were 3350.35 cm^{-1} (OH group), 2924.09 cm^{-1} (alkane stretch), 2852.72 cm^{-1} (alkane) and C=O group 1703.14, 1693.50 cm^{-1} . Four considerable peaks were observed in Chloroform (Fig. 8) and found as an OH, alkane and (C=O) functional groups. Two sharp and strong peaks (2922.16 and 2850.79 cm^{-1}) attributed the existence of alkane group in *n*-hexane extract (Fig. 9) extract and another two peaks values were seen in the region of 3398.57 and 1716.65 cm^{-1} were OH and C=O groups.

FTIR analysis of the *H. rhamnoides* L. leaves extracts strongly represent hydroxyl group which was common in all phenolic compounds. All *H. rhamnoides* L. extracts bands of absorption were credited to (OH) vibrations stretching as of phenols, a cluster of compounds having hydroxyl functional groups (-OH) joint with hydrocarbon of aromatic nature. Antifungal activity was recognized by natural derived sources of phenolic compounds [102]. The amount and site(s) location of OH exhibited on the phenols were connected toward microbes toxicity through confirmation so as to toxicity was increases when hydroxylation increased [103]. Similarly carboxylic acids were found to be connected through numerous antifungal and antibacterial activities which were found to exhibit within different molecular structures of plant metabolite e.g. ursolic acid as reported to be a powerful agent against different bacteria [104]. Many active compounds were produced by plants which contained these bioactive compounds (secondary metabolites). The extracts other chemical compounds as well surely might add, although deficient in the chemical profile has in no way been documented on this. Probably that these bioactive components were primarily accountable for the antimicrobial activities pragmatic in the current investigation.

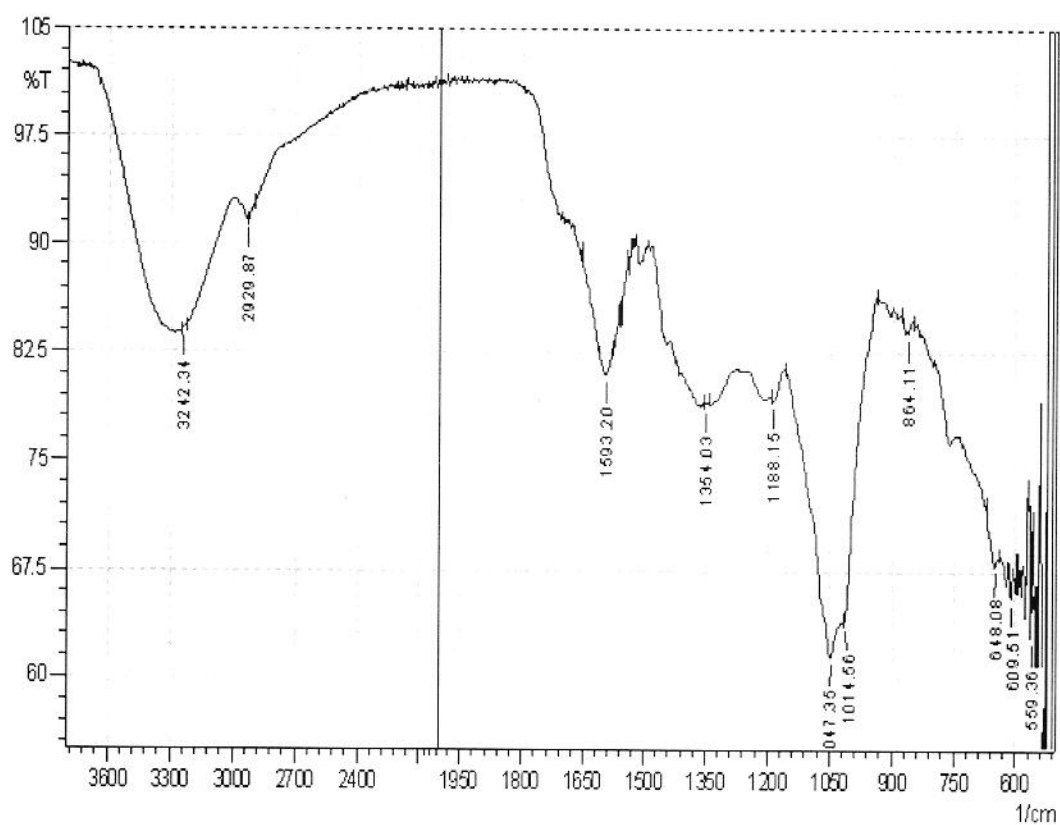


Fig. 3. FTIR Spectra of Aqueous Extract of *H. rhamnoides* L. Leaves.

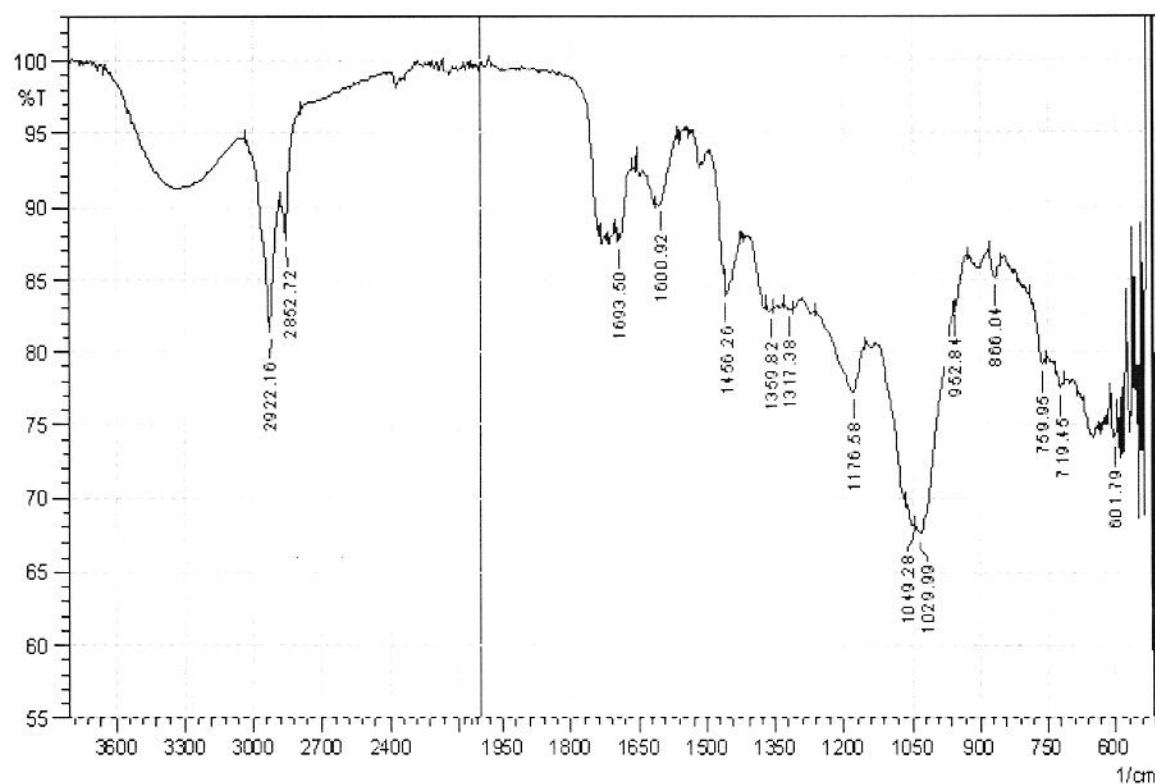


Fig. 4. FTIR Spectra of Methanol Extract of *H. rhamnoides* L. Leaves.

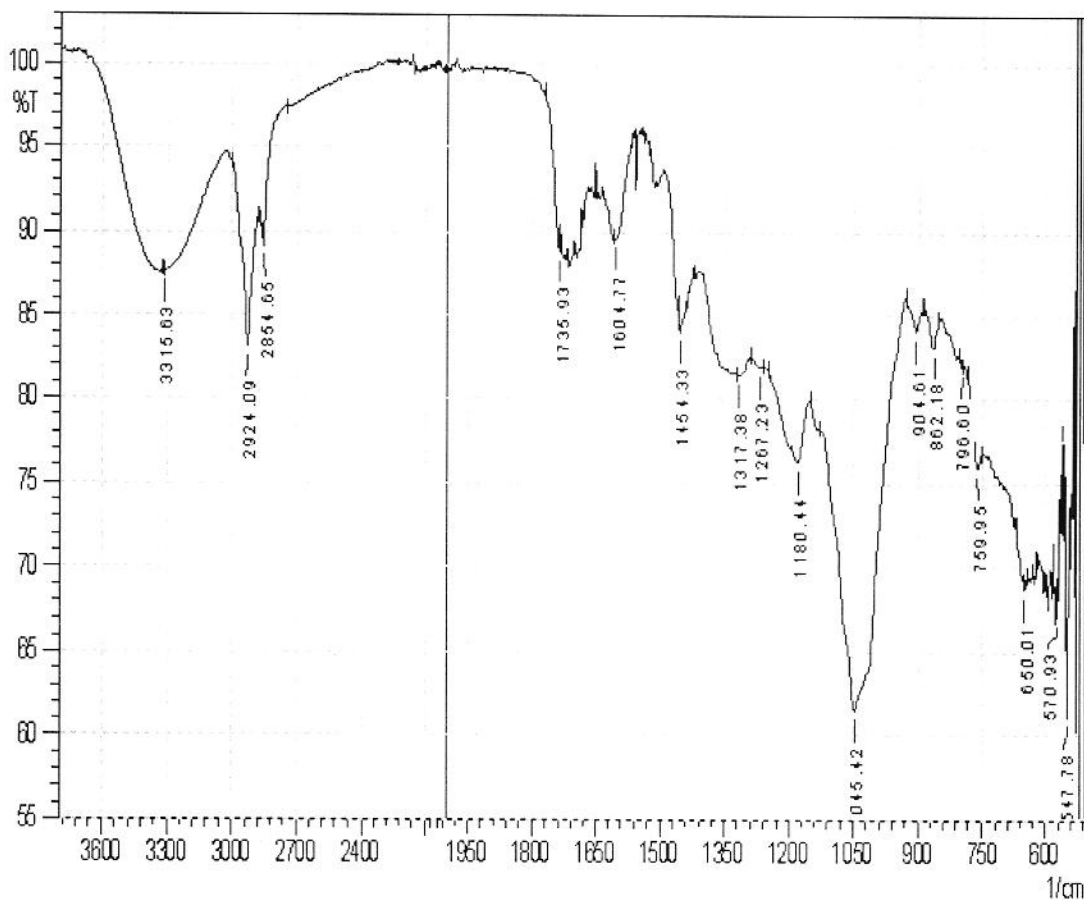


Fig. 5. FTIR Spectra of Ethanol Extract of *H. rhamnoides* L. Leaves.

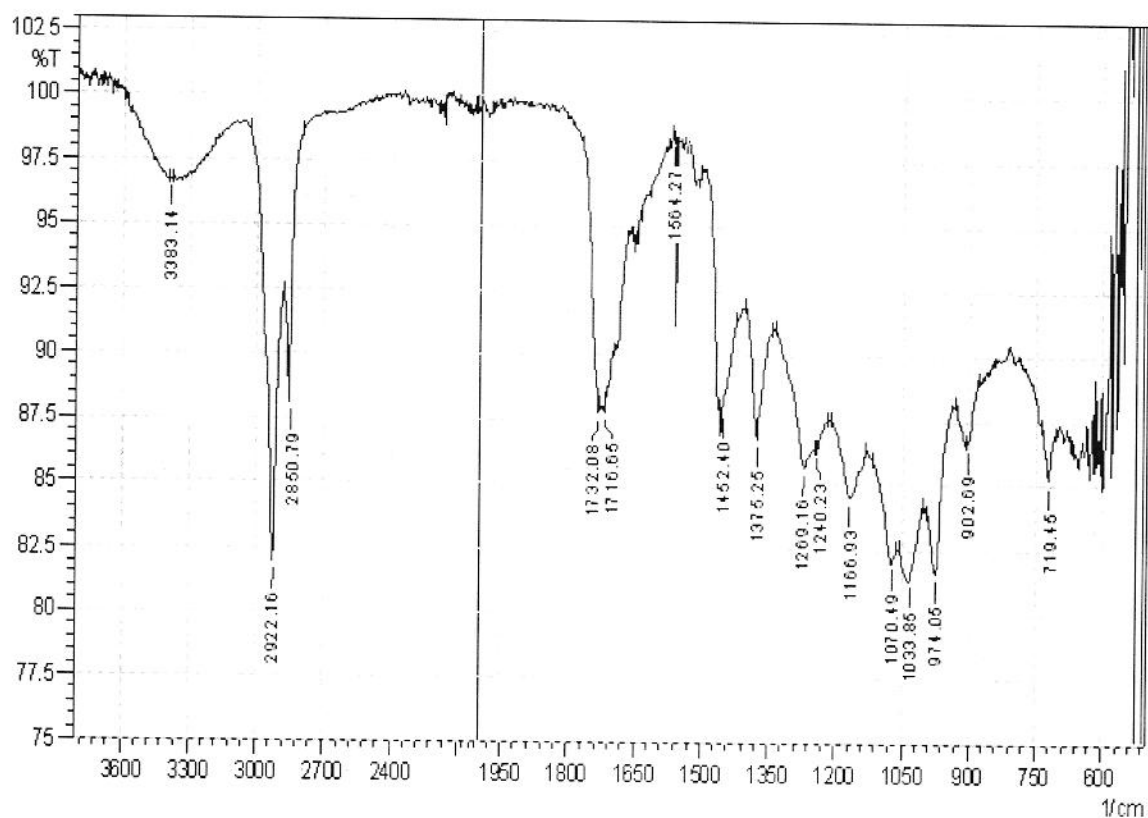


Fig. 6. FTIR Spectra of Ethyl Acetate Extract of *H. rhamnoides* L. Leaves.

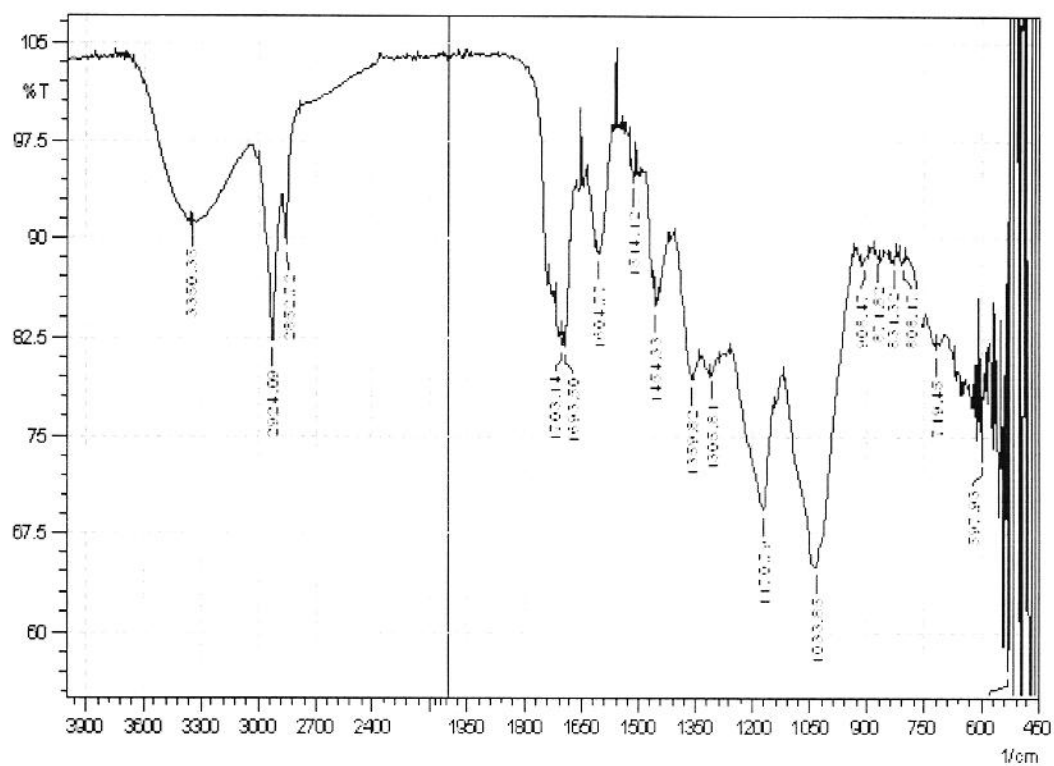


Fig. 7 .FTIR Spectra of Acetone Extract of *H. rhamnoides* L. Leaves.

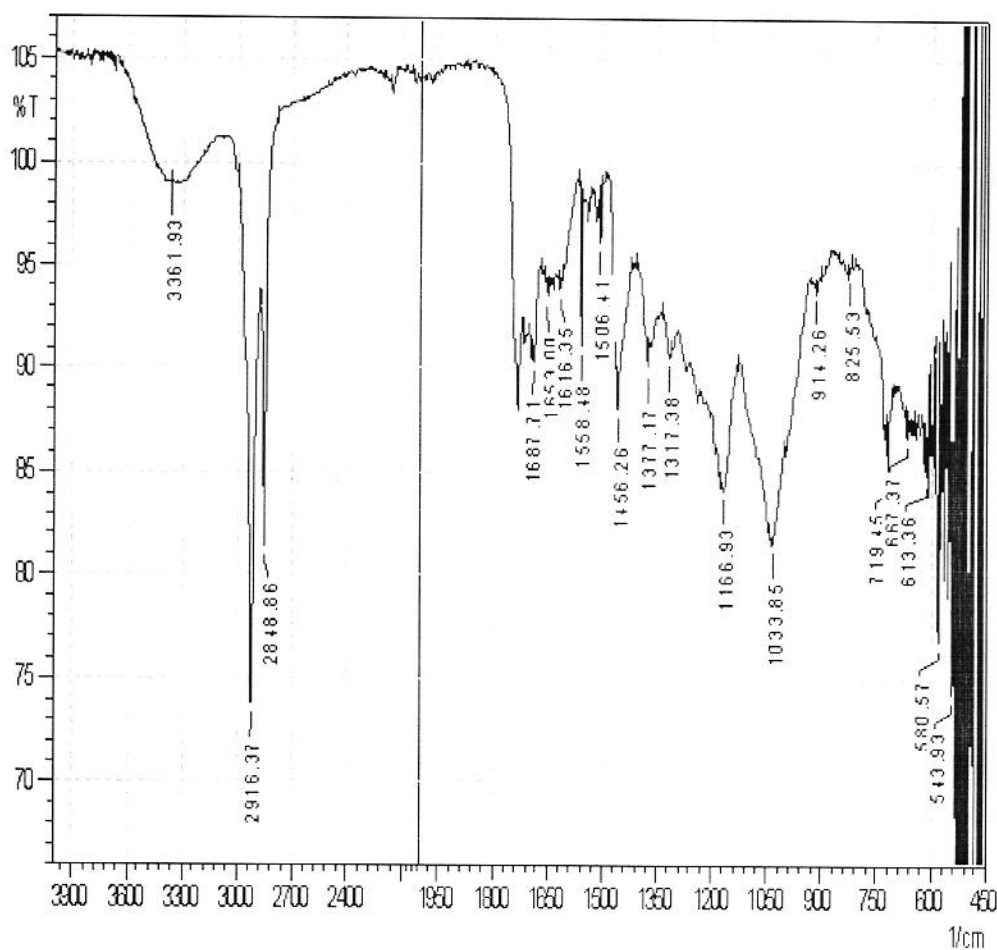


Fig. 8. FTIR Spectra of Chloroform Extract of *H. rhamnoides* L. Leaves.

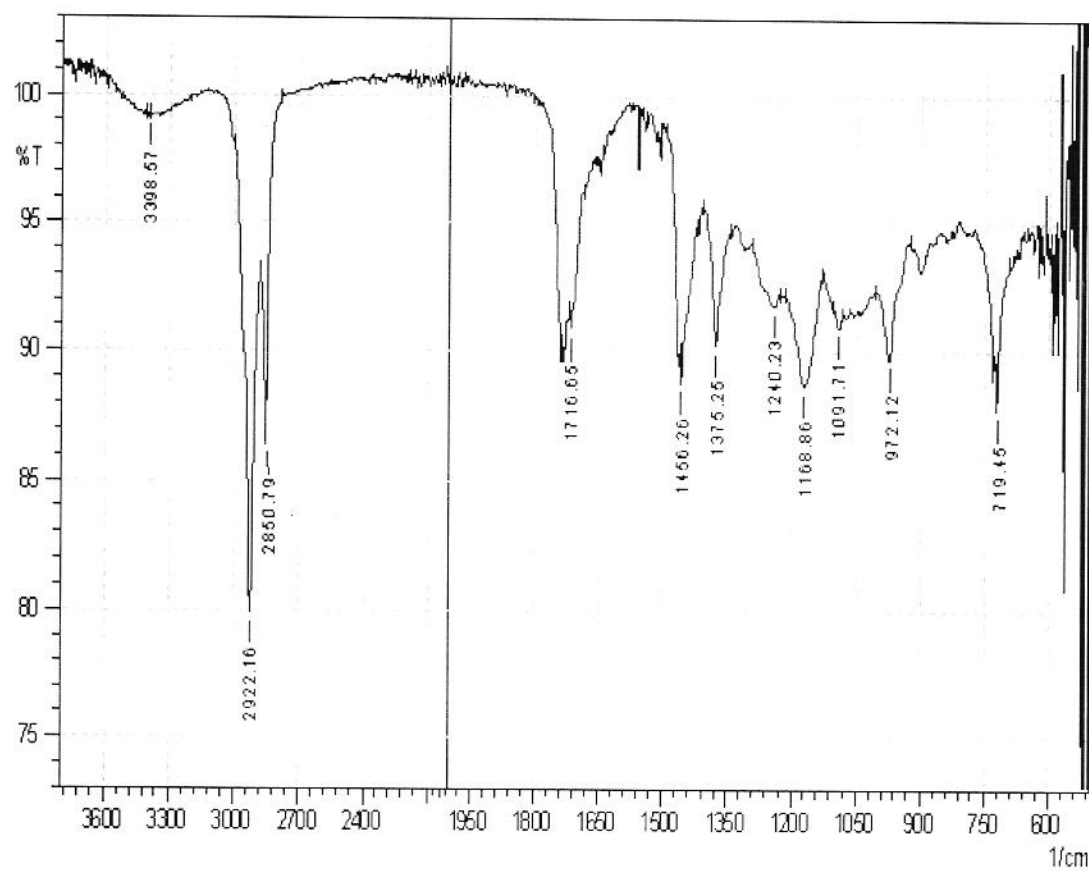


Fig. 9. FTIR Spectra of *n*-hexane Extract of *H. rhamnoides* L. Leaves.

3.5. Antibacterial Activity of *H. rhamnoides* L. Leaves Extracts

The result of antibacterial activity of *H. rhamnoides* L. leaves extracts is presented in table 3. The aqueous extract was found highly effective against *S. aureus* and the zone of inhibition (ZI) was 21 ± 1 mm, while 20 ± 2 mm was noted against *E. coli*. Zone of inhibition 15 ± 1.7 mm was observed against *E. faecalis* followed by 14 ± 1 mm against both *K. pneumoniae*, *P. aeruginosa* and *B. cereus* and 13 ± 0 mm was against *V. cholerae*. *Salmonella typhi* was found more resistant than all tested bacteria with minimum 11 ± 0 mm of the aqueous extract. The second least 12 ± 1 mm of the aqueous extract was found against *B. subtilis* followed by 13 ± 1 mm against *C. freundii*. The methanol extracts showed high activity (21 ± 1 mm) against *V. cholerae*, while 19 ± 1 mm against *E. coli*. The 17 ± 2 mm was noted against *K. Pneumoniae* and 16 ± 1 mm against both *B. cereus* and *C. freundii*. The methanolic extract showed (14 ± 1.7 mm) against *E. faecalis* and *B. subtilis* (14 ± 0 mm) followed by 11 ± 1.7 mm against *P. aeruginosa*, while the least 9 ± 0 mm was determined against *S. typhi*. The ethanolic extract was found more active against *P. aeruginosa* (20 ± 1 mm) where 16 ± 0 mm, 17 ± 1 mm and 18 ± 1 mm were observed against *E. faecalis*, *K. pneumoniae* and *E. coli* respectively. Similarly 15 ± 0 mm was recorded against *B. subtilis*, 15 ± 1 mm against *C. freundii*, 13 ± 0 mm against *S. aureus* and 13 ± 1 mm against *B. cereus*. The *S. typhi* was found more resistance against ethanolic extract having the least 8 ± 0 mm ZI. The ethyl acetate extract of *H. rhamnoides* L. leaves were found less effective against all the test bacterial strains as compared to aqueous, methanol and ethanol extracts. The highest activity 15 ± 1 mm was recorded against *P. aeruginosa* and *B. cereus*, while 13 ± 1 mm against *E. coli*. 12 ± 1 mm against *S. aureus* and *K. pneumoniae*. While 10 ± 0

mm against *S. typhi* and 10 ± 1 mm against *C. Freundii* followed by 9 ± 0 mm and 7 ± 0 mm against *B. subtilis* and *V. cholerae*. The acetone extract was found more active against *P. aeruginosa* with 18 ± 0 mm, while the other values were in the range of 10 - 17 mm. The chloroform extract showed high activity 16 ± 0 mm against *E. coli* while 11 ± 0 mm against *S. aureus*, *K. pneumonia* and *C. freundii*. The least activity 8 ± 0 mm was against *P. aeruginosa* whereas *n*-hexane extract of *H. rhamnoides* leaves was found inactive against all the tested bacteria. The negative control (DMSO) gave no zone of inhibition against all the tested bacteria. While the positive control (Ciproxin) showed 28 ± 1 mm, 26 ± 1 mm, 24 ± 0 mm, 25 ± 1 mm, 23 ± 0 mm, 24 ± 0 mm, 22 ± 0 mm, 25 ± 1 mm, 23 ± 0 mm and 20 ± 1 mm zone of inhibition against *S. aureus*, *E. coli*, *E. faecalis*, *K. pneumoniae*, *P. aeruginosa*, *V. cholerae*, *S. typhi*, *B. cereus*, *B. subtilis* and *C. freundii* respectively.

The *H. rhamnoides* L. aqueous and methanol extracts of seed, pomace and leaves showed low ZI against *B. cereus*, *E. faecalis*, *S. aureus*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* as compared to our study [101]. The antibacterial activity of *H. salicifolia* D. Don reported [105] that seed extract showed high activity as compared to leaf extract against gram-positive bacteria. Whereas, no activity was observed against all test extracts in gram-negative bacteria except in case of *A. tumefaciens* some activity was obtained against seed extract. While Kanamycin (1000 $\mu\text{g/ml}$) exhibited 16 – 23 mm zone of inhibition.

Table 3. Antibacterial Activity of *H. rhamnoides* L. Leaves Extracts.

Bacteria	Zone of Inhibition (mm)								
	Extracts							Control	
	Aq	Meth	Eth	Eth Act	Acet	Chl	n-Hex	C ⁺	C ⁻
<i>S. aureus</i>	21±1	14±1.7	13±0	12±1	11±1	11±0	0.0	28±1	0.0
<i>E. coli</i>	20±2	19±1	18±1	13±1	10±0	16±0	0.0	26±1	0.0
<i>E. faecalis</i>	15±1.7	14±1.7	16±0	08±0	16±1	13±0	0.0	24±0	0.0
<i>K. pneumoniae</i>	14±1	17±2	17±1	12±1	13±0	11±0	0.0	25±1	0.0
<i>P. aeruginosa</i>	14±1	11±1.7	20±1	15±1	18±0	08±0	0.0	23±0	0.0
<i>V. cholerae</i>	13±0	21±1	14±0	07±0	16±0	09±1	0.0	24±0	0.0
<i>S. typhi</i>	11±0	09±0	08±0	10±0	17±0	10±1	0.0	22±0	0.0
<i>B. cereus</i>	14±1	16±1	13±1	15±1	11±0	13±1	0.0	25±1	0.0
<i>B. subtilis</i>	12±1	14±0	15±0	09±0	12±1	12±0	0.0	23±0	0.0
<i>C. freundii</i>	13±1	16±1	15±1	10±1	16±0	11±0	0.0	20±1	0.0

C⁺ = Positive Control (Ciproxin 0.5mg/ml), C⁻ = Negative Control (DMSO), Each value represents mean ± SD (n = 3), 0 = No zone of inhibition.

3.6. MIC and MBC of *H. rhamnoides* L. Leaves Extracts

As can be seen from Fig. 10 and 11, the MIC values were found less than MBC while in some cases the MIC and MBC values were equal. The aqueous extract of *H. rhamnoides* L. leaves showed that activity against *S. aureus* having MIC and MBC values (40 mg/ml and 60 mg/ml) followed by *E.coli* (65 mg/ml and 75 mg/ml), *E. faecalis* (70mg/ml and 80 mg/ml), *K. pneumoniae* (80 mg/ml and 90 mg/ml), *P. aeruginosa* (80 mg/ml and 85 mg/ml), *V. cholerae* (90 mg/ml and 95 mg/ml), *S. typhi* (90 mg/ml and 100 mg/ml), *B. cereus* (75 mg/ml and 80 mg/ml), *B. subtilis* (80 mg/ml and 90 mg/ml) and *C. freundii* (80 mg/ml and 110 mg/ml). The methanolic extract were found less active against *P. aeruginosa* having MIC (mg/ml) and MBC (mg/ml) values of 100 and 110 respectively, while showed high activity against *S. typhi* with values of (50 and 55) respectively. The other values were (60 and 80) against *S. aureus*, (60 and 80) against *E. faecalis*, (60 and 90) against *B. subtilis*, (55 and 70) against *E.coli*, (55 and 70) against *K. pneumoniae*, (55 and 70) against *B. cereus* and (50 and 70) against *C. freundii*. The activity of ethanolic extract against *E. coli* having MIC and MBC values of (40 mg/ml and 60 mg/ml), while the other MIC (mg/ml) and MBC (mg/ml) values against the test bacteria were in the range from (45 and 66 to 75 and 80). Ethyl acetate extract were MIC and MBC values against *E. coli* i.e. 120 mg/ml and 125 mg/ml, while activity 110 mg/ml and 130 mg/ml was determined against *V. cholerae*. Similarly other MIC (mg/ml) and MBC (mg/ml) values were (100 and 120) *E. faecalis*, (90 and 125) *C. freundii*, (80 and 120) *B. cereus*, (70 and 125) *P. aeruginosa*, (70 and 85) *B. subtilis*, (70 and 80) *S. aureus*, (65 and 75) *K. pneumoniae* and (65 and 75) against *S. typhi*. The acetone extract of *H.*

rhmnoides L. leaves was found highly active against *E. coli* with MIC (mg/ml) and MBC (mg/ml) values (60 and 90) followed by (60 mg/ml and 95 mg/ml) against *S. aureus*. The other MIC mg/ml values were in the range of 65 - 115 and MBC (mg/ml) values were 85 – 135 range against the test bacteria. The chloroform extract MIC was 70 mg/ml against *K. pneumoniae*, 85 mg/ml against *E.coli*, *P. aeruginosa*, *V. cholerae*, *S. typhi*, *B. cereus* and *C. Freundii* and 75 mg/ml against *E. faecalis* and 80 mg/ml against *S. aureus* and *B. subtilis*. Similarly the MBC was 125 mg/ml against *S. typhi*, 90 mg/ml against *E. coli*, *E. faecalis* and *K. pneumoniae*. While 95 mg/ml against *B. subtilis*, 100 mg/ml against *S. aureus*, *P. aeruginosa*, *B. cereus* and *C. Freundii*. The MBC was 110 mg/ml against *V. cholerae*. The positive control Ciproxin MIC (mg/ml) was found in the range 0.10 - 0.22 and MBC was found in the range of 0.10 – 0.25 against the test bacteria.

The MIC values of aqueous and methanol extracts of seed, pomace and leaves were found low as compared to our study [101]. The MIC of *H. rhmnoides* root and stem methanol extract against *B. subtilis*, *S. aureus* and *E. coli* were less as compared to the present study findings [3].

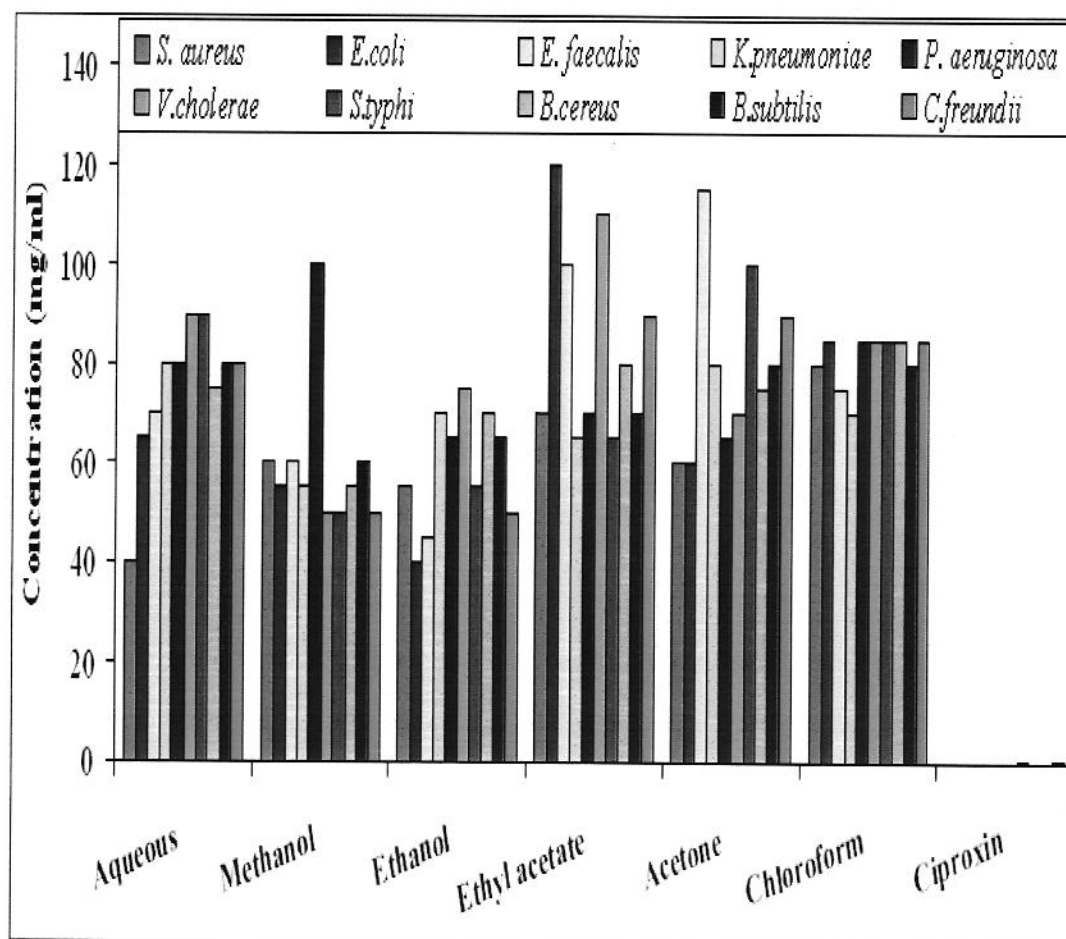


Fig. 10. Antibacterial MIC of *H. rhamnoides* L. Leaves Extracts.

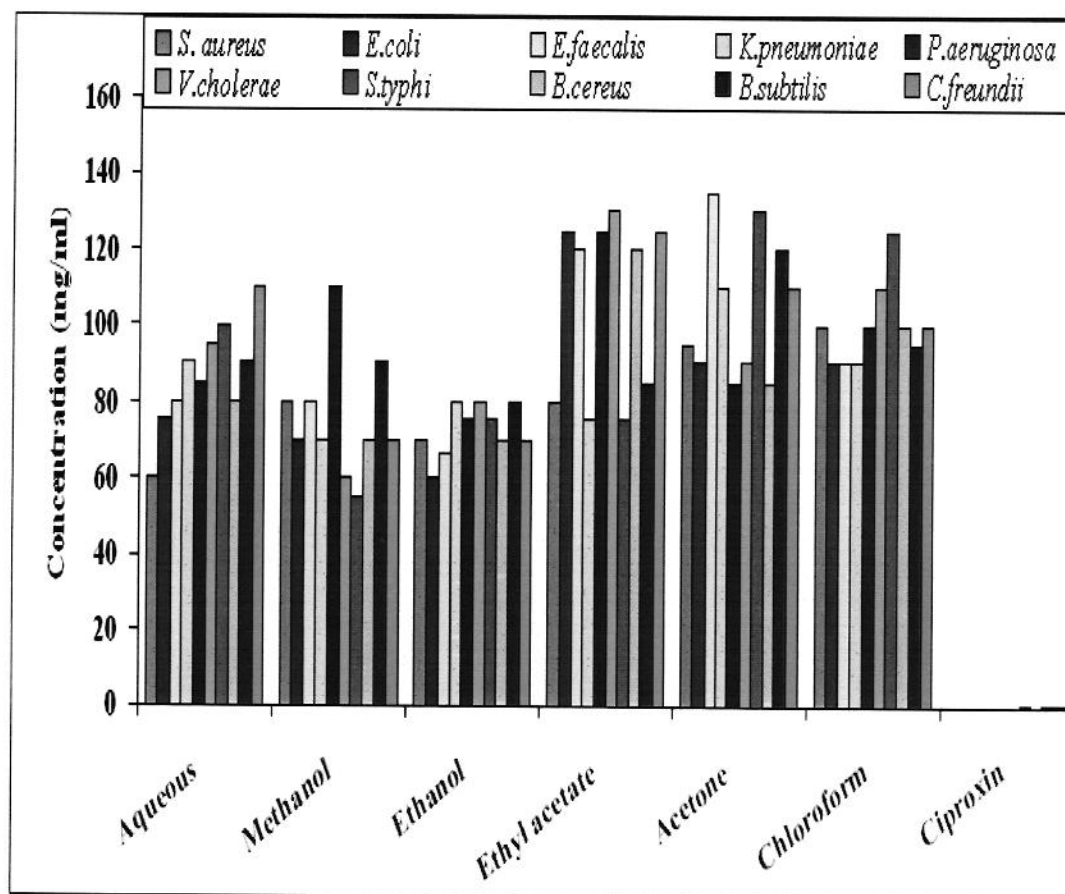


Fig. 11. Antibacterial MBC of *H. rhamnoides* L. Leaves Extracts.

3.7. Antifungal Activity of *H. rhamnoides* L. Leaves

Antifungal activity of *H. rhamnoides* L. leaves different extracts is shown in table 4. Antifungal activity of the aqueous extract was more with zone of inhibition (ZI) 16 ± 1 mm against *F. oxysporum* whereas the second highest 15 ± 0 mm was noted against *R. arrhizus*. Likewise 13 ± 1 mm against *A. niger* and *A. flavus*, while 12 ± 0 mm against *A. oryzae*, 12 ± 1 mm against *C. albicans* and 11 ± 1 mm against *A. alternata*. The minimum ZI 07 ± 0 mm against *P. digitatum*. Methanolic extract were found less active with 09 ± 1 mm ZI against *A. oryzae*, whereas the other values were in the range of 13 to 18 mm. Ethanolic extract were found less active than aqueous and methanolic extract having maximum 14 ± 1 mm against *A. niger* and *C. albicans*. While 10 mm against *A. flavus*, *A. alternata* and *P. digitatum* and the less activity 9 ± 0 mm against *C. albicans*. Ethyl acetate extract were found highly active (15 ± 0 mm) against *R. arrhizus* and 13 – 8 mm in the range against the other test fungal stains. All the fungus was found highly resistant against the acetone extract and the values were in the range of 8 and 12 mm. The chloroform extract of *H. rhamnoides* L. leaves showed nearly the same activity as of acetone. The maximum activity 12 ± 0 mm were recorded against *A. niger* and *P. digitatum* and minimum 07 ± 1 mm was observed against *A. alternata* whereas *n*-hexane extract were found inactive against all the tested fungal strains. The positive control (fluconazole) showed high activity against all the tested fungi, with maximum 24 ± 0 mm against *A. oryzae* and minimum 16 ± 0 against *R. arrhizus*. The negative control DMSO showed no activity against all the test fungi.

3.8. MIC and MFC of *H. rhamnoides* L. Leaves Extracts

The MIC and MFC values of *H. rhamnoides* L. leaves extracts against test fungi are presented in figure 12 and 13. The aqueous extract was found highly active against *F. oxysporum* with MIC and MFC values 50 mg/ml and 65 mg/ml respectively. While *A. niger* and *A. oryzae* with the same MIC and MFC values 60 mg/ml and 75 mg/ml respectively, whereas *A. flavus* showed high resistance against the aqueous extract having MIC and MFC value (120 mg/ml and 145 mg/ml). The other MIC (mg/ml) values were in between 65 – 110 and MFC (mg/ml) values in the range 75 – 130 against different fungi. The methanolic extract was found less effective against *A. oryzae* with MIC and MFC values (90 mg/ml and 135 mg/ml). The ethanolic extracts were found less effective against all the test fungi as compared to aqueous and methanolic extract. The activity was recorded against *P. digitatum* having MIC and MFC values (80 and 90). The other MIC range values were 80 – 130 and MFC range values were 95 – 160. The ethyl acetate extract showed MIC and MFC values against *C. albicans* (120 mg/ml and 180 mg/ml) while found more active against *A. fumigates* having MIC and MFC values (80 mg/ml and 90 mg/ml). The effect of ethyl acetate extract showed that MIC 80 mg/ml and MFC 90 mg/ml against *F. oxysporum* respectively, whereas the MIC and MFC values were observed 120 mg/ml and 180 mg/ml against *C. albicans* respectively. The acetone extract highest MIC and MFC values (120 and 145) were detected against *A. niger*, while MIC and MFC values was 85 mg/ml and 100 mg/ml against *F. oxysporum* and *C. albicans* respectively. The chloroform extract MIC 135 mg/ml and MFC 150 mg/ml against *A. niger*. The MIC (mg/ml) range was 80 – 130 and MFC (mg/ml) was 100 – 150 for the rest of fungal

strains. The positive control fluconazole calculated MIC (mg/ml) values in the range of 0.25 – 0.50 and MFC (mg/ml) values in the range 0.30 – 0.50 against all the tested fungi.

Hippophae salicifolia D. Don seed extract observed considerable activity against *Tilletia indica* and *Mucor indicus*, while in case of *R. oryzae* no activity was showed against all tested extracts [105]. The reported MIC (μ g/ml) of root and stem methanolic extract against *C. albicans* were 125 and 62 respectively, while Ketoconazole MIC was 250 and mycostantin MIC was 500 for *C. albicans* [3].

Table 4. Antifungal Activity of *H. rhamnoides* L. Leaves Extracts.

Fungi	Zone of Inhibition (mm)								
	Extracts							Control	
	Aq	Meth	Eth	Eth Act	Acet	Chl	n-Hex	C ⁻	C ⁺
<i>A. niger</i>	13±1	15±1	14±1	10±1	08±0	12±0	0.0	0.0	18±0
<i>A. parasiticus</i>	14±0	17±1	12±1	09±0	09±0	11±1	0.0	0.0	22±0
<i>A. flavus</i>	13±1	16±1	10±1	08±1	10±0	09±0	0.0	0.0	20±0
<i>A. fumigatus</i>	09±0	13±0	11±0	11±1	09±0	08±1	0.0	0.0	23±0
<i>A. oryzae</i>	12±0	09±1	11±1	12±0	08±0	09±1	0.0	0.0	24±0
<i>F. oxysporum</i>	16±1	13±0	13±0	13±1	12±1	10±0	0.0	0.0	22±0
<i>R. arrhizus</i>	15±0	14±0	09±0	15±0	11±0	8±0	0.0	0.0	16±0
<i>A. alternata</i>	11±1	18±0	10±0	11±1	09±0	07±1	0.0	0.0	19±0
<i>C. albicans</i>	12±1	14±1	14±1	09±0	10±1	10±1	0.0	0.0	17±0
<i>P. digitatum</i>	07±0	13±1	10±0	08±0	09±1	12±0	0.0	0.0	21±0

C⁺ = Positive Control (Fluconazole 0.5mg/ml), C⁻ = Negative Control (DMSO), Each value represents mean ± SD (n = 3). 0 = No zone of inhibition.

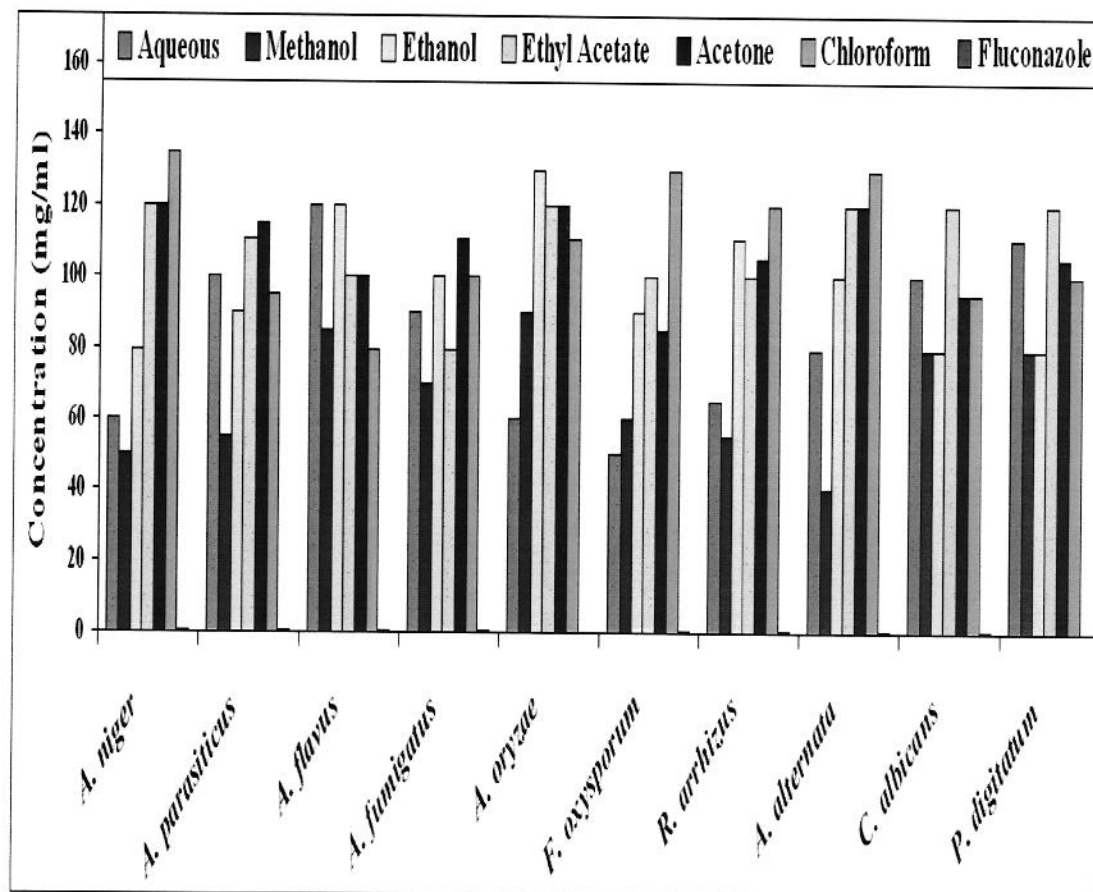


Fig. 12. Antifungal MIC of *H. rhamnoides* L. Leaves Extracts.

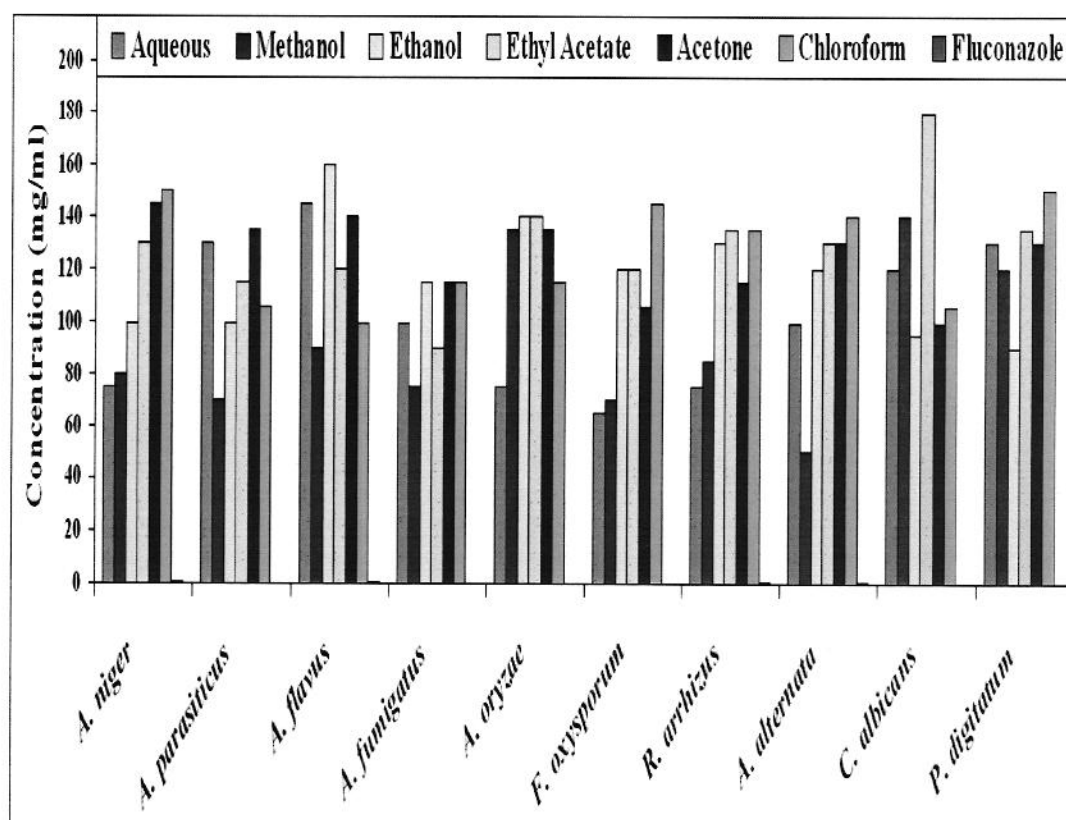


Fig.13. Antifungal MFC of *H. rhamnoides* L. Leaves Extracts.

3.9. Antioxidant Activity of *H. rhamnoides* L. Leaves Extracts

H. rhamnoides L. leaves extracts DPPH radical scavenging activity is presented in Figure 14 and showed a concentration dependent activity in comparison with ascorbic acid (standard). At a concentration ($\mu\text{g/ml}$) of 10, 20, 30, 40, 50, 60, 70 and 80, the scavenging activity (%) of vitamin C was 37.73 ± 0.46 , 53.40 ± 0.23 , 68.07 ± 0.33 , 80.81 ± 0.46 , 82.74 ± 0.56 , 83.02 ± 0.05 , 83.15 ± 0.18 and 84.55 ± 0.47 respectively. The aqueous extract % scavenging activity was 28.09 ± 0.09 , 39.58 ± 0.42 , 53.56 ± 0.09 , 65.14 ± 0.14 , 70.04 ± 0.18 , 74.19 ± 0.05 , 80.12 ± 0.30 and 80.80 ± 0.46 at concentration ($\mu\text{g/ml}$) of 10, 20, 30, 40, 50, 60, 70 and 80 respectively. The methanol extract calculated scavenging activity (%) was 36.33 ± 0.47 at 10 $\mu\text{g/ml}$, 50.34 ± 0.42 at 20 $\mu\text{g/ml}$, 62.33 ± 0.24 at 30 $\mu\text{g/ml}$, 73.78 ± 0.47 at 40 $\mu\text{g/ml}$, 81.70 ± 0.23 at 50 $\mu\text{g/ml}$, 81.96 ± 0.05 at 60 $\mu\text{g/ml}$, 82.02 ± 0.09 at 70 $\mu\text{g/ml}$ and 82.96 ± 0.09 at 80 $\mu\text{g/ml}$. The ethanolic extract DPPH % scavenging activity at concentration ($\mu\text{g/ml}$) of 10, 20, 30, 40, 50, 60, 70 and 80 was 32.12 ± 0.46 , 44.66 ± 0.33 , 59.05 ± 0.23 , 69.82 ± 0.24 , 78.09 ± 0.19 , 81.49 ± 0.05 , 81.50 ± 0.14 and 82.52 ± 0.05 respectively. Similarly ethyl acetate extract of *H. rhamnoides* L. exhibited a % DPPH scavenging activity of 18.07 ± 0.47 at 10 $\mu\text{g/ml}$, 26.50 ± 0.47 at 20 $\mu\text{g/ml}$, 39.92 ± 0.71 at 30 $\mu\text{g/ml}$, 49.72 ± 0.92 at 40 $\mu\text{g/ml}$, 57.72 ± 0.71 at 50 $\mu\text{g/ml}$, 69.07 ± 0.24 at 60 $\mu\text{g/ml}$, 80.06 ± 0.09 at 70 $\mu\text{g/ml}$ and 81.40 ± 0.05 at 80 $\mu\text{g/ml}$. The acetone extract DPPH free radical scavenging activity (%) was 24.22 ± 0.87 , 39.23 ± 0.09 , 48.22 ± 0.28 , 52.40 ± 0.42 , 64.89 ± 0.46 , 76.84 ± 0.15 , 80.59 ± 0.23 and 82.21 ± 0.94 at concentrations ($\mu\text{g/ml}$) of 10, 20, 30, 40, 50, 60, 70 and 80 respectively. Chloroform extract scavenging (%) activity was 19.13 ± 0.44 (10 $\mu\text{g/ml}$), 30.87 ± 0.72 (20 $\mu\text{g/ml}$),

42.57 ± 1.43 (30 $\mu\text{g/ml}$), 51.78 ± 0.47 (40 $\mu\text{g/ml}$), 63.89 ± 0.38 (50 $\mu\text{g/ml}$), 73.78 ± 0.93 (60 $\mu\text{g/ml}$), 80.06 ± 0.09 (70 $\mu\text{g/ml}$) and 81.43 ± 0.05 (80 $\mu\text{g/ml}$). The *n*-hexane scavenging activity (%) at 10 $\mu\text{g/ml}$ was 15.26 ± 1.24 , at 20 $\mu\text{g/ml}$ was 25.56 ± 0.46 , at 30 $\mu\text{g/ml}$ was 34.92 ± 0.46 , at 40 $\mu\text{g/ml}$ was 44.29 ± 0.47 , at 50 $\mu\text{g/ml}$ was 48.97 ± 0.47 , at 60 $\mu\text{g/ml}$ was 59.95 ± 0.23 , at 70 $\mu\text{g/ml}$ was 80.03 ± 0.06 and at 80 $\mu\text{g/ml}$ was 80.15 ± 0.25 .

The EC_{50} ($\mu\text{g/ml}$) values (Fig.15) of ascorbic acid, aqueous, methanol, ethanol, ethyl acetate, acetone, chloroform and *n*-hexane were found to be 15.75, 25.01, 17.72, 20.75, 35.70, 28.62, 32.85 and 40.86 respectively.

The compounds which were holdup or slow down the oxidation of other molecules through stop the beginning or proliferation chain reaction of oxidation known as antioxidant [89]. Medicinal plants have been performing a central role for drug innovation all over the world. Synthetic antioxidants are produced nowadays but the major drawback with these antioxidants is the adverse effect when use *in vivo* [106]. The uses of artificial antioxidant were restricted due to its carcinogenic nature. Therefore, the importance of non-synthetic antioxidants rises. Non-synthetic antioxidants are vitamin C, carotenoids, phenolic compounds (flavonoids, phenolic acids and tocopherols) and nitrogen compounds (amines, chlorophyll derivatives, alkaloids and amino acids) [89]. Plants have polyphenols, which are natural antioxidant because of its ability to donate H^+ or e^- and capturing free radicals [107]. Different extract of *H. rhamnoides* L. pomace [108] showed that the activity was concentration dependent and maximum was 70% in methanolic extract. *H. rhamnoides* L. stem and roots methanol extract showed strong antioxidant activities

(DPPH bioassay) [3]. The *H. rhamnoides* pomace extracts reducing power were rises as the concentration of the extracts increases and maximum was 70% in methanolic extract [109]. Flavonoids (37.50 mg/ml) from seabuckthorn exhibited an outstanding DPPH scavenging activity, which inhibit 46.5% of DPPH in two minutes [110]. DPPH radical scavenging activity RC_{50} ($\mu\text{g/ml}$) of *H. rhamnoides* methanolic extract was 5.0, while fraction of hexane, ethanol, butanol and aqueous were 3.0, 1.5, 2.5 and 9.5 respectively. Similarly stem methanolic extract RC_{50} ($\mu\text{g/ml}$) was 2.7 and fractions of hexane, ethanol, butanol and water were 3.2, 1.0, 1.4 and 3.0 respectively [3]. The current study showed that antioxidant activity is a dose dependent and methanolic extract showed the maximum activity, which is in close agreement of the most cited above research work.

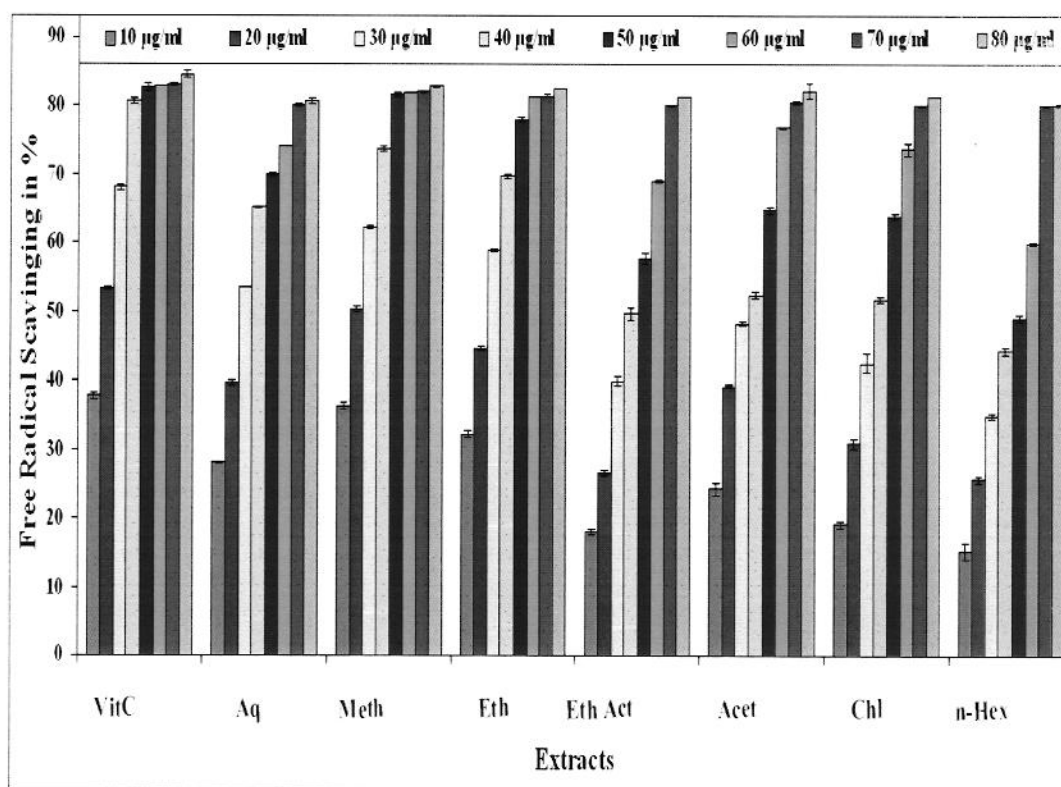


Fig.14. Antioxidant Activity (DPPH) of *H. rhamnoides* L. Leaves Extracts.

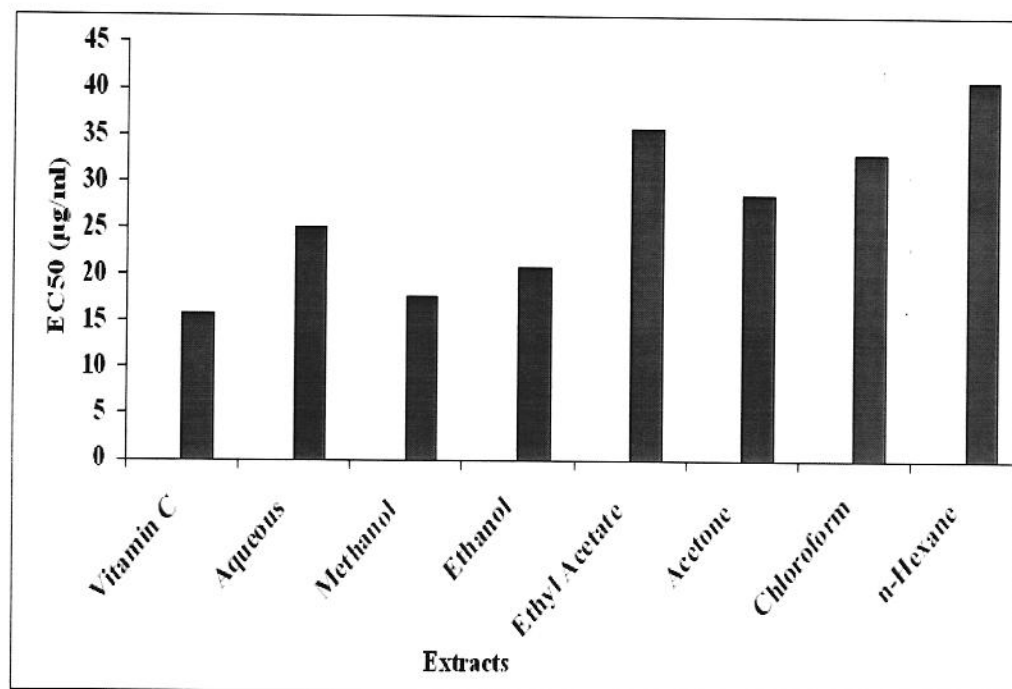


Fig.15. EC₅₀ (µg/ml) of *H. rhamnoides* L. Leaves Extracts.

3.10. Cytotoxic Activity of *H. rhamnoides* L. Leaves Extracts

Cytotoxic activity was the beginning footstep in exploration for development of anti-cancer drug. The brine shrimp cytoxic assay has been widely used in the key screening of the isolated compounds as well as crude extracts to assess the toxicity towards brine shrimps, which could also give a sign of possible cytotoxic properties of the test substances [111]. Because this bioassay is easy simple protocol. The procedure allows the use of smaller amount of the extracts and allowed bigger number of dilutions and samples within shorter time than using the original test vials [112]. Furthermore it has been established that the cytotoxic materials commonly show significant activity in the brine shrimp cytotoxicity assay, and this assay can be suggested as a channel for the discovery of pesticidal and antitumor compounds because of its low cost and simplicity [113].

Cytotoxic effects (% mortality) of the *H. rhamnoides* L. extracts are summarized in Fig.16. Brine shrimp cytotoxicity of all tested extracts of *H. rhamnoides* L. showed that mortality rate was concentration dependent. The mortality was 46.7%, 16.7% and 6.7% at concentration of 1000, 100 and 10 $\mu\text{g/ml}$ respectively in aqueous extract. The methanol extract percent mortality was 46.7, 26.7 and 6.7 at 1000, 100 and 10 $\mu\text{g/ml}$. The ethanolic extract at 1000, 100 and 10 $\mu\text{g/ml}$ concentrations showed % mortality of 50.0, 26.7 and 16.7 respectively. No mortality was found at concentration of 10 $\mu\text{g/ml}$ in ethyl acetate and acetone extract, but these extract showed 6.7 percent mortality at 100 $\mu\text{g/ml}$. While 26.7 and 33.3 mortality (%) were calculated at 1000 $\mu\text{g/ml}$ concentration for ethyl acetate and acetone respectively. Chloroform and *n*-hexane extract exhibited 6.7% mortality at 10 $\mu\text{g/ml}$ concentration. At 1000 and 100

$\mu\text{g/ml}$ concentration the chloroform extract mortality were (40.0 and 23.3%) and *n*-hexane extract mortality were (33.3 and 16.7%) respectively. No mortality was recorded for negative control and 100% mortality was noted in positive control.

The LD_{50} values of *H. rhamnoides* leaves extracts is shown in Fig. 17. The methanol extracts LD_{50} 1199.97 $\mu\text{g/ml}$, while ethanolic extract LD_{50} was 1206.91 $\mu\text{g/ml}$. The *n*-hexane extract was highest LD_{50} values of 6146.53 $\mu\text{g/ml}$. The other LD_{50} ($\mu\text{g/ml}$) values for aqueous, ethyl acetate, chloroform and acetone were 1515.09, 4050.53, 2198.80 and 2282.31 respectively. Alkaloids are commonly antibacterial, cytotoxic, antimalarial and anticancerous agents [114]. While saponins contain antimicrobial and larvicidal characteristics [115]. Anthraquinones exhibits cytotoxicity and antimicrobial activities, whereas terpenoids have larvicidal and antibacterial activities [116]. Flavonoids have been exposed to cover antioxidant, antiviral, anti-thrombotic, anti-inflammatory, vasodilatory, antibacterial, antiallergic and antineoplastic activities [117]. Tannins have shown potential antioxidant, antibacterial, anticancer and antiviral activities [118]. The preliminary phytochemical analysis of *H. rhamnoides* berries claims the existence of saponins, glycosides, alkaloids, flavonoids, tannins and anthraquinones [119]. Plants containing flavonoids, tannins and sterols may be used for antiviral activity [120]. The LC_{50} of methanolic extract [5] of *H. rhamnoides* (twigs) was 1584.89 ppm and the difference in Brine Shrimp cytotoxicity results may be due to the variation in the type and quantity of cytotoxic phytochemicals (e.g. flavonoids, triterpenoids, tannins, or coumarins) found in the plants crude extracts. *H. rhamnoides* L. leaf [121, 122] have found free from heavy metal contamination and no side effect when taken orally.

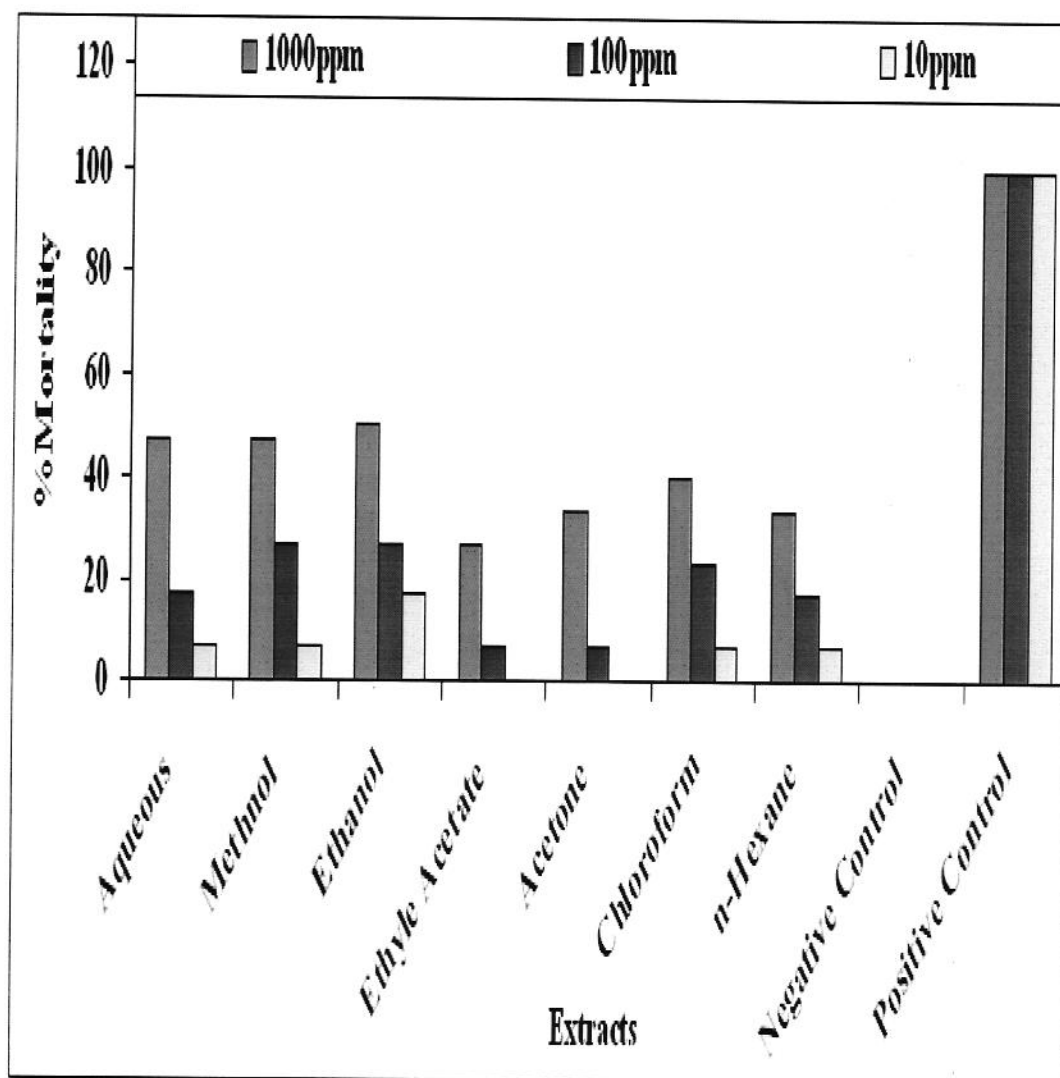


Fig.16.Cytotoxic Activity (% Mortality) of *H. rhamnoides* L. Leaves Extracts.

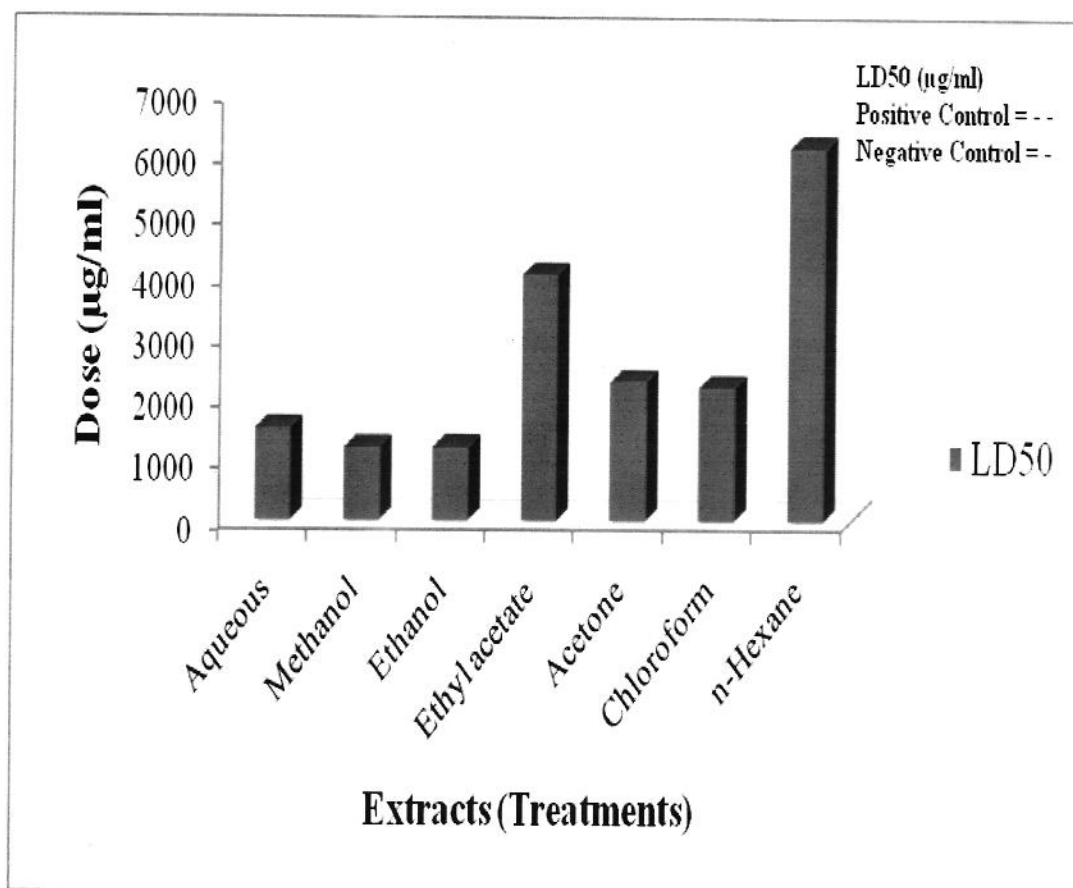


Fig.17.LD₅₀ (Cytotoxic Activity) values of *H. rhamnoides* L. Leaves Extracts.

3.11. Phytotoxic Activity of *H. rhamnoides* L. Leaves Extracts

The phytotoxicity results (Fig. 18 and 19) indicated that all extracts obtained from *H. rhamnoides* L. leaves did not cause any phytotoxicity at the concentration of 5 µg/ml. The methanol extract showed growth regulation of 33.33% at 500 µg/ml and 20.00% at 50 µg/ml, while LD₅₀ was 1344.79 µg/ml. The percent growth regulation of ethanol extract was 33.33 for 500 µg/ml concentration and 16.67 at 50 µg/ml and aqueous extract was 26.76% and 13.33% at 500 and 50 µg/ml respectively. No phytotoxic activity was found in *n*-hexane extract at tested concentrations. The acetone extract %GR at 500 µg/ml was 30.00 and at 50 µg/ml was 10.00. The chloroform and ethyl acetate percent GR at 500 µg/ml was 16.67 and 26.76 and at 50 µg/ml was 10.00 and 6.67 respectively. The LD₅₀ (µg/ml) values of aqueous, ethanol and acetone were 2751.26, 1356.12 and 1619.55 respectively. The LD₅₀ values 15069.88 µg/ml and 2025.26 µg/ml were calculated for chloroform and ethyl acetate respectively.

In plant kingdom allelopathy is an essential ecological factor as well as phytotoxic in medicinal plants. It has been reported that flavonoids work as allelochemicals [123]. They were found in all extracts of *H. rhamnoides* L. leaves. The variations in the inhibition of *Lemna* by plants might be due to solvent and plant material differences. Allelopathic effects are related to plants, their parts and extraction procedures [124]. Glycosides and terpenoids have exhibit phytotoxicity [125]. Several researchers reported that amino acids have allelopathic effect against plants [126, 127]. Allelochemicals inhibit electron transport chain in mitochondria [128]. Phenolic compounds and its derivatives were latent inhibitors of seedling growth and germination and have allelopathic applications in forestry and agriculture as

herbicides [129]. The active constituents detected in the *H. rhamnoides* L. leaves extracts could be accountable for the inhibition of *Lemna minor* in the current study. Phytotoxins [130] affect enzymatic activity, ion uptake, cell division, membrane permeability, electron transport in respiratory chains and photosynthesis. The presence of polyphenolic compounds and tannins, are lethal and simply enter into the host cells leading to fatality of host tissues. The phenolic compounds can arbitrate hazardous connections indirectly or directly by connecting autotrophs toward each other and to herbivores [131].

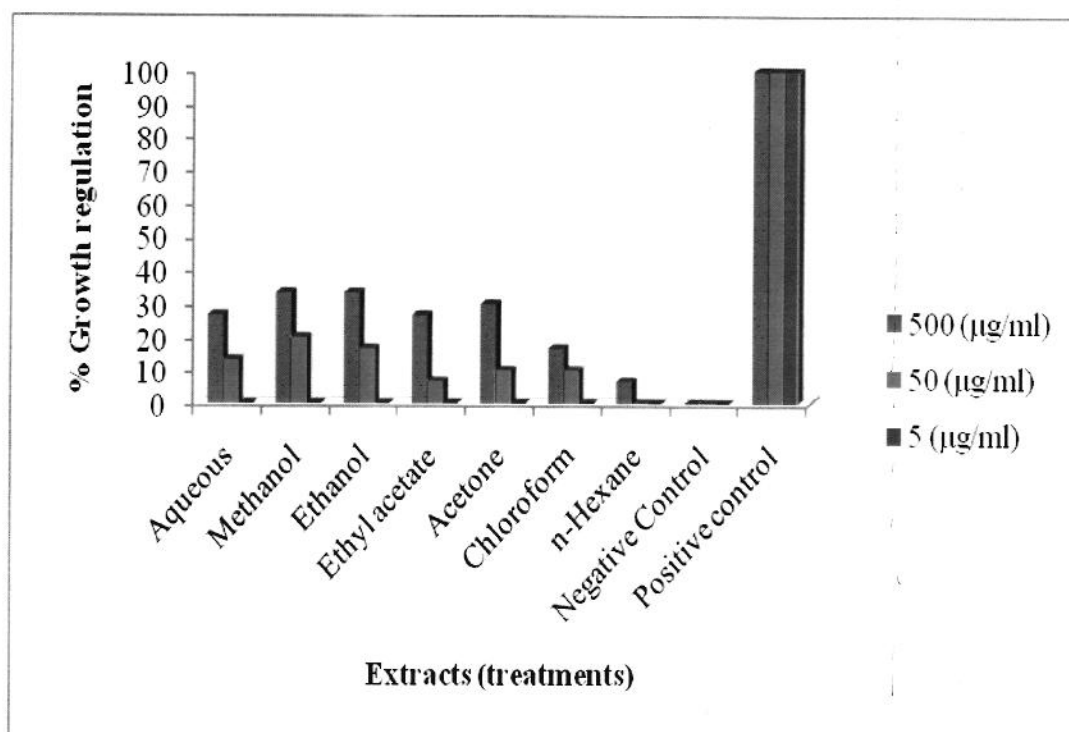


Fig. 18. Phytotoxic Activity of *H. rhamnoides* L. Leaves Extracts against *L. minor*.

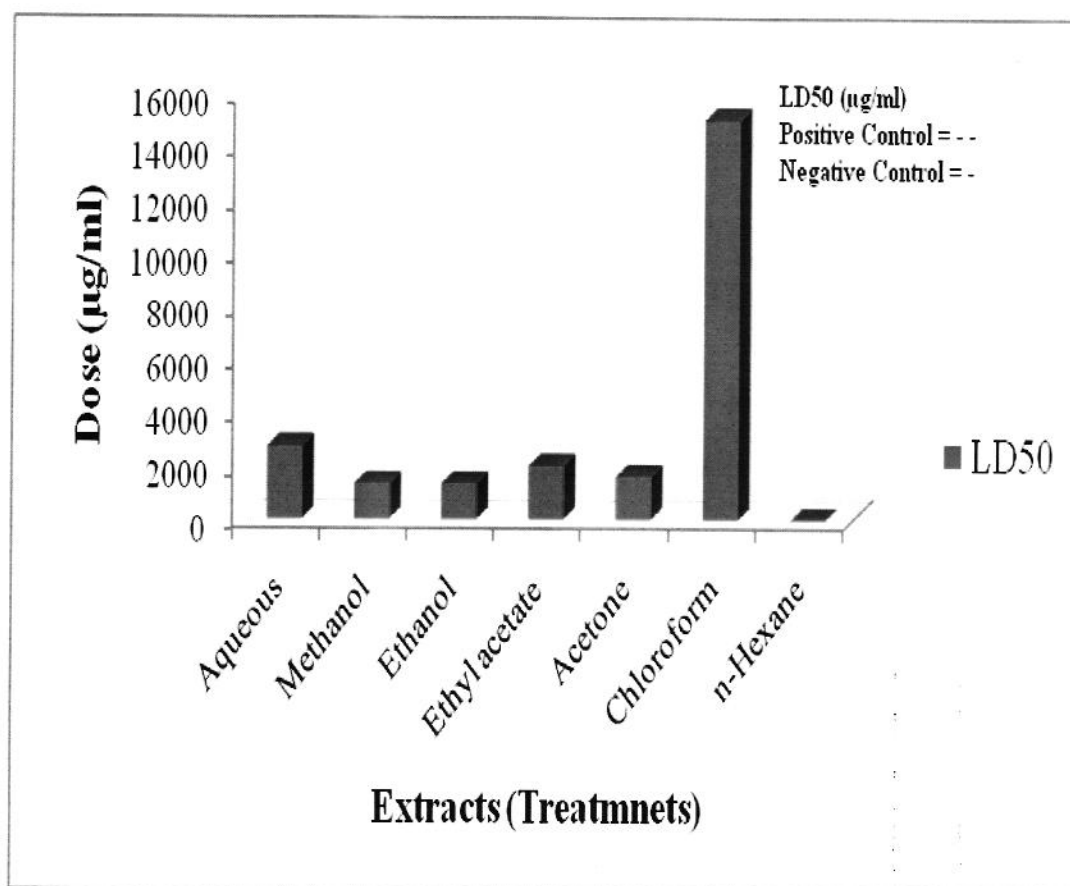


Fig. 19. LD₅₀ of *H. rhamnoides* L. Leaves Extracts against *L. minor*.

3.12. Larvicidal Activity of *H. rhamnoides* L. Leaves Extracts Against *A. aegypti*

The analysis of larvicidal activity of *H. rhamnoides* L. leaves extracts against vector mosquitoes (*Aedes aegypti*) are shown in Fig. 20 and was found dose and time dependent. The percent mortality increased by increasing the dose and time of exposure of larvae for each extract. The aqueous extract at 1000 ppm showed $12 \pm 0.82\%$, $19 \pm 0.96\%$ and $39 \pm 2.5\%$ mortality after 24 hrs, 48 hrs and 72 hrs respectively. When the dose of the same extract were increased to 1500 ppm with respect to its time, the percent mortality was found high as compared to 1000 ppm i.e. $42 \pm 1.29\%$, $45 \pm 0.96\%$ and $47 \pm 1.7\%$. High mortality rate ($77 \pm 0.96\%$) was detected in a concentration of 2000 ppm of the same extract. The methanol extract exhibited 20 ± 0.82 , 28 ± 0.82 and $36 \pm 0.81\%$ larval mortality at 24 hrs, 48 hrs and 72 hrs with exposed time respectively at concentration of 1000 ppm. At high concentration (2000 ppm) the larval mortality were calculated $68 \pm 0.82\%$, $71 \pm 1.30\%$ and $81 \pm 1.71\%$ after 24 hrs, 48 hrs and 72 hrs respectively. The ethanol extract at 1000 ppm with 24 hrs, 48 hrs and 72 hrs incubation time showed $18 \pm 1.30\%$, $26 \pm 1.30\%$ and $32 \pm 1.80\%$ larval mortality respectively. But with high concentration 1500 ppm and 2000 ppm at 72 hrs contact time the larval mortality (%) were 42 ± 1.30 and 84 ± 0.82 respectively. The ethyl acetate extract was found less effective against *A. aegypti* larvae and the results observed that percent larval mortality at 1000 ppm were 13 ± 0.53 (24 hrs), 34 ± 1.91 (48 hrs) and 37 ± 1.26 (72 hrs), at 1500 ppm were 43 ± 1.26 (24 hrs) and at 2000 ppm were 55 ± 0.96 (24 hrs), 64 ± 1.63 (48 hrs) and 73 ± 0.96 (72 hrs). The acetone extract % larval mortality was 15 ± 0.96 , 24 ± 1.63 and 36 ± 2.6 at 1000 ppm at 24 hrs, 48 hrs and 72 hrs respectively. Similarly with high concentration (1500 ppm) at 24 hrs, 48 hrs

and 72 hrs the larval mortality was $51 \pm 0.96\%$, $59 \pm 1.50\%$ and 62 ± 0.56 , while highest percent mortality 79 ± 0.96 was noted at 2000 ppm with 72 hrs incubation period. Fig 20 showed that at a concentration of 1000 ppm high percent mortality 25 ± 1.7 , 46 ± 0.53 and $50 \pm 1.30\%$ were noted in chloroform extract at 24 hrs, 48 hrs and 72 hrs respectively, while at the highest concentration (2000 ppm) the % larval mortality was counted 68 ± 0.82 (24 hrs) and 74 ± 1.30 (48 hrs and 72 hrs). The *n*-hexane extract showed percent larval mortality at 24 hrs, 48 hrs and 72 hrs contact period at 1000 ppm was 13 ± 0.50 , 16 ± 0.82 and 25 ± 1.7 respectively; however at high concentration the 2000 ppm the % larval mortality was increased up to 76 ± 2.94 at 72h incubation time. No mortality was observed for negative control (distilled water).

As can be seen from (Table 5) the lowest LC_{50} (24 hrs) was 1424.45 ppm for ethanol extract and the highest LC_{50} (24 hrs) value 1860.16 ppm was observed for chloroform extract. While other LC_{50} (ppm) 24 hrs values of aqueous, methanol, ethyl acetate, acetone and *n*-hexane extract were 1631.646, 1659.193, 1627.14, 1588.05 and 1832.87 respectively against *A. aegypti*. The chloroform extract showed highest LC_{90} (ppm) 24 hrs value 4053.22 and the aqueous extract was observed lowest LC_{50} 24 hrs value 2783.35 ppm. LC_{50} (ppm) and LC_{90} (ppm) at 48 hrs are display in table 6. The results showed that aqueous, methanol, ethanol, ethyl acetate, acetone, chloroform and *n*-hexane LC_{50} (ppm) 48 hrs were 1563.188, 1506.914, 1104.53, 1525, 1393.10, 1389.74 and 1564.11 respectively. Similarly the lowest and highest LC_{90} (ppm) 48 hrs against *A. aegypti* were noted 2632.08 and 4266.78 respectively. The LC_{50} (ppm) and LC_{90} (ppm) at 72 hrs are shown in table 7. The findings observed that ethanolic extract against *A. aegypti* calculated the lowest LC_{50} (ppm) and LC_{90} (ppm) at 72 hrs

were 1035.21 and 2643.63 respectively. While *n*-hexane and aqueous extract showed the highest LC₅₀ (ppm) and LC₉₀ (ppm) at 72 hrs were 1513.22 and 4374.21 respectively. The aqueous, methanol, ethyl acetate, acetone and *n*-hexane extracts revealed that LC₅₀ (ppm) at 72 hrs were 1375.185, 1268.71, 1322.13, 1250.74 and 1467.78 respectively against *A. aegypti*. The LC₉₀ (ppm) at 72 hrs were found 2871.44, 3461.05, 3163.00, 3068.31 and 3061.26 at methanol, ethyl acetate, acetone, chloroform and *n*-hexane respectively.

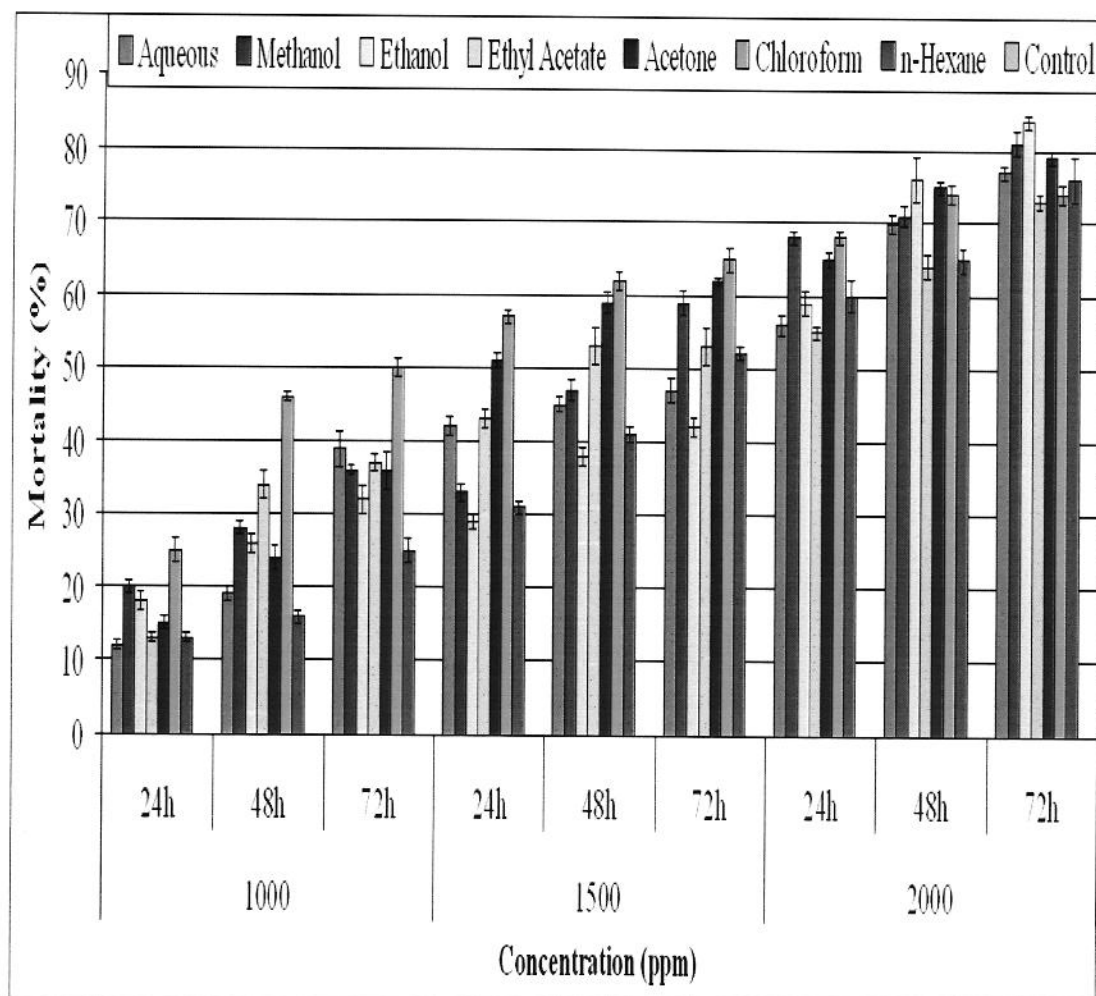


Fig.20. Larval Mortality (%) of *H. rhamnoides* L. Leaves Extracts against *A. aegypti*. Control (distilled water, nil mortality). h = hours.

Table.5.Probit Analysis of Larvicidal Activity of *H. rhamnoides* L. Leaves Extracts against *A. aegypti*.

Extracts	*LC ₅₀ (ppm) 24 hrs	**LC ₉₀ (ppm) 24 hrs	Regression equation	Chi-square value (X ²)
Aqueous	1631.646	2783.35	Y= -12.75+5.53x	0.00
Methanol	1659.193	3294.58	Y= -8.85 +4.30x	2.08
Ethanol	1424.45	4395.42	Y= -8.01+4.13x	0.315
Ethyl Acetate	1627.14	2841.20	Y=-12.00 +5.29x	0.01
Acetone	1588.05	2963.47	Y= -10.14+4.73x	1.12
Chloroform	1860.16	4053.22	Y= -7.39+3.79x	2.20
n-Hexane	1832.87	3518.84	Y= -9.76+4.52x	0.62

*LC₅₀: Lethal concentration needed to kill 50% of the exposed population.

**LC₉₀: Lethal concentration needed to kill 90% of the exposed population.

Table.6.Probit Analysis of Larvicidal Activity of *H. rhamnoides* L. Leaves Extracts against *A. aegypti*.

Extracts	*LC ₅₀ (ppm) 48 hrs	**LC ₉₀ (ppm) 48 hrs	Regression equation	Chi-square value (X ²)
Aqueous	1563.188	2903.35	Y= -9.84+4.65x	0.095
Methanol	1506.914	3268.89	Y= -5.98+3.45x	0.228
Ethanol	1104.53	4266.78	Y= -1.65+2.18x	0.008
Ethyl Acetate	1525.43	3480.17	Y= -6.39+3.58x	0.512
Acetone	1393.10	2632.08	Y= -9.58+4.64x	0.264
Chloroform	1389.74	2718.32	Y= -8.82+4.39x	0.040
n-Hexane	1564.11	2671.16	Y= -12.61+5.51x	0.881

*LC₅₀: Lethal concentration needed to kill 50% of the exposed population.

**LC₉₀: Lethal concentration needed to kill 90% of the exposed population.

Table.7. Probit Analysis of Larvicidal Activity of *H. rhamnoides* L. Leaves Extracts against *A. aegypti*.

Extracts	*LC ₅₀ (ppm) 72 hrs	**LC ₉₀ (ppm) 72 hrs	Regression equation	Chi-square value (X ²)
Aqueous	1375.185	4374.21	Y= -3.00+2.55x	1.310
Methanol	1268.71	2871.44	Y= -6.21+3.61x	0.047
Ethanol	1035.21	2643.63	Y= -4.49+3.14x	0.422
Ethyl Acetate	1322.13	3461.05	Y= -4.57+3.07x	0.363
Acetone	1250.74	3163.00	Y= -4.85+3.18x	0.110
Chloroform	1467.78	3068.31	Y= -7.67+4.00x	0.608
<i>n</i> -Hexane	1513.22	3061.26	Y= 4.18+-8.32x	1.271

*LC₅₀: Lethal concentration needed to kill 50% of the exposed population.

**LC₉₀: Lethal concentration needed to kill 90% of the exposed population.

3.13. Larvicidal Activity of *H. rhamnoides* L. Leaves Extracts Against *A. stephensi*

The percent larval mortality of *H. rhamnoides* L. leaves extracts against *A. stephensi* are shown in Fig.21. The results showed that aqueous extract percent mortality at 1000 ppm were 12 ± 1.2 (24 hrs), 16 ± 0.82 (48h) and 27 ± 6.75 (72 hrs), at 1500 ppm were 41 ± 0.96 (24 hrs), 61 ± 0.96 (48 hrs) and 67 ± 0.50 (72 hrs), at 2000 ppm were 71 ± 1.30 (24 hrs), 84 ± 0.82 (48 hrs) and 88 ± 0.82 (72 hrs) respectively. The methanolic extract exhibited mortality (%) of 13 ± 0.50 (24 hrs), 19 ± 0.50 (48 hrs) and 21 ± 0.96 (72 hrs) at 1000 ppm, while at 1500 ppm maximum mortality $60 \pm 0.82\%$ was observed and the highest mortality (%) reported at 2000 ppm was 89 ± 0.50 in 72 hrs incubation time. The ethanolic extract exhibited % mortality of 77 ± 0.96 , 86 ± 0.56 and 90 ± 1.30 at 24 hrs, 48 hrs and 72 hrs exposure time at 2000 ppm. The ethyl acetate observed larval mortality (%) at 1000, 1500 and 2000 ppm at 72 hrs exposure were 41 ± 0.96 , 63 ± 0.96 and 79 ± 0.96 respectively. Acetone extract at concentration of 1500 and 2000 ppm at 24 hrs, 48 hrs and 72 hrs time showed mortality (%) of (45 ± 0.96 , 50 ± 1.30 and 62 ± 1.30) and (68 ± 0.82 , 77 ± 0.96 and 81 ± 1.71) respectively. The chloroform extract at 2000 ppm for 24 hrs, 48 hrs and 72 hrs exposure time % mortality were 72 ± 0.96 , 76 ± 2.94 and 82 ± 1.30 respectively. The *n*-hexane at high concentration i.e. 2000 ppm showed $79 \pm 0.96\%$ larval mortality. No mortality was found in control (distilled water).

Probit analysis of larvicidal activity of *H. rhamnoides* L. leaves extract against *A. stephensi* is presented in table 8. The results indicated that the aqueous extract LC_{50} (ppm) at 24 hrs was 1601.29, methanolic extract was 1566.65, ethanolic extract was

1494.30, ethyl acetate extract was 1509.58, acetone was 1631.69, chloroform extract was 1725.80 and *n*-hexane extract was 1636.23. The lowest and highest LC₉₀ (ppm) 24 hrs was recorded 2292.18 and 2895.69 in ethyl acetate and chloroform extracts. But the aqueous, methanol and ethanol extracts LC₉₀ (ppm) 24 hrs values were 2630.36, 2621.11 and 2605.78 respectively.

At 48 hrs, the LC₅₀ (ppm) and LC₉₀ (ppm) values of different leaves extract of *H. rhamnoides* L. against *A. stephensi* are presented in table 9. The acetone and chloroform extract exhibited highest LC₅₀ (ppm) 48 hrs i.e. 1508.97 and 1505.49 respectively. The lowest LC₅₀ (ppm) 48 hrs was observed as 1320.38 in ethanol extract. Similarly the lowest and highest LC₉₀ (ppm) 48 hrs were found 2142.63 and 2697.33 in ethanol and acetone extract. The other moderate values of LC₉₀ (ppm) 48 hrs were 2158.66, 2274.36, 2165.49, 2452.96 and 2571.96 at aqueous, methanol, ethyl acetate, chloroform and *n*-hexane respectively.

The LC₅₀ (ppm) and LC₉₀ (ppm) 72 hrs data is presented in table 10. The data revealed that the maximum LC₅₀ (ppm) 72 hrs was 1361.07 in chloroform extract followed by 1310.64 (methanol), 1295.49 (*n*-hexane), 1266.99 (aqueous), 1252.71 (acetone), 1170.87 (ethyl acetate) and 1117.36 (ethanol). While the lowest LC₉₀ (ppm) 72 hrs were 2042.06 at methanol extract followed by 2057.01 (ethanol), 2081.43 (aqueous), 2176.37 (ethyl acetate), 2289.54 (chloroform), 2409.09 (*n*-hexane) and 2641.97 (acetone).

Plants are the main reservoir of bioactive compound which are less toxic, minimum chance to cause resistance and easily biodegradable [68]. Phytochemicals can be used as suitable alternative over synthetic insecticides which are comparatively safe,

less expensive and easily available through out the world. *Azadirachta indica* is a rich source of phytochemicals mainly containing high amounts of steroids, tannins, saponins and alkaloids which was responsible for the high mosquitocidal activity [132]. The phytochemicals present in twigs of *H. rhamnoides* extracts i.e. cold chloroform/ methanol (1:1) and methanol showed glycosides, terpenoids, steroids, flavonoids, reducing sugars and tannins [5]. The phytochemicals including alkaloids, terpenoids and phenols may act independently or jointly as ovicidal activity and were responsible as skin repellent against *A. Stephensi* [68]. It has been reported that the phenolic constituents showed high activity against larvae (mosquito) and the extract especially tannin from *Eclipta prostrate*, *Hemidesmus indices* and *Gymnema sylvestre* was responsible for the mortality in *C. quinquefasciatus* larvae [133]. Total phenolic content (mg GAE/g extract) of *H. rhamnoides* L. extracts of leaves methanolic, leaves aqueous, seed methanolic, seed aqueous, pomace methanolic and pomace aqueous were found 278.80, 184.89, 162.56, 109.57, 107.01 and 87.35 respectively [101]. Mosquito larvicidal activities are mainly due to tannins, phytosterols, flavonoids, phenols, saponins and carbohydrates [68]. The phenolic and flavonoids contents in methanolic extract of *H. rhamnoides* leaves were 34.6 (mg quercetin equivalent/100 g dry leaf) and 18.1 respectively [6]. In fact the leaves and twigs of the plant species are most commonly used for malarial therapy which are very useful to sustain the harvesting of plants for long time [30]. Chloroform extract was found more effective against *A. aegypti* larvae, while the ethanolic and petroleum ether extracts showed less larvicidal activity [29]. Less mortality [134] was observed in the larvae and adult stages as compared to pupae stage. Ethanol extract from garlic bulb [135] exhibited

effective larvicidal properties. Bioassays of leaf and fruit showed [136] that the extract of petroleum ether has been found more effective than methanol and chloroform extract against larvae. The *Aedes* mortality [137] was the highest followed by *Culex* with moderate mortality rate, while the mortality of the *Anopheles* was found very low as compared to the former two genera. It has been documented that [135] biological activity and chemical composition of the plant depend upon the geographical origin of plant, the plant tissues, plant age, the age and species of the tested pest organism. Crude extract [66] (hexane, ethyl acetate, dichloromethane and diethyl ether) of *Abutilon indicum* were found highly effective against larvae while the hexane extract showed maximum larvicidal activity against *A. aegypti*. Larval toxicity effect of *Euphorbia hirta* leaf extract [69] against malarial vector *A. stephensi* (IV instar) at 250 ppm observe percent larval mortality of 66.2, while LC_{50} (197.40) and LC_{90} (371.34). Beside it was observe that mortality was increased as the concentration increased. A study [70] revealed that larvicidal activity of *P. tithymaloides* aqueous stem extract against the *A. aegypti* (instar IV) at 1.0% concentration calculated the percent mortality was 16 with LC_{50} (2.210 %). Plant extracts [66] can injured both the eggs and egg shells, which may be by endosmosis. The eggs become desiccated after the initial phase of bump and shrink followed by the death of larvae.

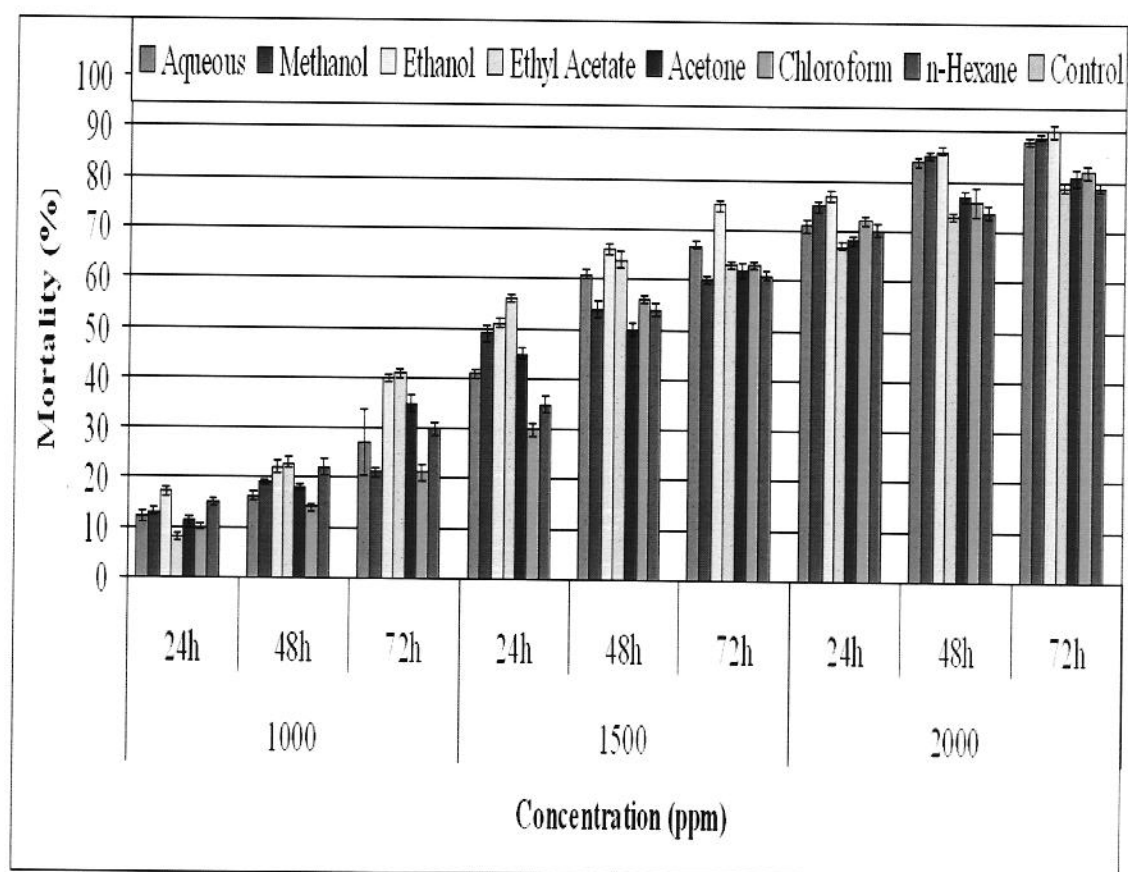


Fig.21. Percent Larval Mortality of *H. rhamnoides* L. Leaves Extracts against *A. stephensi*. Control (distilled water, Nil Mortality), h = hours.

Table. 8. Probit Analysis of Larvicidal Activity of *H. rhamnoides* L. Leaves Extracts against *A. stephensi*.

Extracts	*LC ₅₀ (ppm) 24 hrs	**LC ₉₀ (ppm) 24 hrs	Regression equation	Chi-square value (X ²)
Aqueous	1601.29	2630.36	Y= -14.05+5.94x	0.202
Methanol	1566.65	2621.11	Y= -13.32+5.73x	0.392
Ethanol	1494.30	2605.78	Y= -12.19+5.41x	0.013
Ethyl Acetate	1509.58	2292.18	Y= -17.46+7.06x	1.614
Acetone	1631.69	2786.68	Y= -12.71+5.51x	0.346
Chloroform	1725.80	2895.69	Y= -13.45+5.70x	1.885
<i>n</i> -Hexane	1636.23	2834.38	Y= -12.26+5.37x	1.873

*LC₅₀: Lethal concentration needed to kill 50% of the exposed population.

**LC₉₀: Lethal concentration needed to kill 90% of the exposed population.

Table. 9. Probit Analysis of Larvicidal Activity of *H. rhamnoides* L. Leaves Extracts against *A. stephensi*.

Extracts	LC ₅₀ (ppm) 48 hrs	LC ₉₀ (ppm) 48 hrs	Regression equation	Chi-square value (X ²)
Aqueous	1399.08	2158.66	Y= -16.40 +6.80x	0.232
Methanol	1408.33	2274.36	Y= -14.38+6.16x	0.210
Ethanol	1320.38	2142.63	Y= -14.02+6.10x	0.18
Ethyl Acetate	1330.39	2165.49	Y= -13.92+6.05x	0.067
Acetone	1508.97	2697.33	Y= -11.15+5.08x	0.010
Chloroform	1505.49	2452.96	Y= -14.21+6.04x	0.010
<i>n</i>-Hexane	1427.86	2571.96	Y= -10.82+5.01x	0.001

LC₅₀: Lethal concentration needed to kill 50% of the exposed population.

LC₉₀: Lethal concentration needed to kill 90% of the exposed population.

Table.10. Probit Analysis of Larvicidal Activity of *H. rhamnoides* L. Leaves Extracts against *A. stephensi*.

Extracts	*LC ₅₀ (ppm) 72 hrs	**LC ₉₀ (ppm) 72 hrs	Regression equation	Chi-square value (X ²)
Aqueous	1266.99	2081.43	Y= -13.44+5.94x	0.001
Methanol	1310.64	2042.06	Y= -15.75+6.65x	0.084
Ethanol	1117.36	2057.01	Y= -9.74+4.84x	0.070
Ethyl Acetate	1170.87	2176.37	Y= -9.60+4.76x	0.964
Acetone	1252.71	2641.97	Y= -7.25+3.95x	0.001
Chloroform	1361.07	2289.54	Y= -12.78+5.67x	0.335
<i>n</i> -Hexane	1295.49	2409.09	Y= -9.80+4.76x	0.020

*LC₅₀: Lethal concentration needed to kill 50% of the exposed population.

**LC₉₀: Lethal concentration needed to kill 90% of the exposed population.

3.14. Characterization of Bioinspired Synthesized AgNPs

3.14.1. UV-Visible spectroscopy Analysis of AgNPs

Recently plant extract utilization for the biosynthesis of NPs has gained much attention, because nanotechnology was engaged in latest and precise way for ailments beating. UV-visible spectroscopy is an essential method to verify the stability and synthesis of NPs in aqueous solution. In the current study AgNPs were prepared using *H. rhamnoides* L. leaves (Fig. 22). The incubation time was one hour and colour (yellowish brown) was appeared by 1 mM AgNO₃ aqueous solution addition to the plant extract, this was due to the phenomenon of surface plasmon resonance. *H. rhamnoides* L. leaves aqueous extract without AgNO₃ did not show any color change (Fig. 23). The AgNPs synthesis confirmation was carried out by a spectrum in visible range peak broadening indicated that the nature of particles (polydispersed) and the maximum absorbance occurs at 435 nm as shown in Fig.24. Two control reactions were kept as shown in Fig. 23 i.e. 1 mM AgNO₃ aqueous solution which is colorless and leaves extract solution which demonstrate pale yellow colour. However there was no broadening of peak absorption in to the leaves extract because NPs and AgNO₃ were absent (Fig. 25). The maximum absorption peak occurs at 435 nm representing AgNPs as previous research finding [39, 138, 139]. The development of colour (yellowish-brown) assumed the preparation of AgNPs [46]. The thickness and incubation period for color change depend on plant to plant. These are due to the presence of H⁺ ions for silver reduction or the formation of AgNPs. Due to the excitation of surface plasmon vibrations in AgNPs it was found that it have yellowish brown colour in aqueous solution [47].



Fig. 22. *H. rhamnoides* L. Leaves Used in the Biosynthesis of AgNPs.

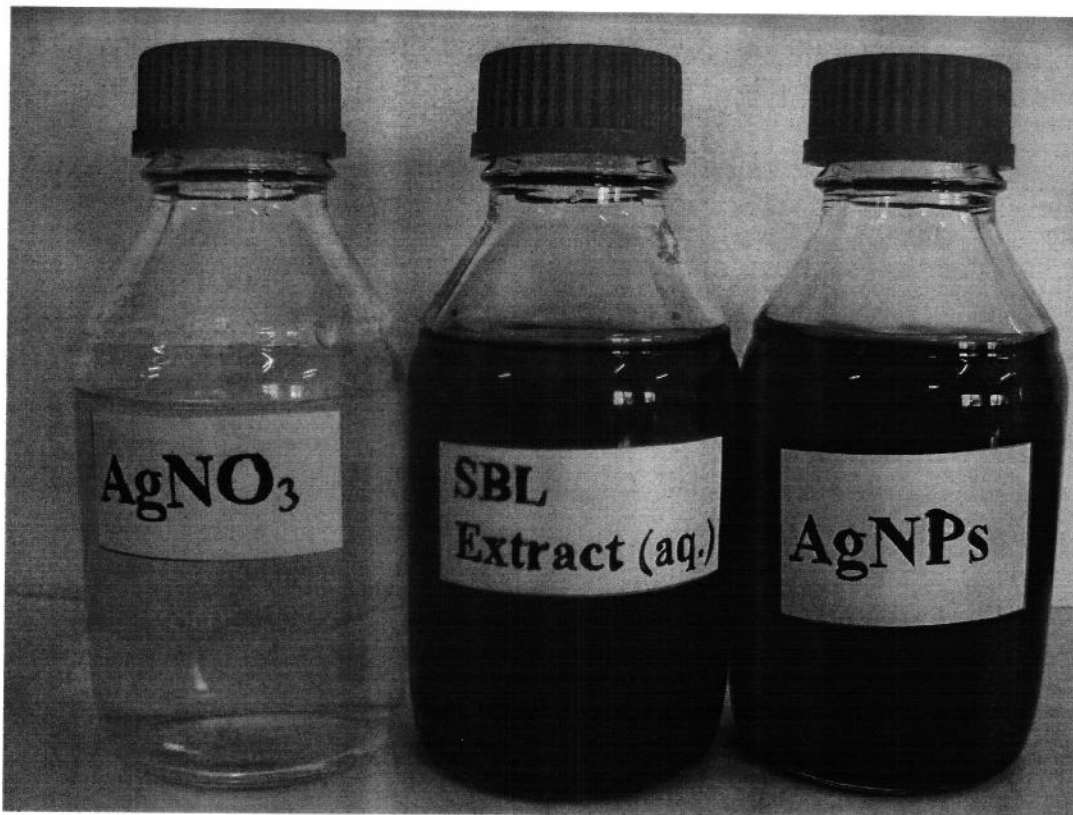


Fig. 23. Digital Photographs of (AgNO₃) = 1 mM Silver Nitrate Solution, (SBL Extract aq.) = Seabuckthorn Leaves Aqueous Extract, (AgNPs) = Biosynthesized Silver Nanoparticles.

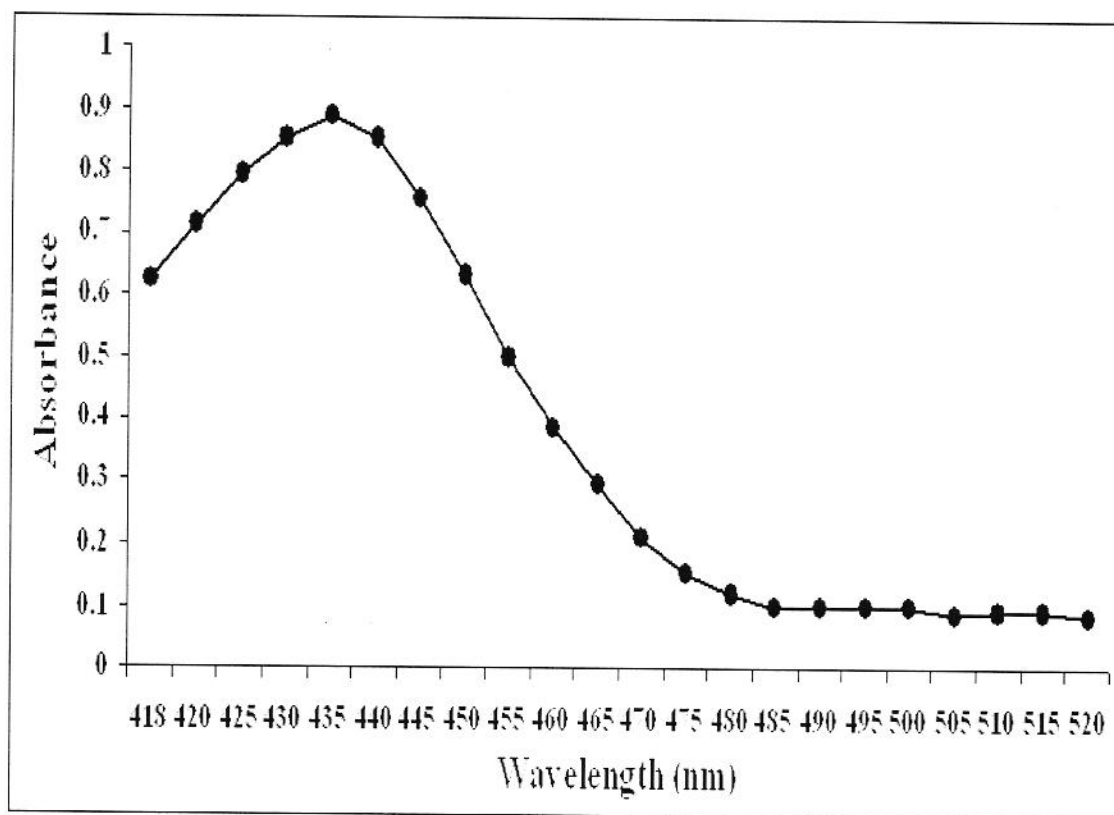


Fig.24. UV-VIS Absorption Spectra of Biosynthesized AgNPs.

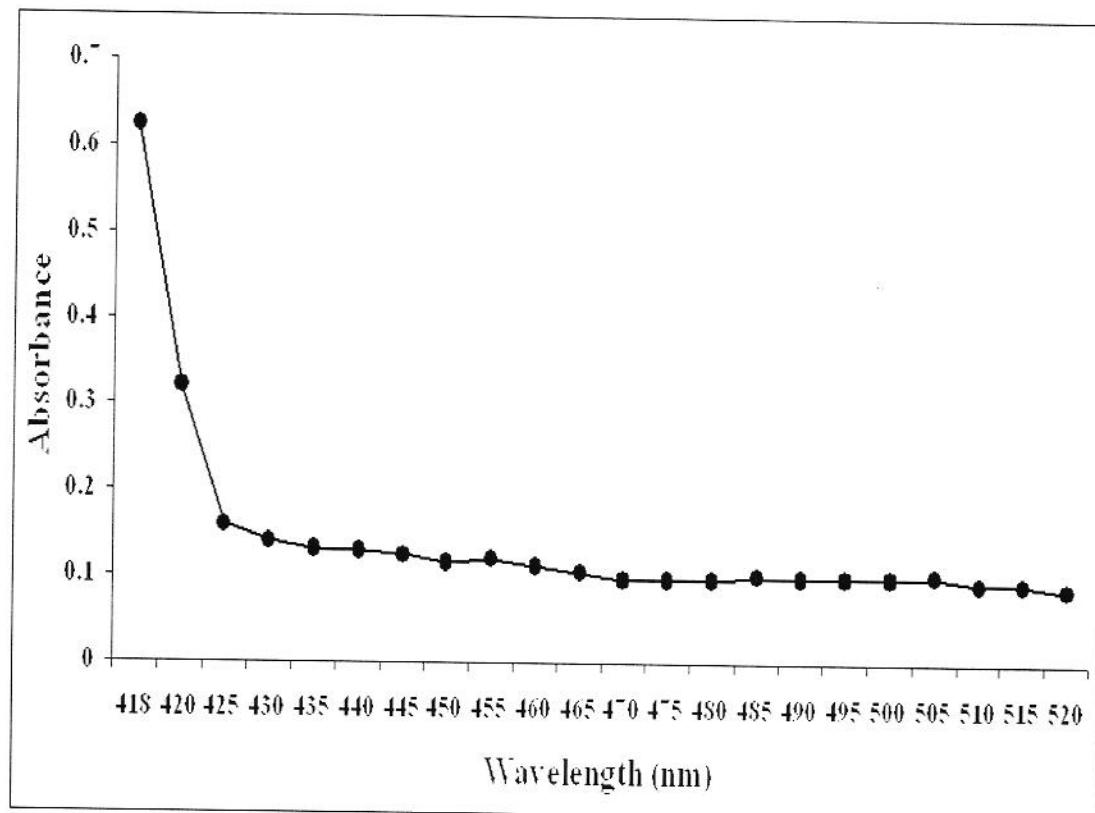


Fig.25. UV -VIS Absorption Spectra of *H. rhamnoides* L. Leaves Extract (Aqueous).

3.14.2. FTIR Analysis of AgNPs

FTIR peaks measurements were intended for the identification of responsible suitable biocomponents to reduce Ag^{+2} ions and capping of the bio-reduced synthesized AgNPs. *H. rhamnoides* L. leaves powder FTIR spectra showed the presence of alkenes, nitrates, amino acids, organic halogen compounds, carbohydrates and ethers (Fig. 26). Dried AgNPs were analyzed by FTIR to determine capping ligand of AgNPs which play a role as stabilize agent. FTIR spectrum of prepared AgNPs is visually shown in Fig. 27). The peaks values of 3309.85 cm^{-1} corresponds to hydroxyl compound, band at 2918.30 and 1708.93 cm^{-1} represent the carbonyl groups (compounds), similarly the other lower values corresponds to aldehyde and alkynes groups. It was clear that synthesized AgNPs were bounded by metabolites and proteins. Carbonyl group [140] form amino acid and proteins residues were powerful affinity to fasten through metal demonstrating that the proteins may be responsible to form a film to cover the metal NPs (i.e., capping of AgNPs) to stabilize the medium and prevention of agglomeration. In our finding (Fig. 23) it was confirmed that *H. rhamnoides* L. leaves have carbonyl group and performed the same function. Phenolic compounds [141] determination had revealed that these in the leaves extract might improve the reduction mechanism and the occurrence of these ingredients in biological synthesis of NPs solution may act as stabilizing or capping components. These compounds also act as antimicrobial agents. The phenolic constituents were occurred in the solution of AgNPs, which may enhance the antioxidant activity. Probably the biomolecules may perhaps dual action i.e. bioinspired synthesis of AgNPs and stability.

The most important absorbance peaks exhibited in the spectrum of gum ghatti [142] were at 3425 (stretching vibrations of O-H groups), 2928, 1406, 1234 (methylene groups), 2122 (carbonyl species), 1635 (carboxylate group), 1311 (carboxylate group), 1068 (ether group) and 1028 (alcoholic group) cm^{-1} . While, the spectrum of AgNPs denoted distinctive absorbance peaks at 1024, 1260, 1385, 1632, 1728, 2304, 2345, 2964 and 3431 cm^{-1} . The FTIR [37] absorbance spectra at 3402, 2925, 1610, 1420, 1059, 640 cm^{-1} were associated with the stretch vibration of O-H, CH₂, -C=C, -C-H, -C-O respectively. These absorbance signals could be accredited to the proteins, saccharides, flavonoids and reducing sugars in the extract, as the silver ions reduction take place due to flavonoids and reducing sugars. The FTIR peaks (cm^{-1}) documented for AgNPs [36] at 3418, 2920, 1384, 1076 and 569 were corresponding functional groups of -OH structural polymeric association, chelating compounds, germinal methyls, ether linkages and C=C groups or from aromatic rings respectively. Some of which originate to be subsequent to spectra in flower extracts and recommended the occurrence of terpenoids or flavanones lying on the surface of NPs. The FTIR spectra (cm^{-1}) for AgNPs [53] were 3402 (O-H stretching), 2920 (C-H stretching alkyl group), 2856 (C-H aldehyde group), 1645 (N-H stretch of amines), 1252 (C-N aromatic amines), 1029 (C-N aliphatic amines) and 668 (N-H primary and secondary amines).

FTIR spectra of AgNPs [40] revealed the occurrence of bonds due to germinal methyl group (around 1,380 cm^{-1}), C=C group (around 1,600 cm^{-1}), aldehydic C-H stretching (around 2,910 cm^{-1}) and O-H stretching (around 3,430 cm^{-1}). FTIR spectrum of AgNPs showed [138] the existence of bands (cm^{-1}) at 3419 (primary amine O-H),

1650 (primary amine N-H band), 1489 (methylene from protein) and 1059 (C-N stretching of protein). These proofs propose that the molecule of protein could possibly accomplish the role of the AgNPs synthesis well as stabilization in the aqueous medium.

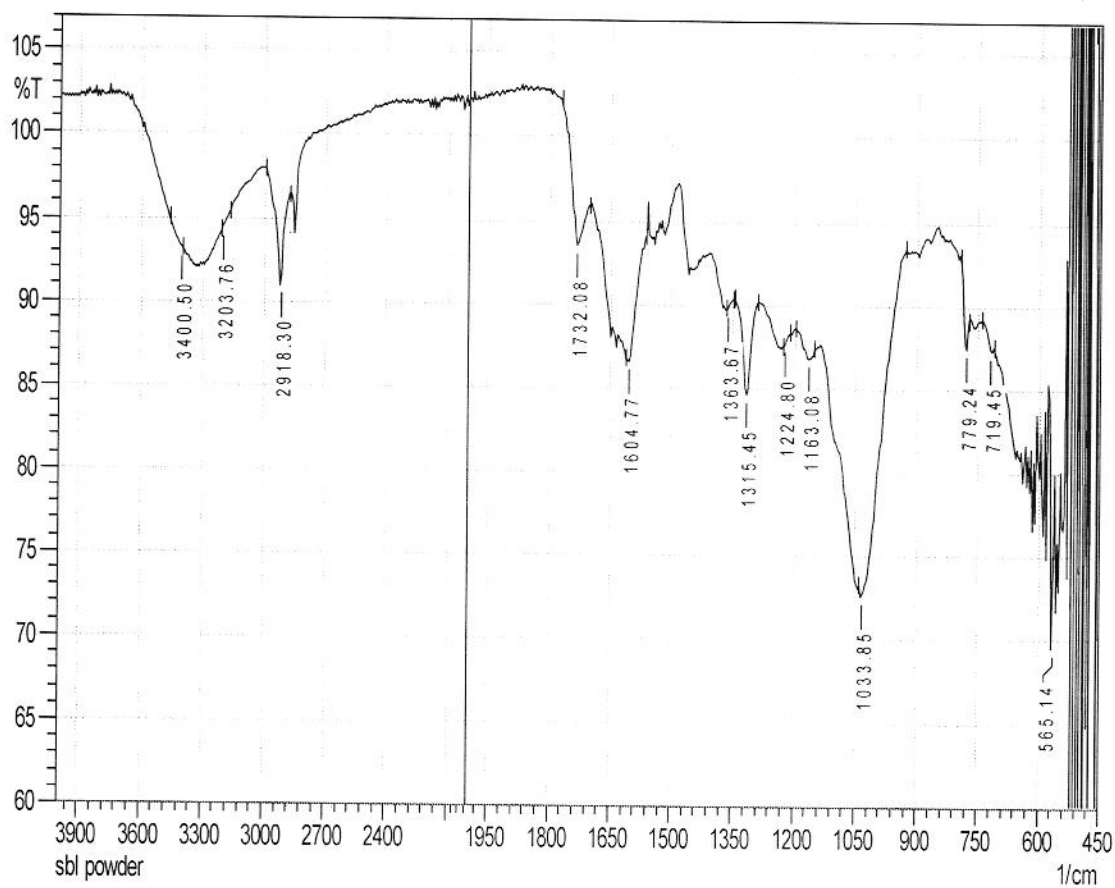


Fig. 26. FTIR Spectrum of *H. rhamnoides* L. Leaves Powder.

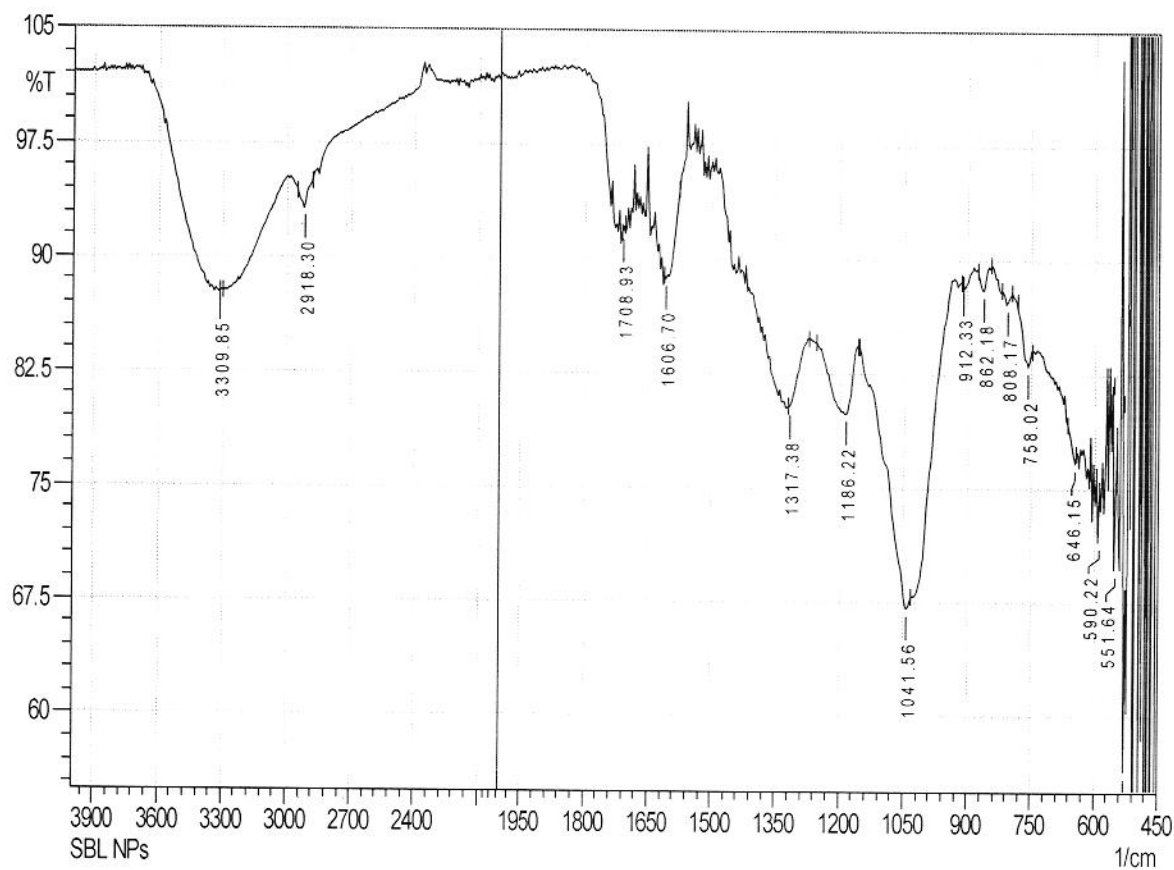


Fig. 27. FTIR Spectrum of Biosynthesized AgNPs.

3.14.3. SEM Analysis of AgNPs

The SEM technique was used to determine the size and morphology of the synthesized AgNPs. Fig. 28 shows the SEM image of plant leaves powder as a control. Four images of AgNPs were taken with four different magnification powers (X15, 000, X30, 000, X50, 000 and 100,000). As the magnification increases the images of the NPs was seen more prominent. The images showed (Figs. 29, 30, 31 and 32) that AgNPs found in a number of aggregates and also in individual identity. It was also seen that although the AgNPs spherical shaped (round) and aggregate into bigger round distinct shape. It was also noted that AgNPs were not in straight make contact with even the aggregations, indicating that proteins secreted by plant leaf extracts are the capping agent to stabilize the NPs. The SEM analysis showed that the prepared AgNPs were spherical in shapes and size in the range (135- 300 nm). The occurrence of bioactive compounds as capping agent to AgNPs may be come from leaves extracts [14]. Many authors have also prepared AgNPs having different sizes with diameter range 40-50 nm [143], 40-100 nm [141], 40-70 nm [19] and 29-68 nm [33]. SEM studied [34] showed that the formation of agglomerated AgNPs due to the capping agent in the range of 100 – 200 nm. SEM analysis showed the plant mediated (nyctanthes leaves extract) TiO_2 NPs size in the range from 100 to 150 nm [144].

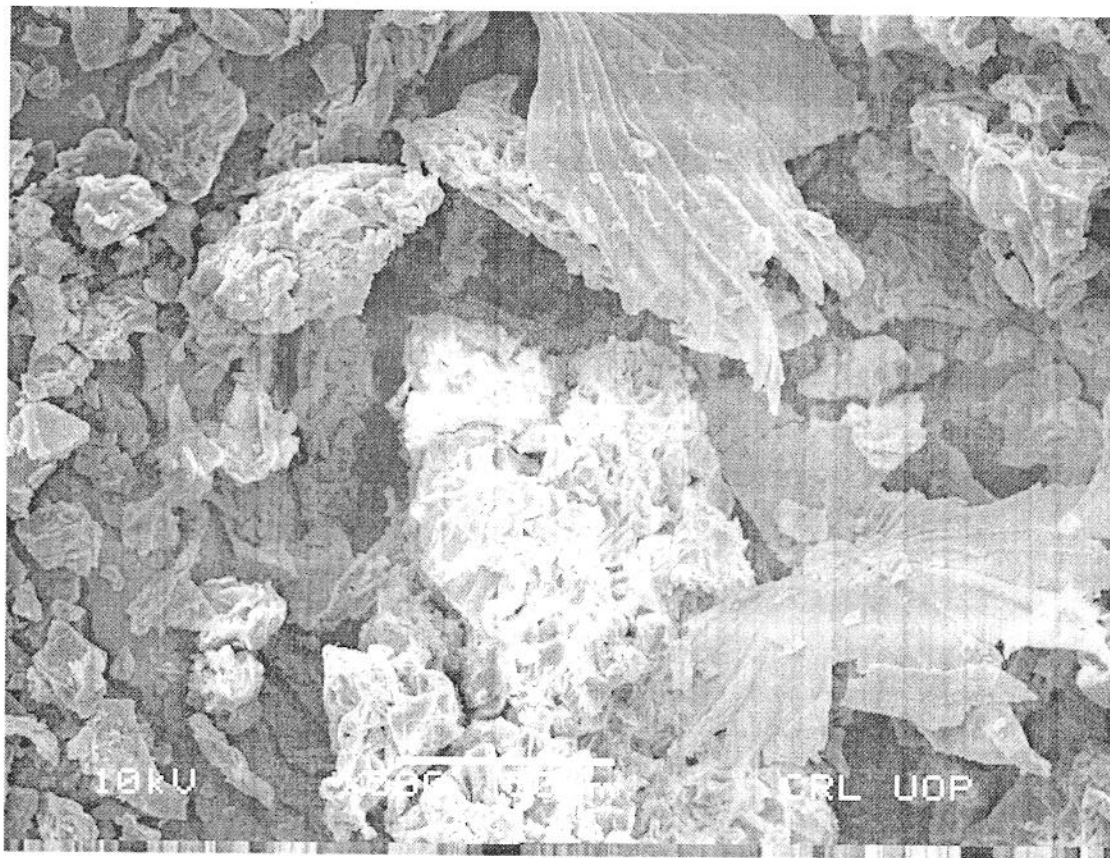


Fig. 28. SEM Micrograph of Leaves Powder of *H. rhamnoides* L.



Fig. 29. SEM Micrograph of AgNPs under X15, 000 magnification.



Fig.30. SEM Micrograph of AgNPs under X30, 000 magnification.



Fig. 31. SEM Micrograph of AgNPs under X50, 000 magnification.

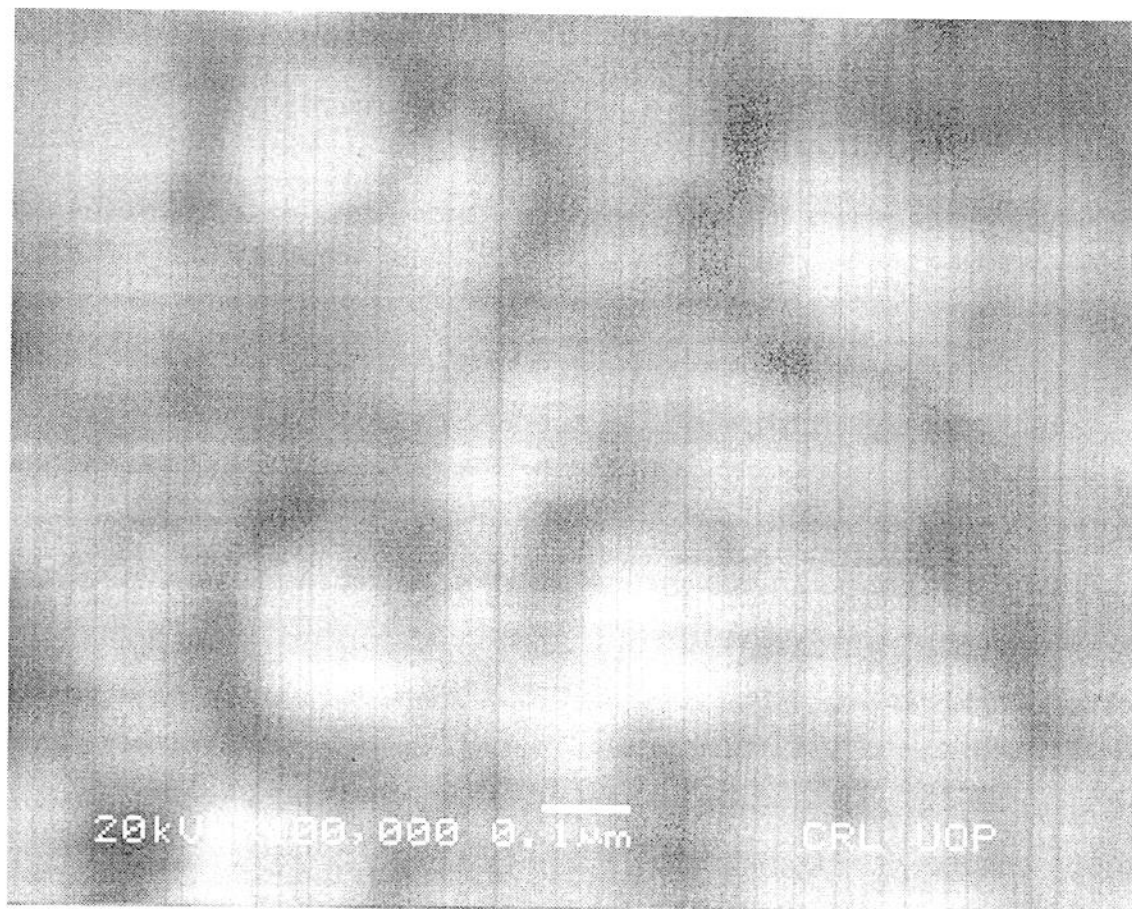


Fig.32. SEM Micrograph of AgNPs under X100, 000 magnification.

3.14.4. EDX Analysis of AgNPs

The EDX technique is utilized to determine the amount of silver as well as to identify additional elementary compositions in the prepared NPs. The EDX pattern of spectrum (Fig. 33) recorded high signals for silver. Elemental composition of AgNPs showed that besides silver other elements such as carbon, oxygen, sodium and potassium were also detected. The counts of x-ray is showing on perpendicular while energy (keV) on the parallel axis. The bright and sharp signals of silver correspond to the peaks in the graph confirm the formation of AgNPs. The AgNPs indicate an optically absorb band peaking at 3 keV which was the identification absorption peaks of AgNPs [145, 11, 53, 54, 35]. Figure 34 is showing the elemental composition of AgNPs. The silver composition revealed that by weight it was 40.33% and 9.06% (by atomic). Similarly C, O, Na and K by weight were 9.95%, 43.60%, 2.48% and 3.64% respectively. While by atomic percent composition of C, O, Na and K were 20.07, 66.01, 2.62 and 2.25 respectively. The elemental analysis other than silver showed that these elements come from the leaves aqueous extract.

The EDX spectrum analysis of AgNPs [31] showed that C, O and Ag atomic percent were 34.48, 47.71 and 17.01 respectively. The EDX spectra revealed [57] a prominent signal for Ag along with carbon and oxygen bands that may be from the protein residue which act as stabilizing agent on its surface, the incident of an extra peak for copper was because of copper grids on which the NPs film were formed. In our findings the elements C, O, Na and K were found which come from the extract source. EDX showed [34] the presence of AgNPs in high quantity besides calcium, magnesium and chloride traces. The EDX analysis revealed sharp silver signal

confirming AgNPs synthesis [35]. Copper signal was also seen in EDX spectrum, which has appeared from Copper TEM grid. Additional signals of elements (Zn, Mn, Ca and Cl) were recorded probably due to elements from proteins or enzymes occurring inside the *Jatropha curcas* latex. The EDX analysis of AgNPs showed [73] that C, O, Cl and Ag were present (Atomic %) in 18.14, 5.55, 20.08 and 56.22 respectively. The elemental composition (wt %) of AgNPs [138] were 47.10, 11.91, 08.38, 02.46 and 30.15 for C, O, P, Cl and Ag respectively, the first three elements low peaks were probably because of X-ray discharge from the *Gelidiella sp.* A sharp signal [31] from the Ag atoms (17.81%) in the AgNPs and weak signal from C (34.48%) and O (47.71%). These weak signals were attributed from the organic ingredients of plant. The EDX spectroscopy observed [18] peaks for O, C (35.58% and 40.14 % in wt %) which were due to the occurrence of poly vinyl alcohol (PVA). The weak peaks, derived for silver atom was 24.27 % in weight %. The weaker peaks were credited to the dispersed nature of AgNPs in the Ag/PVA nanocomposite membrane. The signals of silver [49] were detected and assigned. Signals for C and Cu were come from the utilized grid and nitrogen, sulfur and phosphorus peaks were correspond on the surface of AgNPs as protein capping. The strong peaks [146] in the Ag region was found 33.30 atomic %. The extra signals for Pt (5.80%), Cl (0.59%), K (3.61%) and Al (17.79%) were propose that they were occurred in extract of plant as mixed precipitates.

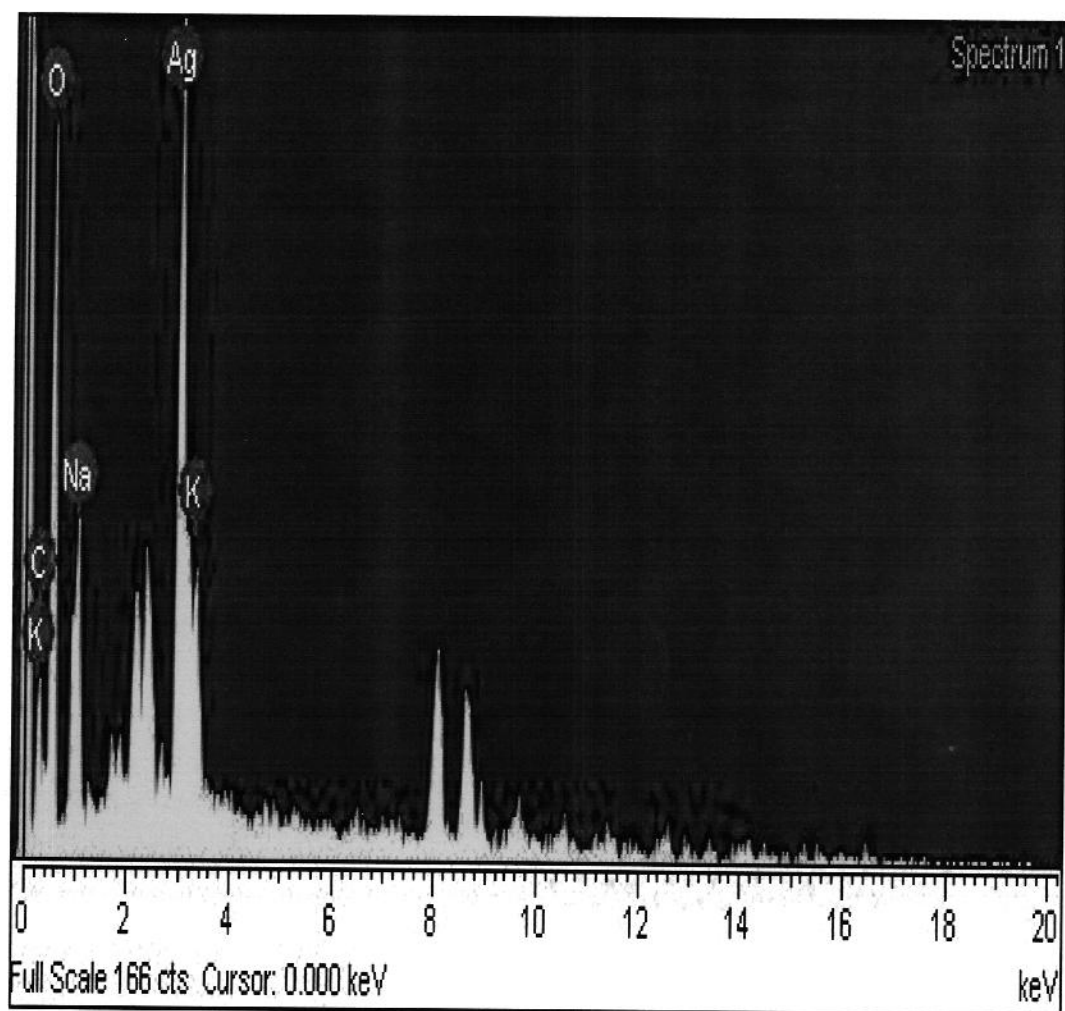


Fig. 33. EDX Spectrum of AgNPs.

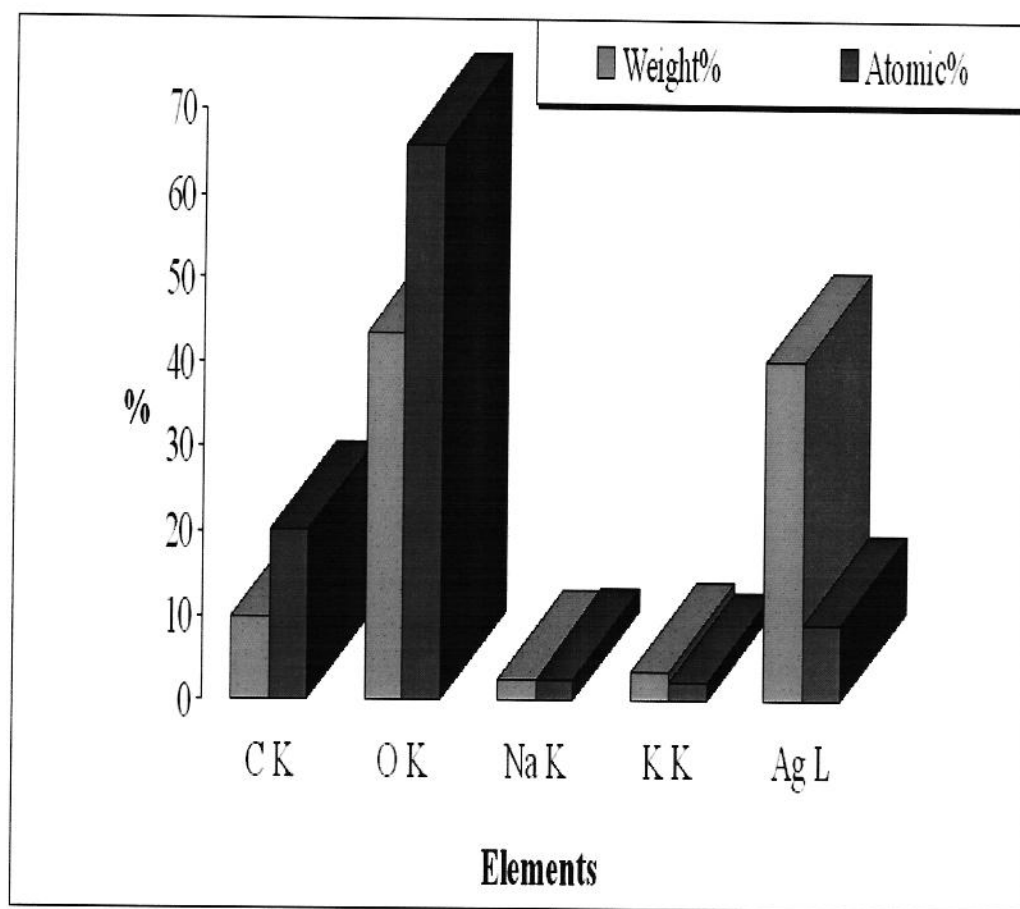


Fig. 34. Elemental Composition of AgNPs.

3.14.5. XRD Analysis of AgNPs

The XRD technique is used to study the nature and crystal size of NPs. XRD pattern of the prepared AgNPs is shown in Fig. 35. XRD pattern of the synthesized AgNPs revealed the presence of sharp diffraction lines at 10° to 70° , showing four strong peaks in the entire spectrum. The AgNPs have peaks of Ag at $2\theta = 34.24^\circ$, 38.12° , 44.32° and 64.52° . XRD peaks clearly illustrate that the AgNPs in the current study are crystalline in nature. There are no peaks observed for the impurities in the XRD pattern is a sign of the purity of the AgNPs. The *H. rhamnoides* leaves powder XRD pattern is shown in Fig.36. The FWHM and β values along with respective 2θ are shown in table 11. The AgNPs crystal size was determined using Debye-Scherrer's equation. The average size was 21.909 nm. Table 12 is describing the 2θ values, d-values and intensity. At 2θ value 34.24° d-value were 2.621 with 63 intensity, 38.12° d-value (2.362), intensity 227, 44.32° d-value (2.046), intensity 71 and 64.52° d-value (1.448) with intensity 42.

The patterns of XRD at 2θ were 38.08, 32.96, 32.22 and 15.3 (instantly) and 32.94 (one day after). A sharp peak of diffraction situated at 38.2 was attributed to (111) facets of AgNPs respectively with crystalline by nature. No peaks were recorded for the contamination in the XRD spectrum proving that the synthesized AgNPs were of high purity [146]. These findings are a close agreement to our prepared AgNPs in which the same sharp peak (38.12°) was occurred. The XRD studies revealed the average grain size to be 41.9 nm [141].

The observed patterns of XRD for the biosynthesized AgNPs using *Solanum torvum* leaves extract [147] indicate the occurrence of clear sharp peaks of AgNPs

corresponding to the 2 2 0, 2 0 0 and 1 1 1, which were represented as crystalline silver face-centered cubic (fcc) phase. Similarly the XRD pattern obtained [148] for AgNPs observed a distinctive spectra close to the 2θ value of 38.02° . A Bragg reflection exhibited to the (111) lattice planes sets evaluated which may be indexed based on the fcc silver structure. Beside to the Bragg peak of fcc AgNPs, the biomolecules layer place on the AgNPs surface of the AgNPs as unassigned and extra peaks were witnessed. The XRD peaks observed [149] for biosynthesized AgNPs using *Eucalyptus hybrida* leaf extract show characteristic peaks (at $2\theta = 30.8^\circ$), assigned with (111) and indicates the synthesized AgNPs were crystalline character. According to Scherrer formula the calculated AgNPs size from the FWHM of the (111) peak of Ag was 50 - 150 nm. The study showed [35] that XRD patterns of biosynthesized AgNPs using latex of *J. curcas*. A Bragg reflections by means of 2θ values were 77.18° , 63.43° , 46.18° and 38.03° sets of lattice planes were estimated which might be indexed to the (3 1 1), (2 2 0), (2 0 0) and (1 1 1) facets of AgNPs respectively and obviously display that the fabricated AgNPs were crystalline behavior.

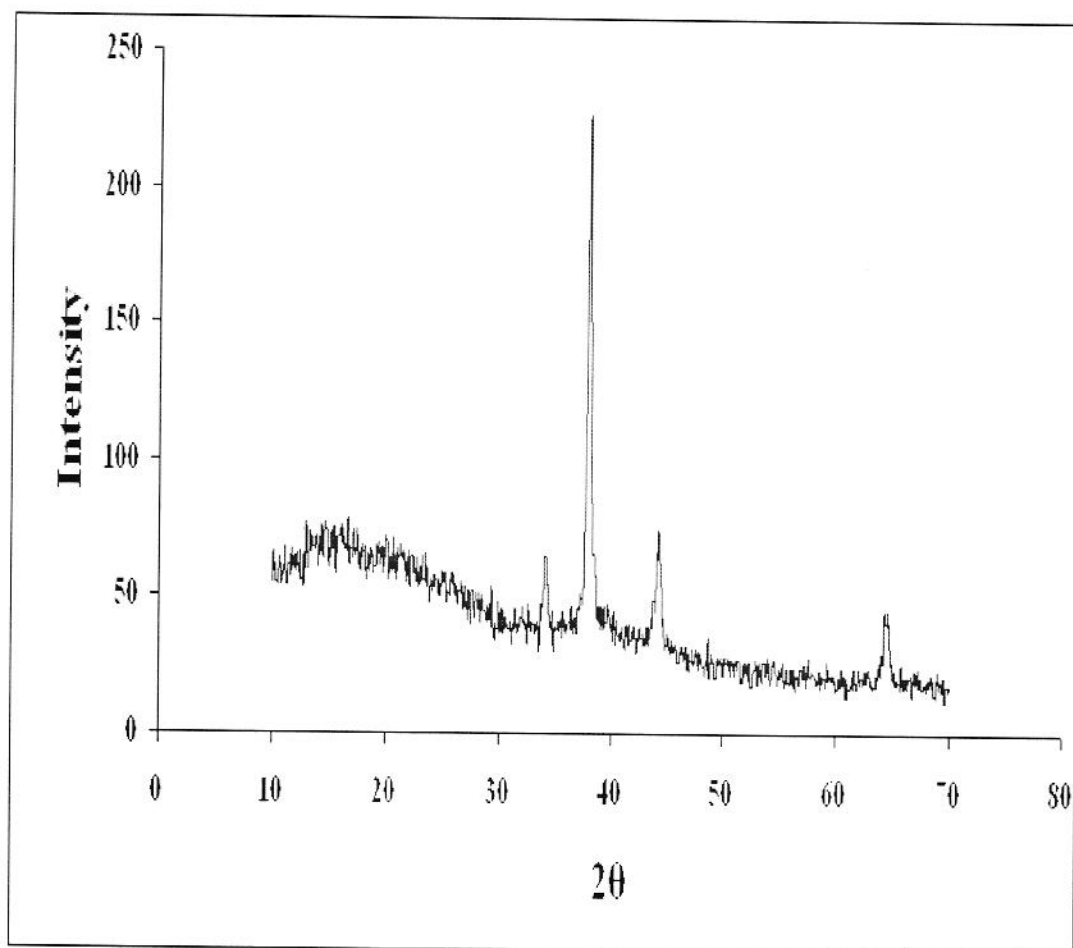


Fig. 35. XRD Analysis of AgNPs.

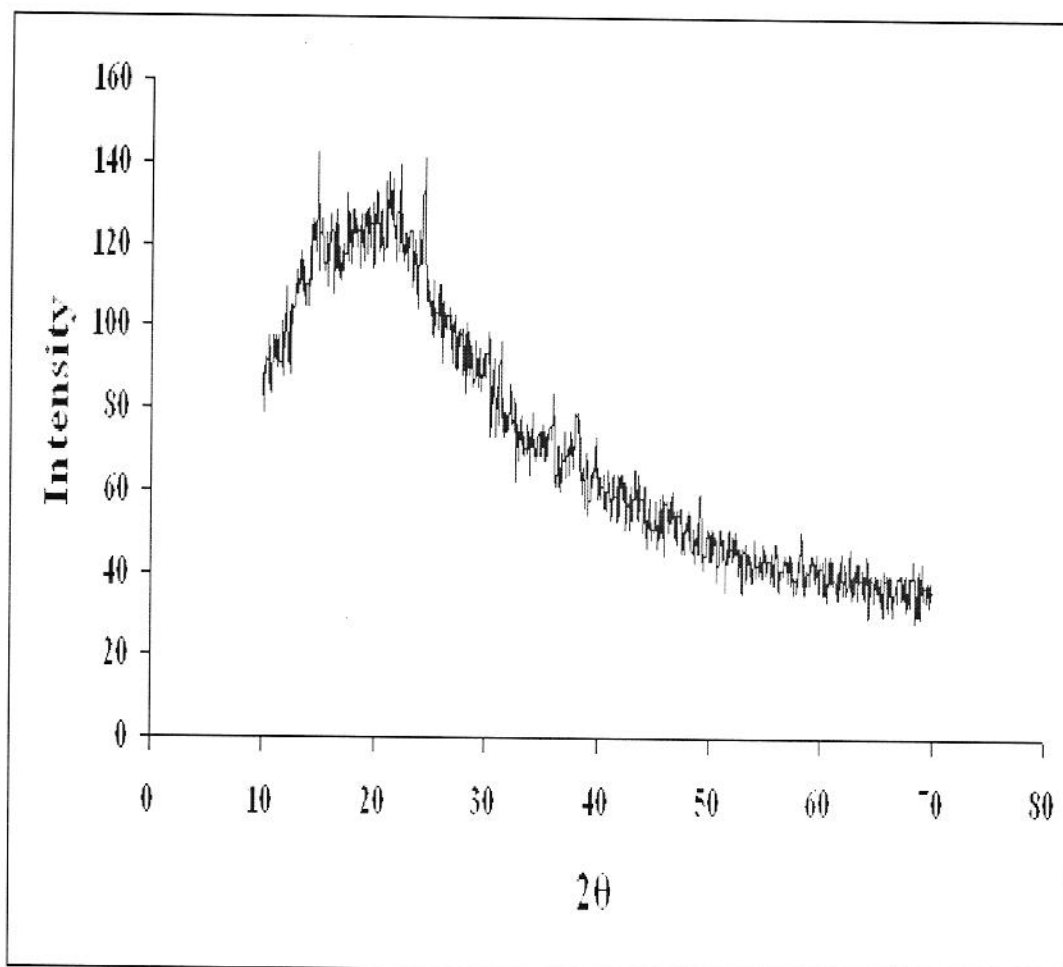


Fig. 36. XRD Pattern of *H. rhamnoides* L. Leaves Powder.

Table. 11. Crystalline Size Determination of AgNPs using Debye-Scherrer's equation.

S#	2 θ	FWHM	$\beta = \pi * \text{FWHM} / 180$	$D = k\lambda / \beta \cdot \cos\theta$
1	34.24	0.279	0.004867	327.92 Å°
2	38.12	0.402	0.00732	218.40 Å°
3	44.32	0.518	0.01011	154.49 Å°
4	64.52	0.559	0.00975	175.55 Å°
Average crystalline size of AgNPs = 219.09 Å° = 21.909 nm				

$\pi = 3.14$, $K = 0.94$, $\lambda = 1.54 \text{ Å}$, FWHM = full width at half maximum

Table. 12. XRD Pattern of AgNPs.

S#	2 θ value	d-value	INT.
1	34.24	2.621	63
2	38.12	2.362	227
3	44.32	2.046	71
4	64.52	1.448	42

3.14.6. Thermogravimetric-differential Thermal Analysis (TG-DTA) of AgNPs

The prepared AgNPs were characterized by TG-DTA technique. The recorded TGA and DTA spectra were carried out in the range of temperature as of 44.3241°C to 910.5593°C using equipment Diamond TG/DTA Parkin Elmer USA. A ceramic (Al_2O_3) crucible was used for heating purposes and in nitrogen atmosphere measurements were carried out in at the heating rate of 10°C/min. TGA and DTA curves of bioinspired AgNPs is given in Figure 37.

The TGA curve in Figure 37 shows that at 50.724°C, 70.034°C and 131.198°C the weight reduction of AgNPs were 8.532 mg, 8.484 mg and 8.191 mg respectively, which demonstrate no significant weight reduction. While prominent weight reduction footstep starting from 194.027°C till to 588.531°C, which showed that in this temperature range, weight was reduced from 7.601 mg to 1.002 mg, but no extra loss of weight was recorded at beyond 600°C. The loss of weight was correlated to the organic matrix ignition, which was present in the prepared AgNPs, acting as a cap and stabilizing agent. The DTA curve (Fig.37) is showing that an exothermic peak was detected between 400 to 607 °C with a maximum at about 500 °C. It was concluded that the thermal processes be able to be linked through the burnout of organic matrices occurred in the *H. rhamnoides* leaves aqueous extract and of the left over carbon residue.

In the DTA curve, at 750 °C an exothermic peak was recorded for magnetic NPs, because of transformation of the maghemite-hematite [150]. On behalf of NPs a sintering procedure has to acquire place prior to the transformation of a superior symmetry phase into further by inferior symmetry [151]. For this transition the

documented temperature ranges from 200 to 500 °C for NPs and among 500 and 600 °C for bulkiness samples. Temperature of transition decline by declining particle size because of the reduction in activation energy of the system although the impurities exists on the surface, like Na, might delay the transformation up to 750 °C [152]. As a result, impurities at the surface could be accountable for the high transformation temperature for magnetic NPs [150].

It was observed [153] that main loss of weight of the sample happened in temperature between 200 and 300°C. Almost there was zero weight reduction beneath 200°C and over 300°C. This was usually accredited to the organic constituents and water evaporation. The display plot of DTA was strong exothermic peak involving 200°C and 300°C which mostly credited to AgNPs crystallization. The (TG-DTA) curves of as-prepared In_2O_3 precursor observed a greater weight loss from 190°C up to about 350°C with slightly loss weight from 350°C to 600°C and no more loss weight was calculated above 600°C. The weight loss and combustion of organic matrix are correlated. Curve on the DTA a major exothermic effect was seen between 260°C and 360°C with a maximum at about 320°C, assuming that the thermal activity can be related with the burnout of organic matter involved in the precursor powders (remaining organic matter from *Aloe vera* extract), due to direct crystallization of nanocrystal In_2O_3 from the residual carbon or from amorphous component [154]. The DTA curve of YFeO_3 nanocrystalline [155] exhibits a wide peak in the temperature range 220-350 °C, probably linked with the removal of bound organics and water. The decomposition and dehydration processes were apparent in the TG curve as supported by a nonstop reduce in the NPs weight. The TGA plot of the AgNPs [36]

observed a stable weight failure in the 0-100 °C temperature due to loss of moisture. Between 100-750 °C, weight reduction was due to organic compounds degradation. Subsequently, over 750 to 1100 °C degradation was absent and residual substance accounts for Ag weight. TGA study exposed that preliminary substance was 4.16 mg and overall reduction of organic material was 1.81 mg (35%).

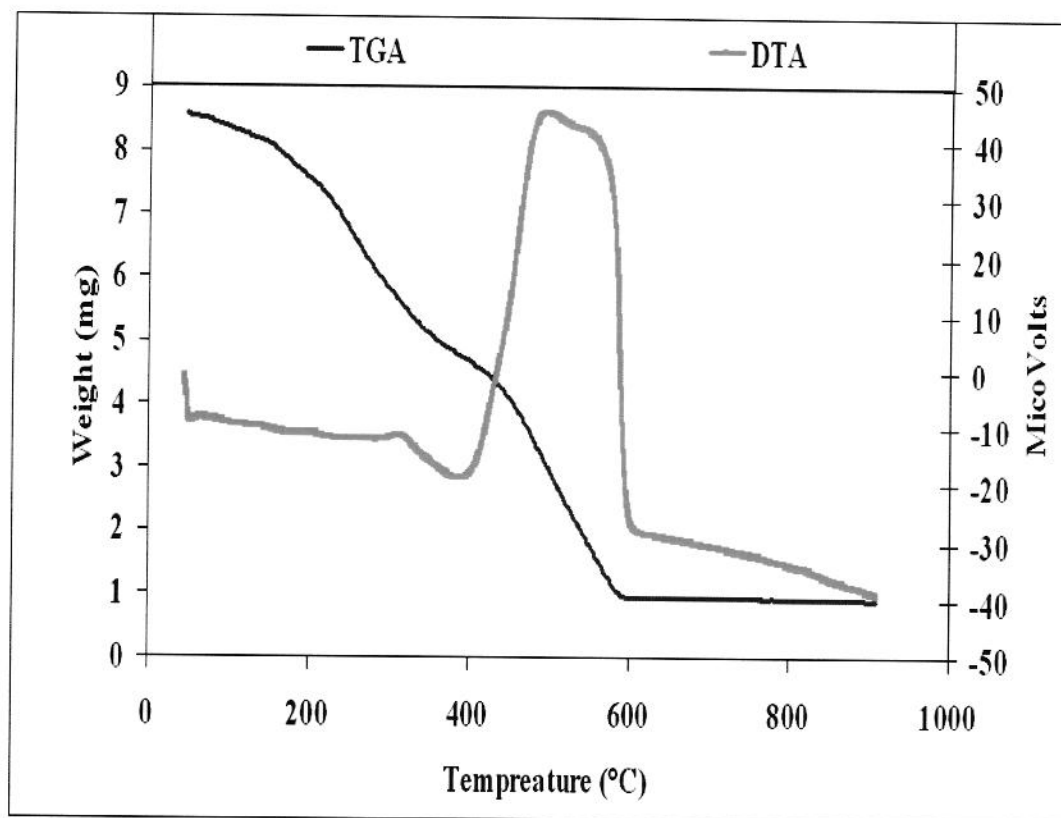


Fig. 37. TG/DTA Curves of AgNPs.

3.15. Optimization of Different Reactions Conditions for the synthesis of AgNPs

3.15.1. Temperature

Figure 38 is showing the UV-Visible spectra of AgNPs at different reaction temperature. Reaction temperatures play an important role to control the nucleation process of NPs configuration. Absorbance increased with raise in the temperature from 25 to 75 °C and thereafter decreased at higher temperatures. Experimental temperature range is from 25°C to 85°C, maximum synthesis was obtained at 75°C. Our study indicates that the most favorable temperature i.e. 75°C is quite precise for AgNPs synthesis by *H. rhamnoides* L. leaves giving maximum absorbance at 435 nm.

Increase in absorbance of reaction mixture with the increase in incubation temperature obviously depicts the higher synthesis of AgNPs at high temperatures [156]. Moreover, NPs synthesized at higher temperature exhibit surface plasmon resonance at narrow absorption range indicating monodispersity. Increase in temperature increased the rate of formation of AgNPs from silver ions, retarding the secondary reduction process. A study reported that absorbance increased with increase in the temperature from 25 to 45°C and thereafter decreased at higher temperatures [36]. These observations follow the same pattern as in our study. Our study finding was in close agreement of the previous study [38] in which the raise in temperature, increased the rate of AgNPs formation also increased. Because of the reduction in aggregation of the growing NPs the size was reduced initially. Rising the temperature to 75 °C help the crystal formation around the nucleus which causes the absorption. It was observed [11] that maximum formation of AgNPs was at 50°C. At

low temperature the reaction was sluggish and at 4°C, 20°C and 30°C no major dissimilarity was found. But raise in temperature was directly proportional to boosting the reaction rate. The UV-Visible absorption spectra showed [157] that maximum absorption peak was observed at 40°C during the study temperature range (25-45°C). The absorption was decreased at 45°C, our study reported that maximum absorption was showed at 75°C, increase in temperature (85°C) decreased the absorption.

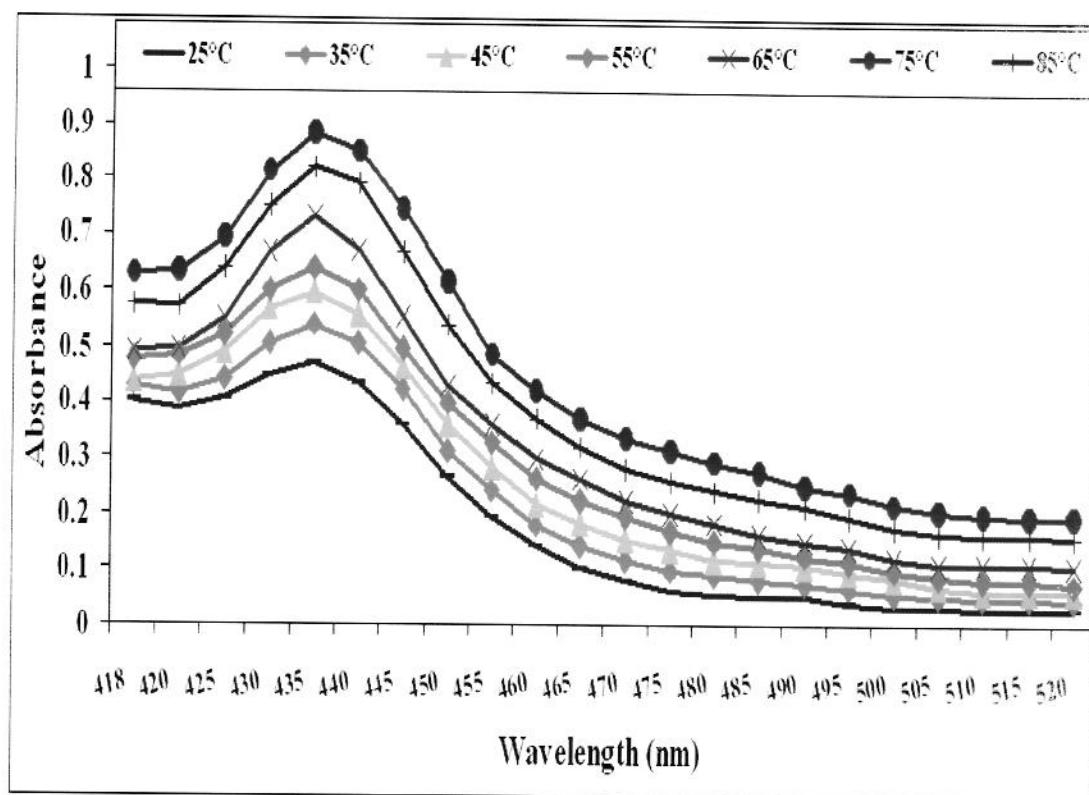


Fig. 38: UV-Visible Spectra of AgNPs Showing Effect of Different Reaction Temperature.

3.15.2. Optimization of pH

Effect of pH on the bioinspired fabrication of AgNPs by means of *H. rhamnoides L.* leaves were tested over a wider pH range (4, 5, 6, 7 and 8) as shown in Fig. 39. The UV-Visible absorption spectra are clearly indicating that the maximum absorption was at pH 7.0. Increase in pH shows decrease in absorption. So current study reveals that optimized pH was 7. It was observed that pH play a significant role during AgNPs synthesis to control shape and size. Larger size NPs were synthesized at low pH (acidic), whereas, at higher pH (alkaline), smaller size NPs was synthesized [36]. To study [158] the effects of pH on the shape and size of AgNPs showed that NPs aggregation seems to outdo the nucleation process in acidic conditions. At alkaline pH, though, the great numbers of nuclei formation, instead of aggregation, more NPs with smaller diameter were synthesized. Blue shift in absorption pattern confirmed formation of relatively smaller NPs. It was described [38] that low pH (acidic) decreases the synthesis of AgNPs, while high pH (basic) increases the synthesis of AgNPs. At lower pH (4) large NPs were synthesized, where as at high pH (8) highly dispersed and small NPs were synthesized. At acidic pH (low), the aggregation of AgNPs to prepared bigger NPs was supposed to be preferred over the nucleation. At alkaline pH (higher), though, the great number of functional groups accessible for silver binding to create a higher number of AgNPs. As a result small size NPs formed. But agglomeration of NPs took place at alkaline pH. The optimum conditions (pH and temperature) were found to be 7.0 and 80°C respectively [159]. In our study the same pH effect is found. It was pointed out that at low pH (2 and 5) NPs synthesis don't take place. But at high pH (9 and 11) rapid colour appearance was observed but

shifted peak to 500 nm. At pH 13 agglomerations was seen instantly following addition of AgNO_3 into the leaf extract. The reaction happened immediately when silver nitrate was supplemented in the leaf extract at pH 7 (neutral) and the synthesis was found in 30 min [51]. It was observed [53] that at 0.5 hrs (reaction time), red shift in the SPR observed in acidic pH in the colloidal solution of AgNPs. This means that the large size particles were formed in acidic pH. In alkaline medium blue shift in SPR clearly indicated the formation of small particles. At pH 2, there was no indication of AgNPs formation in UV-Vis spectra while flocculation observed in reaction medium. This was due to the fact that higher positive charges at the surface of NPs attracted negative charge biomass, which lead to flocculation. A great fall in the flocculation factor in high pH (alkaline) was also observed. Thus, change in SPR was a function of solution pH. In alkaline pH, because of the occurrence of high OH concentration on the surface of NPs, repulsive force dominated in colloidal solution, and thus, particles aggregation reduced. This leads to the size reduction and blue shift in SPR spectra. The finding shows that a maximum absorption sharp peak was show at pH 7.0 [157], which is a close agreement to the present study.

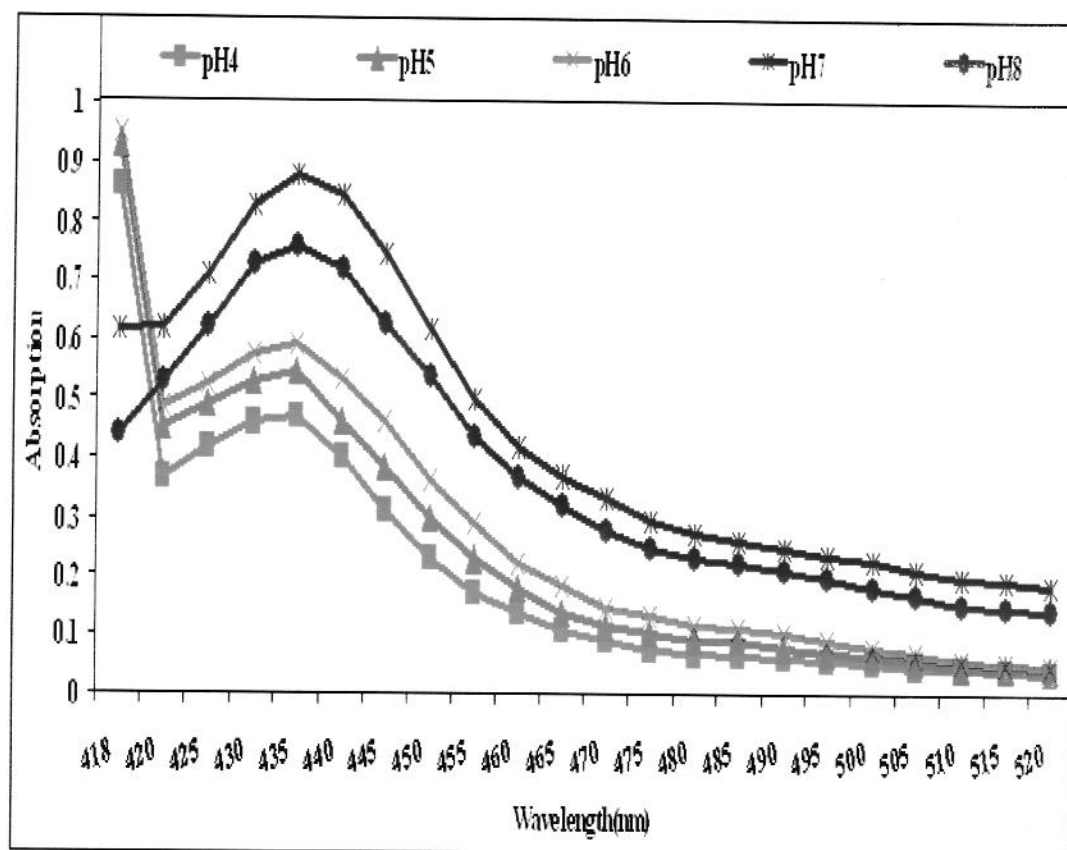


Fig. 39. UV-Visible Spectra of AgNPs Showing Effect of Different Reaction pH.

3.15.3. Time

Absorbance of AgNPs increased with increase in incubation time and maximum absorbance was recorded at incubation time of 1 hrs (Fig.40). But increasing incubation times beyond 1 hrs observe no significant increase in absorption. The study found that absorbance of silver colloidal solution increased with span of time and maximum absorption was observed after 12 hrs of reaction. Aggregation of NPs after 36 hrs of reaction was observed which a sign of instability. Reaction time of more than 10 hrs was due to less redox potential of silver ions [36]. These conclusions were in conformity with explanation drawn by [160]. More AgNPs was formed as the period of reaction increases. An optimum period is required due to the unsteadiness of the AgNPs synthesis, as AgNPs agglomeration after the optimum incubation resulting in larger sizes of particles. The optimum duration necessary for the end of reaction was 1 hrs [38]. In the present study the same duration is observed (Fig.40). It was found that at 0 and 30 minutes no formation of NPs occurred but formation start quickly at 60 minutes and ended in 5 hrs [11]. It was observed that the reaction was happening as rapidly as the AgNO_3 was mixed with leaves extract and the synthesis was achieved within 30 min. After 30 min increasing duration of the reaction absorption also increase but AgNPs take place at 30 min [51]. The formation of AgNPs started within 0.5 hrs incubation and reaction mixture color was changed from translucent to orange-yellow because of the SPR vibration excitation. The SPR intensity and absorbance rise as the reaction period increased up to 8 hrs. There was no change in SPR, means that the stability of the AgNPs colloidal solution within the reaction time period. SPR peak centered at 435 nm (λ_{max}) [53], which is a close

agreement to our finding. Silver nanoparticles were fabricated quickly within 30 min of incubation time with brown color. It may be due to the reduction of AgNO_3 and surface plasmon resonance excitation effect [69]. The study reported that the absorption peaks increased as the incubation time increased (24 hrs - 120 hrs) [157]. Silver nanoparticles were prepared from 0.5% gum at 1 mM AgNO_3 with varying the reaction time (10–60 min). It was noticed from the UV peaks that absorbance increased when the time increased. Perhaps more and more hydroxyl groups were converted to C=O groups through the oxidation of air, as a results silver ions reduction take place [142]. These observations were a close agreement to our findings.

The SPR band increases as the time increases (0, 3, 6, 9 and 12 hrs) confirming that the synthesis of AgNPs was accomplished in 9 hrs. The UV absorbance did not vary considerably with additional reaction time for 12 hrs; so, optimize time condition was 9 hrs for AgNPs synthesis using water extract 5% (v/v) [161]. These findings support our results, as in our investigation the optimize time was 60 min, but increase in absorption and then no significant increase in absorption phenomena were the same. The UV absorbance spectra [40] of AgNPs observed from thirty 30 min to 4 hrs 15 min, a red move in λ_{max} from 420 to 444 nm was indicated. When the incubation time prolong to 24 hrs, the value of λ_{max} was not change drastically, although values of absorbance were rises. At 24 hrs the maximum absorbance was 0.77.

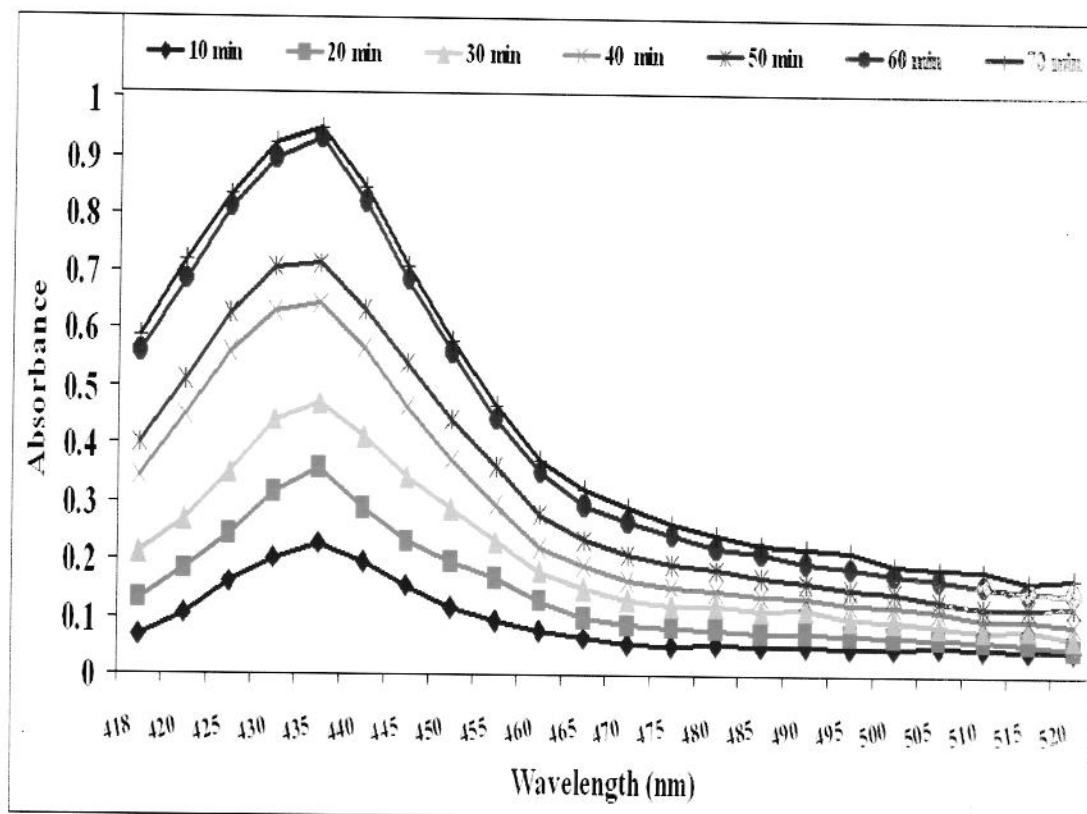


Fig. 40. UV-Visible Spectra of AgNPs Showing Effect of Reaction Time.

3.15.4. Concentration of Silver Nitrate Solution

Effect of initial AgNO_3 concentration was investigated through changing the silver nitrate concentration (0.25, 0.5, 0.75, 1.0 and 2 mM). Increased in yield was observed when metal salts concentration was increased from 0.25 – 1 mM. Beyond this there was again fall in absorbance (Fig. 41). A study found that increase in yield of AgNPs was observed when metal salt concentration was increased from 0.5 - 4 mM. Beyond this there was again fall in absorbance; hence 4 mM concentration of AgNO_3 was selected for further experiments [36]. Similar effect of varying concentration of plant extract and silver salt on yield, size and disparity of AgNPs was investigated [162]. But in the present study 1 mM was the optimum condition for AgNPs. The study has found that the maximum peak was observed in 0.5 mM silver nitrate [31]. In another study a maximum yield of AgNPs was found with silver nitrate (1 mM) solution [38], support our results (Fig. 41). The rate of plant extracts based synthesis of AgNPs was reported showing slowest rate of synthesis were found with AgNO_3 (0.3 mM), but low rate of bioreduction was observed with the higher concentrations and the 0.7 mM was the optimum concentration for the maximum synthesis (very close to our findings) [11]. Study showed [51] that 1 mM AgNO_3 observed fast synthesis while at 2 mM and 3 mM concentrations of AgNO_3 shifted of peak take place.

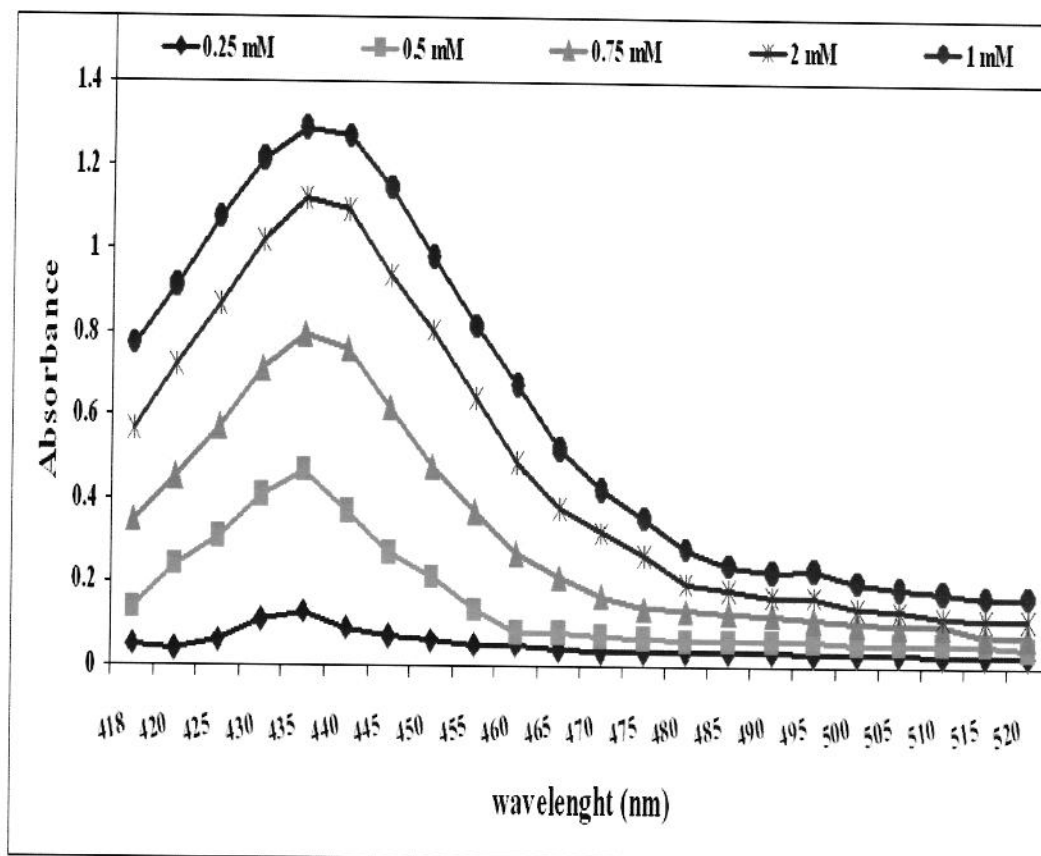


Fig. 41. UV-Visible Spectra of AgNPs Showing Effect of Concentration of AgNO_3 .

3.15.5. Concentration Ratio of Silver Nitrate and Leaves Extract

Optimization of concentration ratio of silver nitrate and leaves extract are shown in Fig. 42. It was observed that ratio of 5:95 give maximum absorption and at high leaf extract (10 ml) with 90 ml of 1 mM AgNO_3 , the absorption decreased. It was reported that optimal extract and metal salt ratio was necessary for the symmetrical NPs preparation [163]. Diluted extract of flower containing 2 ml extract in water (100 ml) i.e. 0.02 dilution was capable to fabricate the highest concentration of AgNPs as shown by maximum absorbance at 420 nm. Sharp peak was observed at 0.02 dilution of flower extract as matched with other peaks [36]. The study was found that the optimum ratio for the reaction was 1:19 based on the number of trials and the optimum yield, these observations was a close agreement to our results [38]. It was concluded that 50 ml reaction mixture containing leaves extract (6 ml) and 44 ml AgNO_3 (1 mM) was changed to brown colour in incubation time (30 min), signifying fast synthesis of AgNPs. Increasing the leaves extract shifted the peaks towards 500 nm [51]. It was reported that [162] the resultant color changes were noticed from reddish-yellow to deep red with rising ratio of leaves extract. It was observed that the 1:30 of leaves extract in the mixture of reaction were efficient for the synthesis of AgNPs and AuNPs.

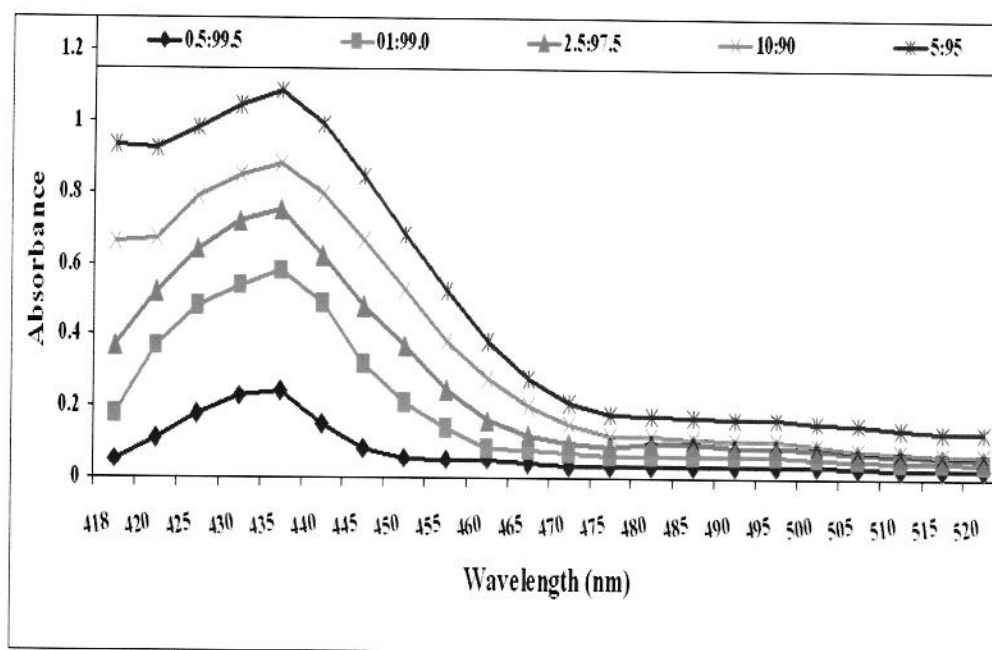


Fig. 42. UV-Visible Spectra of AgNPs Showing Effect of Leaves Extracts and AgNO₃.

3.16. Mechanisms of AgNPs Synthesis

Several possible mechanisms of phenolic compounds with metals have been proposed by different researchers and widely discussed in literature. Bors [164] first proposed the three important criteria for effective activity. (1) The ortho dihydroxy structure in B ring in flavonoids can give higher stability and involved in delocalization. (2) The 2-3 double bonds in configuration with a 4-oxo function in the C ring is responsible for electron delocalization from the B ring. (3) The additional presence of 3-OH and 5-OH groups in the A and C ring largely accounts the activity potential. The number and position of OH also directing the potency of scavenging metal ions as shown in Fig. 43 [164, 165]. Quercetin a member of flavonoids family exhibits potent scavenging and strong antioxidant activity, because it fulfilled all the above required conditions. Flavonoids can chelate metal ion (Ag^+) by removing H-atom. It was also proposed that the 3-OH group is the site to be attached first and then the artho-OH group and finally 3-OH group. For a single flavonoid molecule there are several possible sites that could bind silver (Ag^+) ions [166,167]. These are shown in Fig. 44. The oxidation of phenolic quercetin into quinone form can be shown by the following mechanism Fig. 45.

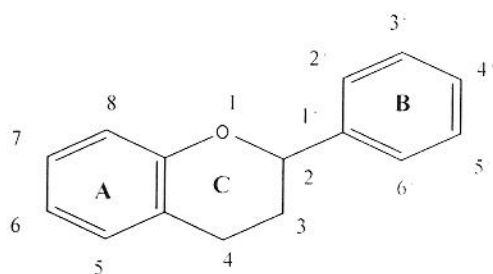


Fig.43. Structure of Flavonoids.

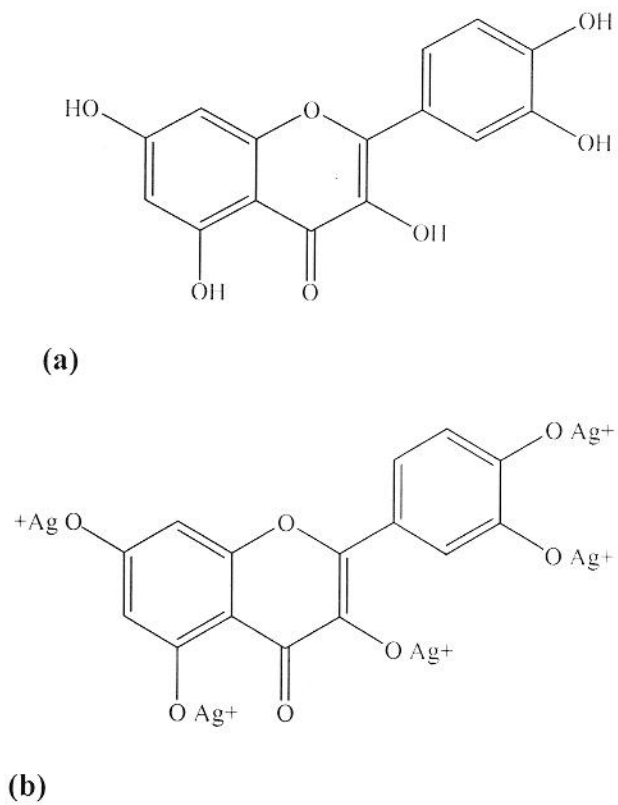
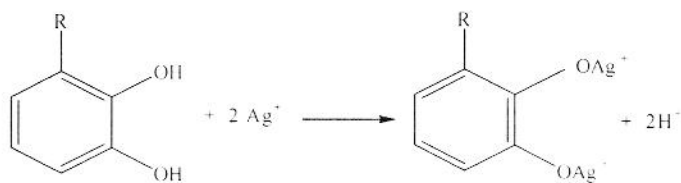
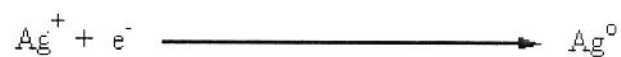
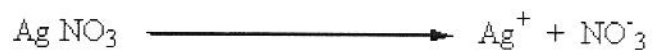
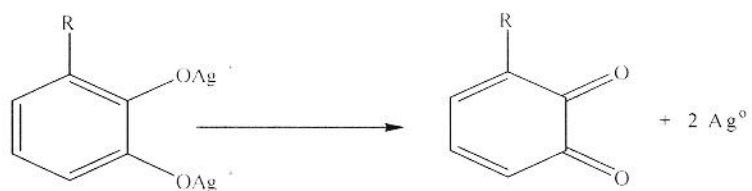


Fig.44. (a) Quercetin, (b), Possible sites to be attacked by Ag^+ .



Phenolic form

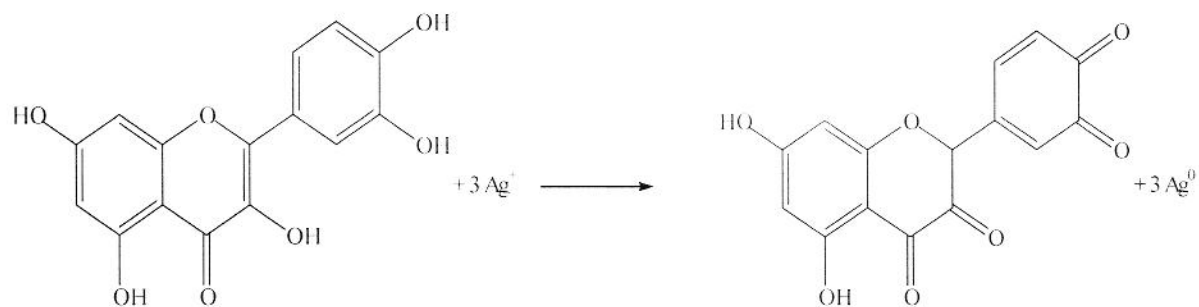
Ag complex



Ag complex

Quinone form

AgNPs



Phenolic form (Quercetin)

Quinone form

AgNPs

Fig.45. Formation of AgNPs.

3.17. Biological Evaluation of Bioinspired Synthesized AgNPs

3.17.1. Antibacterial Activity of AgNPs

The antibacterial activity of bioinspired synthesized AgNPs is shown in Table.13. The biosynthesized AgNPs antibacterial activity indicates that it have strong activities against test bacterial strains. The synthesized AgNPs showed highest zone of inhibition 19 ± 0 mm against *E. coli*, followed by 18 ± 0 mm against *C. freundii* and 17 ± 0 against *P. aeruginosa*, while the lowest was observed 12 ± 0 mm against *E. faecalis*. The other zone of inhibition 14 ± 0 mm, 13 ± 0 mm, 14 ± 1 mm, 16 ± 0 mm, 15 ± 0 mm and 16 ± 1 mm were presented against *S. aureus*, *B. cereus*, *B. subtilis*, *K. pneumoniae*, *V. cholerae* and *S. typhi* respectively. In wide-ranging, G-positive bacteria were found to be more tolerant to AgNPs than G-negative strains. The AgNO_3 at tested concentration showed a high zone of inhibition as compared to AgNPs and the highest zone of inhibition was observed 22 ± 0 mm against *C. freundii*. The 2nd highest zone of inhibition was calculated (21 ± 0 mm) against *E. coli*, followed by 20 ± 1 mm against *P. aeruginosa* and *S. typhi*. The lowest 15 ± 0 mm was found against *B. cereus*, the 18 ± 0 mm was reported against *S. aureus*, *V. cholerae* and *K. pneumoniae*. The zone of inhibition against *E. faecalis* and *B. subtilis* was 17 ± 1 mm and 16 ± 0 mm respectively. The control (sterile distilled water) exhibited no zone of inhibition against all the test bacteria. The positive control streptomycin showed a high zone of inhibition 23 ± 0 mm against *K. pneumoniae* followed by 21 ± 0 mm against *V. cholerae*, while 19 ± 0 mm was observed against *S. aureus* and *B. cereus*. But against *E. coli*, *S. typhi* and *C. freundii* was found 20 mm. The *E. faecalis* and *B.*

subtilis showed 18 mm zone of inhibition and 17 ± 0 mm was seen against *P. aeruginosa*.

The increase of resistance to synthetic antibiotics/drugs possesses a severe long-standing trait to community health. Nowadays, there is a growing concentration for the replacement of artificial drugs and their appliance in pharmacy and medicine [168]. The biosynthesized AgNPs zones of inhibition were found to be 16 mm for *E. coli*, 15 mm for *K. pneumoniae* and 30 mm for *S. aureus* [139]. The current study finding results showed that prepared AgNPs showed zone of inhibition for *E. coli* (19 ± 0 mm) and *K. pneumoniae* (16 ± 0 mm), while low zone of inhibition against *S. aureus* (14 ± 0 mm). Low zone of inhibition against *S. aureus*, *V. cholerae* and *P. aeruginosa* was found [12] as compared to our prepared AgNPs (Table 13). The highest ZI [14] was calculated to be 3 mm against *S. aureus* and lowest of 2 mm among the whole test bacterial strains. The antimicrobial activity was checked against *E. coli* (ampicillin resistant), *E. coli*, *S. aureus*, and *S. typhi* (multi-drug resistant) and concluded that the result was dosage dependent and was more distinct against gram-negative than gram-positive bacteria [169]. The antibacterial activity of AgNPs with a concentration of 20 μ l (0.002 mg) against *S. typhi*, *S. aureus*, *P. aeruginosa*, *E. coli* and *K. pneumonia* measured zone of inhibition (mm) were 40, 36, 35, 34 and 30 respectively [170]. These zones of inhibition were high as compared to our prepared AgNPs. The AgNPs were efficient antibacterial activity against G (-) bacteria as compared with G (+) bacteria [139], which was close agreement to our findings. The antibacterial activities of AgNPs ($100\text{ }\mu\text{g disk}^{-1}$) observed that *E. coli* inhibition zone was 22.20 mm (high as compared to our findings) and *B. subtilis* was 13.93 mm [48]

low as compared to the current study. Silver nanoparticles synthesized from *Boswellia*, *Shorea* and *Svensonia* reported strong antibacterial activities against *Bacillus*, *Pseudomonas*, *E. coli*, *Proteus* and *Klebsiella* in the range 8-12 mm zone of inhibition [43]. The biosynthesized AgNPs reported antibacterial activity (zone of inhibition) in the range of 11-20 mm (2-8 mg) [141] but the present study revealed that biosynthesized AgNPs at low concentration (1 mg/ml) were give a high zone of inhibition as compared to these findings. The recorded AgNPs antibacterial activity against *P. aeruginosa*, *E. coli* and *S. aureus* zone of inhibition were 10 mm, while *B. subtilis* have found no zone of inhibition [50], but in the current study the prepared biosynthesized AgNPs were show more potent antibacterial activities as compared to these chemically synthesized nanoparticles.

Table 13. Antibacterial Activity of AgNPs.

S#	Tested Bacteria	Zone of Inhibition (mm)				
		Gram (-/+)	AgNO ₃	AgNPs	C ⁺	C ⁻
1	<i>S. aureus</i>	+ve	18±0	14±0	19±0	0
2	<i>E. faecalis</i>	+ve	17±1	12±0	18±0	0
3	<i>B. cereus</i>	+ve	15±0	13±0	19±0	0
4	<i>B. subtilis</i>	+ve	16±0	14±1	18±1	0
5	<i>E. coli</i>	-ve	21±0	19±0	20±0	0
6	<i>K. pneumoniae</i>	-ve	18±0	16±0	23±0	0
7	<i>P. aeruginosa</i>	-ve	20±1	17±0	17±0	0
8	<i>V. cholerae</i>	-ve	18 ±0	15±0	21±0	0
9	<i>S. typhi</i>	-ve	20±1	16±1	20±0	0
10	<i>C. freundii</i>	-ve	22±0	18±0	20±1	0

Each value represents mean ± SD (n = 3), 0 = No zone of inhibition.

C⁺ = Positive Control (Streptomycin), C⁻ = Negative control (Sterile distilled water).

3.17.2. MIC and MBC of AgNPs

The MIC of bioinspired synthesized AgNPs are shown in Fig. 46. The MIC ($\mu\text{g/ml}$) of AgNPs for *S. aureus*, *E. faecalis*, *B. cereus*, and *B. subtilis* was 300, 450, 400 and 350 respectively. While 150, 180, 160, 200, 190 and 150 were recorded for *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *V. cholerae*, *S. typhi* and *C. freundii* respectively. The MIC of streptomycin were high i.e. 320, 300, 200 and 100 for *B. subtilis*, *B. cereus*, *E. coli* and *S. typhi* respectively. However MIC in case of *S. aureus* and *P. aeruginosa* as 50 $\mu\text{g/ml}$, for *C. freundii* and *E. faecalis* was 80 $\mu\text{g/ml}$, for *K. pneumoniae* was 60 $\mu\text{g/ml}$ and for *V. cholerae* was 70 $\mu\text{g/ml}$. The silver nitrate MIC proved that it has strong inhibitory activity against all the test bacterial strains and *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. typhi* and *C. freundii* showed lowest MIC 10 $\mu\text{g/ml}$. While 15 $\mu\text{g/ml}$ MIC was found against *V. cholerae* and highest MIC 20 $\mu\text{g/ml}$ for *S. aureus*, *B. cereus* and *B. subtilis*.

The MBC data of synthesized AgNPs are shown in Fig. 47. An analysis of MBC values for cultures strains indicated that the *E. faecalis* and *B. cereus* showed 900 $\mu\text{g/ml}$, *S. aureus* MBC was 1000 $\mu\text{g/ml}$, *B. subtilis* was 800 $\mu\text{g/ml}$. The MBC 220 $\mu\text{g/ml}$ and 250 $\mu\text{g/ml}$ against *E. coli* and *C. freundii* respectively, followed by 300 $\mu\text{g/ml}$ for *P. aeruginosa*, 450 $\mu\text{g/ml}$ for *K. pneumoniae*, 500 $\mu\text{g/ml}$ for *S. typhi* and 600 $\mu\text{g/ml}$ for *V. cholerae*. Our results showed that all the test bacterial strains were most sensitive against AgNO_3 . The MBC observed in this study for silver nitrate were 25 $\mu\text{g/ml}$ for *S. aureus*, *B. cereus* and *B. subtilis*, 20 $\mu\text{g/ml}$ for *E. faecalis* and *V. cholerae*, 15 $\mu\text{g/ml}$ for *E. coli*, *P. aeruginosa* and *S. typhi* and 10 $\mu\text{g/ml}$ for *C. freundii* and *K. pneumoniae*. The MBC for streptomycin were 400 $\mu\text{g/ml}$ against *B.*

cereus and *B. subtilis*, followed by 350 µg/ml for *E. coli*, 200 µg/ml for *S. typhi* and *E. faecalis* and 100 µg/ml for *S. aureus*, *P. aeruginosa* and *V. cholerae*. The other MBC values were 130 µg/ml and 120 for *C. freundii* and *K. pneumoniae*.

The study reported that MIC (µg/ml) range of silver nitrate was 3.9 – 15.6 and MBC range were 7.8 – 31.2. Similarly MIC (µg/ml) of silver nanocomposite was 62.5 – 500 and MBC (µg/ml) were 125 – 1000 [45]. These findings were a close agreement to the present study, but AgNO₃ observed some variation. The study showed the zone inhibition (mm) of AgNO₃ (5 mg/ml) were 18, 15 and 14 against *S. aureus*, *E. coli* and *P. aeruginosa* respectively, while MIC (mg/ml) were 1.25, >2.50 and 2.50 against *S. aureus*, *E. coli* and *P. aeruginosa* respectively and MBC (mg/ml) was >2.50 for all the test bacteria [171]. These findings suggested that in current study synthesized AgNPs were more potent antibacterial activities. The MIC (µg/ml) values ranged 3.125 - 25.0 and MBC (µg/ml) values in the ranged 3.125 - 50.0 against test bacteria [48]. Similarly in another study [49] MIC (µg/ml) value of AgNPs against *Staphylococcus spp*, *E. coli* and *B. subtilis* found 53, 27 and 54 respectively. The MIC and MBC values were low in these studies as compared to *H. rhamnoides* L. leaves AgNPs. It was concluded that nano colloidal silver does not have considerably diverse antimicrobial activity against Gram positive and negative bacteria [25]. The antibacterial activity of nanosilver concluded that Gram negative bacteria were more sensitive than Gram positive bacteria. The Gram positive cells were thicker due to the peptidoglycan than Gram negative. The thicker cell wall of *S. aureus* was responsible for protecting the cell from penetration of Ag⁺ into the cytoplasm, where its react with DNA and cause death of the bacteria [2]. Theses observations were consistent to

the present study findings. Silver nanoparticles MIC ($\mu\text{g/ml}$) against *P. aeruginosa*, *B. subtilis*, *E. coli* and *S. aureus* were >4096, >4096, 2048 and 1024 respectively and MBC ($\mu\text{g/ml}$) were >4096, >4096, >4096 and 4096 against *P. aeruginosa*, *B. subtilis*, *E. coli* and *S. aureus* respectively [50]. These MIC and MBC values were higher as compared to our biosynthesized AgNPs (MIC & MBC). It has possible due to the development and synthesis of AgNPs to utilize silver once again strong antibacterial agents [170]. Research proofs that bacteria have been loses its DNA replication when treated with silver ions [172]. The AgNPs may contact to the cell membrane surface and deteriorate its work power such as respiration and permeability. The particles binding to the microbes depend on the availability of surface area for interaction. Nanoparticles which have characteristically large surface area availability for attachment will show high antibacterial effect than the macro particles [173]. Research study [174] demonstrates that AgNPs were found inside the bacteria as well as on the cell membrane surface. These conclude the possibility that the AgNPs may enter into the cell of bacteria and fungi, causing deterioration by interfering with sulphur and phosphorus components compounds (DNA). Silver exhibit have a strong affinity to react with such compounds. Another possible conclusion about the antimicrobial activities of AgNPs would be the releasing of Ag^+ from AgNPs and act against microbes. Antibacterial activity [175] was probably due to electrostatic attraction between positive charged nanoparticles and negative of cell membrane of microorganisms.

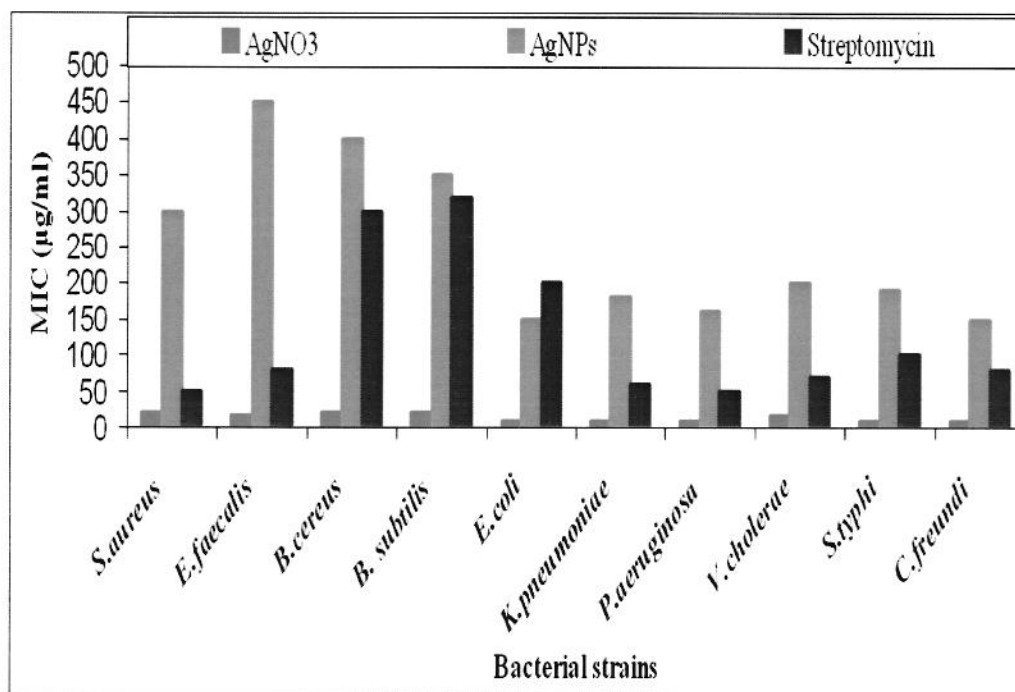


Fig. 46. Antibacterial MIC (µg/ml) of AgNPs.

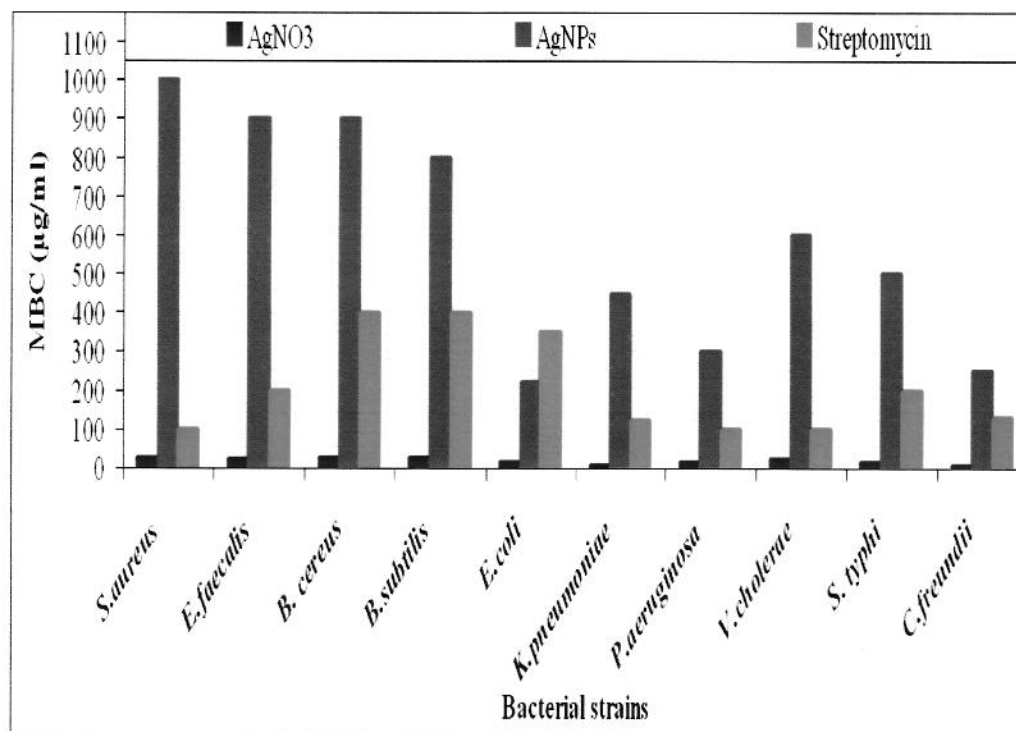


Fig. 47. Antibacterial MBC (µg/ml) of AgNPs.

3.17.3. Antifungal Activity of AgNPs

The antifungal activity of bioinspired AgNPs are summarized in table 14. The prepared AgNPs showed low zone of inhibition i.e. 10 ± 1 mm and 11 ± 0 mm for *R. arrhizus* and *P. digitatum*. The highest ZI (mm) were 18 ± 0 , 17 ± 1 , 16 ± 1 and 15 ± 1 detected against *A. alternata*, *F. oxysporum*, *A. flavus* and *A. paraciticus* respectively. The inhibition zone for *C. albicans* and *A. fumigatus* was 13 ± 0 mm and 12 ± 0 mm, while 14 ± 0 mm was measured for *A. oryzae* and *A. niger*. Silver nitrate (2 mg/ml) showed great antifungal activities with the highest activity against *A. alternata* (20 ± 0 mm), *F. oxysporum* (19 ± 1 mm) and *A. oryzae* (18 ± 1 mm), followed by *A. niger* and *A. flavus* (17 mm each), *A. paraciticus* (16 ± 0 mm), *A. fumigatus* (15 ± 0 mm), *R. arrhizus* and *C. albicans* (14 mm each) and *P. digitatum* (13 ± 0 mm). Bifonazole showed lowest zone of inhibition 10 ± 0 mm against *P. digitatum* and the highest activity against *A. oryzae* and *F. oxysporum* (17 mm each) followed by *F. oxysporum* (16 ± 0 mm), *A. flavus* and *R. arrhizus* (15 ± 0 each), *A. paraciticus* (14 ± 0 mm), *A. niger* (13 ± 0 mm), *C. albicans* and *A. fumigatus* (12 ± 0 mm each). Negative control (distilled water) observed no zone of inhibition against all the tested fungi.

Silver nanoparticles ($100 \mu\text{g/ml}$) activity against *C. albicans* was 13.5 mm [176]. The AgNPs activity against *C. albicans*, *A. niger*, *A. flavus* and *A. fumigatus* exhibited were 15.22 mm, 12.24 mm, 06.04 mm and 07.05 mm respectively [177]. The AgNPs recognized superior antifungal activity ($5 \text{ mg}/10 \mu\text{l}$) against plant pathogenic fungi [51]. The biosynthesize AgNPs showed antifungal activities against *A. niger* was 13.24 mm, *A. flavus* 08.02 mm and *A. fumigatus* 09.02 mm [143] which demonstrate that in the present study (Table 14) synthesized AgNPs were more potent activity

against these fungus. The AgNPs (8 mg/ml) activities against *Fusarium* sp., *C. albicans* and *Aspergillus* sp. were 5 mm, 14 mm and 15 mm [141]. Our prepared AgNPs were more active (2 mg/ml) towards all test fungal strains. Antifungal activity of AgNPs (50 μ L) against *A. flavus* and *A. niger* were 10.7 mm and 11.5 mm [147]. Antimicrobial activity of AgNPs prepared from stem *Shorea tumbuggaia* revealed that *A. niger*, *A. flavus*, *fusarium* and *rhizophus* were 10, 12, 12 and 8 mm zone of inhibition respectively [178]. It was concluded that 15 mg AgNPs showing high activity (ZI) against all phytopathogens [51].

The silver ions strongly interfere with group of sulfur related enzymes and deteriorate its enzymatic function [179]. The toxic effect of nanoparticles can be due to their stable colloidal nature in the medium, which modulates the phosphotyrosine pattern of the microbes proteins and stop its growth [41]. The molecular rout for the biological synthesis of these crystals silver is speculated that the biological substances contain silver binding proteins that give amino acid moieties that act as the nucleation sites [12]. The leaf extracts NPs were more toxic than that of bark extracts, due to more production of AgNPs of leaves. Besides photosynthesis are take place in green leaves and it have more availability for H⁺ ions to reduce the AgNO₃ into AgNPs [43]. It is also found that AgNPs have the capability to interact with metabolic pathways [180]. Antifungal activity of Photosynthesized AgNPs were found highly toxic against *Curvularia lunata* (10 mm), *A. niger* (11 mm), *R. arrhizus* (10 mm), and *F. oxysporum* (8 mm) [181]. The present study demonstrated that the prepared AgNPs were more effective against all the tested fungal strains due to its low concentration (1mg/ml).

Table. 14. Antifungal Activity of AgNPs.

S#	Tested Fungus	Zone of Inhibition (mm)			
		AgNO ₃	AgNPs	Bifonazole	Sterile distilled water
1	<i>A. niger</i>	17±1	14±0	13±0	0
2	<i>A. paraciticus</i>	16±0	15±1	14±0	0
3	<i>A. flavus</i>	17±0	16±1	15±0	0
4	<i>A. fumigatus</i>	15±0	12±0	12±0	0
5	<i>A. oryzae</i>	18±1	14±0	17±0	0
6	<i>F. oxysporum</i>	19±1	17±1	16±0	0
7	<i>R. arrhizus</i>	14±0	10±1	15±0	0
8	<i>A. alternata</i>	20±0	18±0	17±0	0
9	<i>C. albicans</i>	14±1	13±0	12±0	0
10	<i>P. digitatum</i>	13±0	11±0	10±0	0

Each value represents mean $\pm$ SD (n = 3). 0 =No zone of inhibition (at tested quantity), Positive Control = (Bifonazole), Negative control = (Sterile distilled water).

3.17.4. MIC and MFC of AgNPs

The MIC of silver nanoparticles is shown in Fig. 48. Silver nanoparticles showed variable MIC of fungal cultures which differ according to the tested strains. The MIC was against *A. fumigatus* and *P. digitatum* (1700 µg/ml each) followed by *A. niger*, *A. flavus*, *A. oryzae* and *C. albicans* (1500 µg/ml each), *R. arrhizus* (1450 µg/ml), *A. paraciticus* and *F. oxysporum* (1400 µg/ml each) and *A. alternata* (1200 µg/ml). The MIC of AgNO₃ against *P. digitatum* and *R. arrhizus* was 150 µg/ml and against *A. alternata* 50 µg/ml. The MIC same value 100 µg/ml were observed against *A. niger*, *A. paraciticus*, *A. flavus*, *A. fumigatus* and *A. oryzae*. While MIC (µg/ml) value 120 against *C. albicans* and 80 was against *F. oxysporum*. The MIC value of Bifonazole was 8100 µg/ml against *P. digitatum*, followed by *F. oxysporum* (1700 µg/ml), *A. niger* and *C. albicans* (1600 µg/ml each), *A. paraciticus*, *A. flavus*, *A. fumigatus* and *R. arrhizus* (1500 µg/ml each), *A. alternata* (1400 µg/ml) and against *A. oryzae* was 1300 µg/ml.

The MFC of silver nitrate are shown in Fig.49. The MFC revealed that, the AgNPs showed sensitivity at the concentration of 2000 µg/ml against *A. niger*, *A. fumigatus* and *P. digitatum*, 1800 µg/ml against *A. flavus*, *A. paraciticus* and *R. arrhizus*, 1700 µg/ml against *A. oryzae* and *C. albicans*, 1600 µg/ml against *F. oxysporum* and 1500 µg/ml against *R. arrhizus*. The Bifonazole had a MFC of 2000 µg/ml for *A. niger*, *A. paraciticus*, *A. flavus*, *A. fumigatus*, *F. oxysporum*, *R. arrhizus*, *C. albicans* and *P. digitatum*, 1700 µg/ml against *A. oryzae* and 1600 µg/ml against *A. alternata*. The MFC of AgNO₃ for *A. niger*, *A. paraciticus*, *A. flavus*, *A. fumigatus* and *A. oryzae*

was found to be 200 $\mu\text{g/ml}$, for *F. oxysporum* 120 $\mu\text{g/ml}$, for *R. arrhizus* 250 $\mu\text{g/ml}$, for *A. alternata* 100 $\mu\text{g/ml}$, for *C. albicans* 280 $\mu\text{g/ml}$ and for *P. digitatum* 300 $\mu\text{g/ml}$. Silver nanoparticles zone of inhibition against *C. albicans* was 16 mm. The MIC and MFC values of AgNPs (fungus mediated) were 5.8 $\mu\text{g/ml}$ and 9.7 $\mu\text{g/ml}$ [182]. Calculated MIC and MFC of AgNPs against *C. albicans* was 12 $\mu\text{g/ml}$ and 20 $\mu\text{g/ml}$ respectively [176]. The study observed that silver nitrate MIC and MFC against *C. albicans* was 31.2 $\mu\text{g/ml}$ and 250 $\mu\text{g/ml}$ respectively and MIC against *A. niger* was 15.6 $\mu\text{g/ml}$. While MIC and MFC of silver nanocomposite of *C. albicans* were 125 $\mu\text{g/ml}$ and 2000 $\mu\text{g/ml}$ respectively and against *A. niger* MIC was 2000 $\mu\text{g/ml}$ [45]. The AgNPs showed effective activity against *Trichophyton mentagrophytes* and *Candida* species were IC_{80} , 1-7 $\mu\text{g/ml}$ [24]. MIC_{50} and MIC_{90} of AgNPs on *C. albicans* were 0.5 mg/ml and 2 mg/ml respectively. Similarly MIC_{50} and MIC_{90} of AgNPs on *Saccharomyces cerevisiae* were 4 mg/ml and 32 mg/ml respectively [52]. The colloidal silver exhibited antifungal activity with MIC and MFC values range from 2.0 to 3.0 $\mu\text{g/ml}$ [168]. Thermally synthesized AgNPs MFC against *C. krusei* and *C. tropicalis* were 0.03 mg/ml and against *C. albicans* was 0.5 mg/ml [183]. The prepared AgNPs showed a compatible antifungal activity as compared to a standard drug (bifonazole). Moreover our AgNPs required no toxic chemicals during preparation process, low cost and raw materials (precursor) abundantly available.

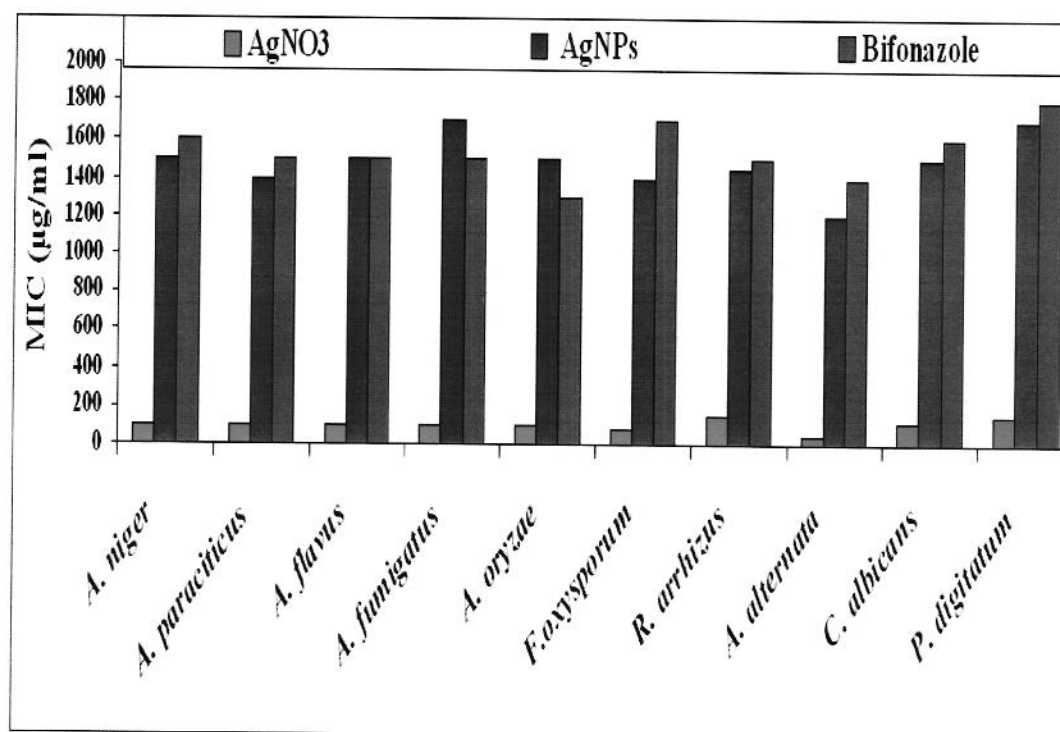


Fig. 48. Antifungal MIC of AgNPs.

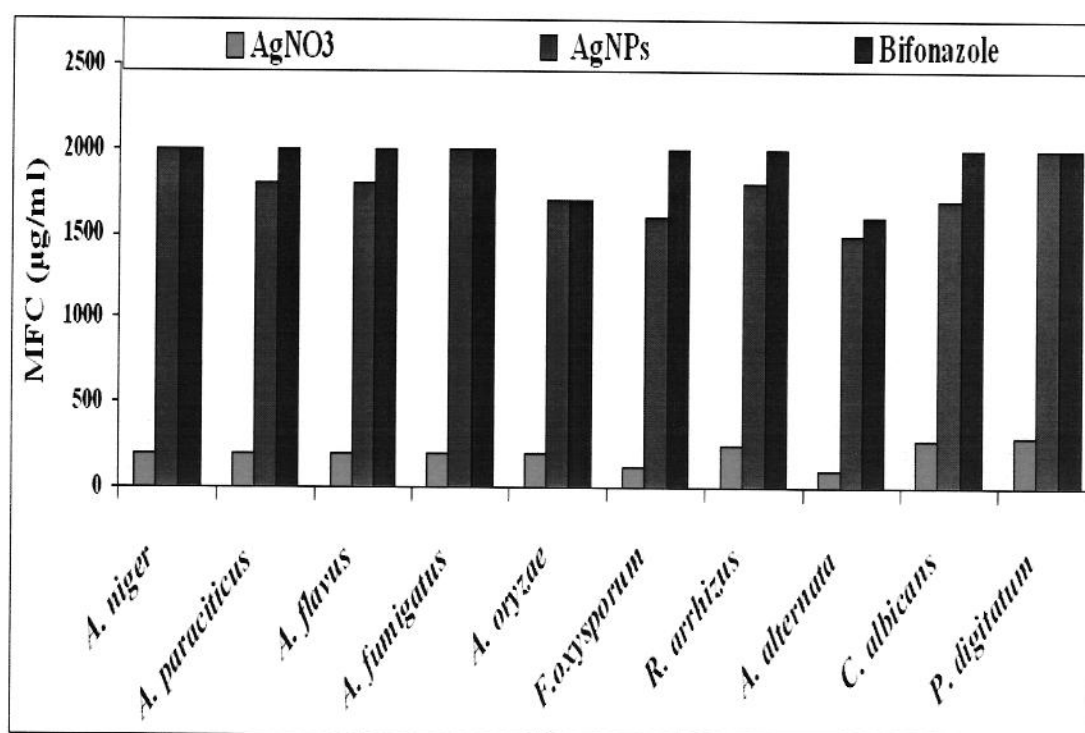


Fig. 49. Antifungal MIC of AgNPs.

3.17.5. Antioxidant Activity of AgNPs

The inorganic NPs have oxygen based free radicals scavenging activity [27]. Due to active surface of NPs the antioxidants compounds might get adsorbed onto it. High surface area to volume ratio and surface reaction phenomenon (due to adsorbed antioxidant moiety onto the surface) of NPs make an affinity to scavenged and interact with free radical [53].

Percent scavenging activity of DPPH is shown in Fig. 50. The AgNPs antioxidant activity increased as the concentration of the substrate increased. On the other hand, the *H. rhamnoides L.* leaves extracts % scavenging activity of DPPH was less as compared to AgNPs. The most interesting finding was that standard, *tert*-butyl-1-hydroxytoluene (BHT) has DPPH scavenging activity nearly equal to that of AgNPs. The DPPH scavenging effects of AgNPs were $84.02 \pm 0.05\%$ at of $80 \mu\text{g/ml}$, followed by $82.99 \pm 0.05\%$ at $70 \mu\text{g/ml}$, $82.33 \pm 0.06\%$ at $60 \mu\text{g/ml}$, $81.93 \pm 0.09\%$ of $50 \mu\text{g/ml}$, 76.06 ± 0.05 at $40 \mu\text{g/ml}$, $63.01 \pm 0.28\%$ at $30 \mu\text{g/ml}$, $52.06 \pm 0.19\%$ at $20 \mu\text{g/ml}$ and $36.64 \pm 0.51\%$ at $10 \mu\text{g/ml}$. Similarly the BHT % scavenging DPPH activity was $38.01 \pm 0.09\%$, $53.03 \pm 0.11\%$, $65.01 \pm 0.05\%$, $78.03 \pm 0.14\%$, $82.49 \pm 0.09\%$, $83.21 \pm 0.05\%$, $83.49 \pm 0.11\%$ and $84.14 \pm 0.05\%$ at a concentration ($\mu\text{g/ml}$) of 10, 20, 30, 40, 50, 60, 70 and 80 respectively. The results of EC_{50} ($\mu\text{g/ml}$) are shown in Fig. 51. The EC_{50} values of AgNPs and BHT were $17.073 (\mu\text{g/ml})$ and $15.945 (\mu\text{g/ml})$ respectively.

The DPPH free radical scavenging activity showed that free radicals % inhibition increased with increase in concentration of AgNPs. As at 100, 200 and $300 \mu\text{g/ml}$ concentration % inhibition was below 80, 80 and above 80 respectively [27]. The

biosynthesized AgNPs were found to have DPPH activity increasing in a dose-dependent manner and exhibited more than 70% scavenging activity of DPPH (inhibition) as compared to *Iresine herbstii* aqueous leaf extracts [54]. It has been reported that free radicals percent inhibitions increased as substrate concentration increased [55]. The antioxidant activity of the extract and AgNPs is a dose dependant manner. Total antioxidant activity of the flower extract was low, L-ascorbic acid was moderate and AgNPs was highest [56]. DPPH scavenging potential of NPs increases from 10.29 to 62.45 % in case of AgNPs and 18.35 to 75.70 %for AuNPs [53].

It has been reported that IC_{50} value of AgNPs was 183.24 $\mu\text{g/ml}$ [55]. The antioxidant potential (IC_{50}) of AgNPs synthesized from *Ricinus communis* fruit was 351, *Ricinus communis* leaf extract was 518 and *Salvia officinalis* L. leaf extract was 745 [59]. Silver nanoparticles DPPH activity was observed to rise in a dose-dependent manner [56]. The DPPH scavenging activity increases in the following order: > plant extract >L ascorbic acid >AgNPs.

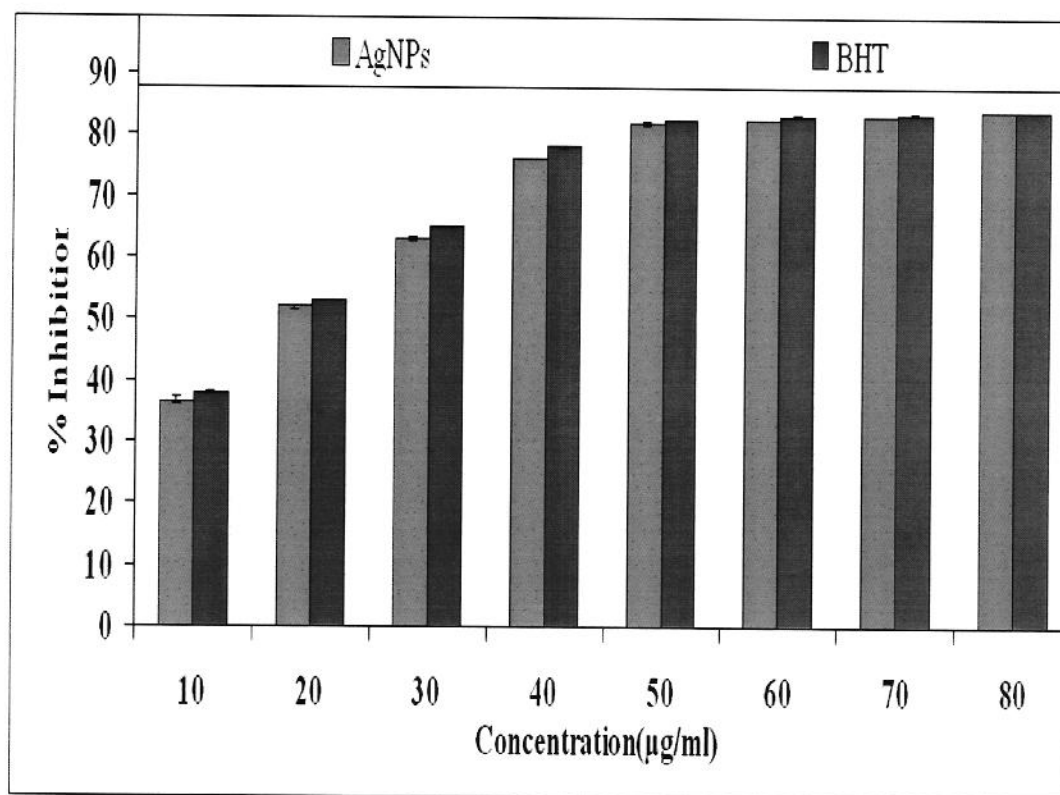


Fig.50. DPPH Free Radical Scavenging Assay of AgNPs.

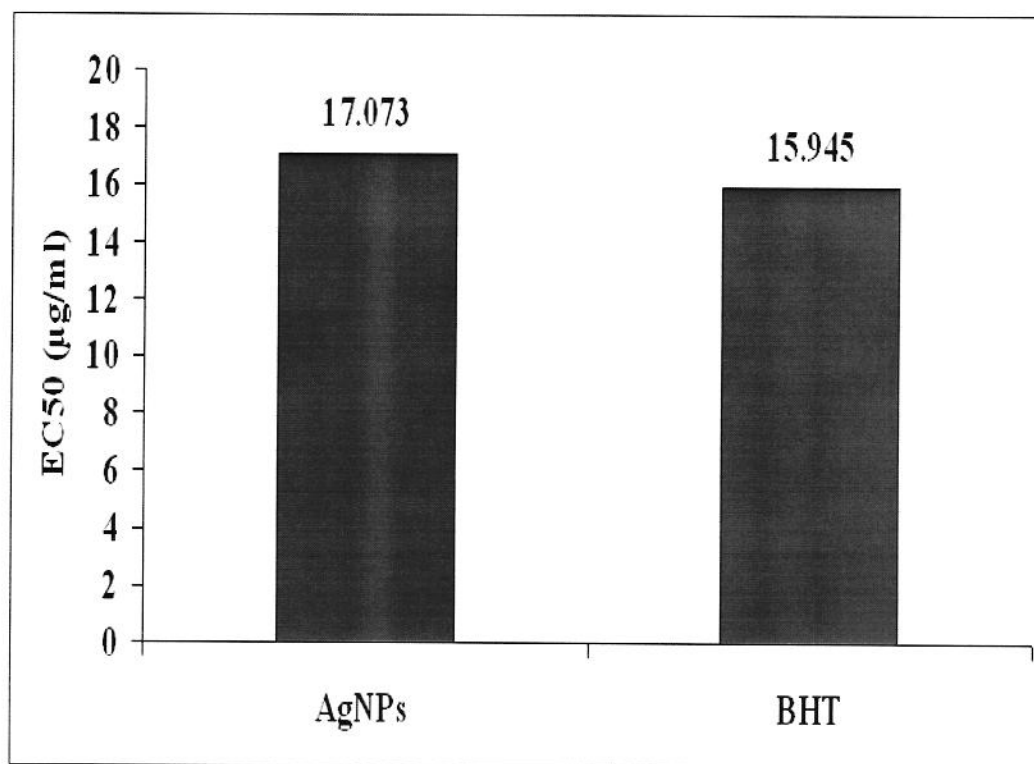


Fig. 51. EC₅₀ (µg/ml) of AgNPs.

3.17.6. Cytotoxic Activity of AgNPs

The cytotoxicity of AgNPs is shown in Fig. 52. The results demonstrated that % mortality increased with increases in AgNPs concentration. The highest mortality was 73.33% at 1000 $\mu\text{g/ml}$, lowest was 26.67% and moderate was 40.00%. The LD_{50} ($\mu\text{g/ml}$) was 145.03. The silver nitrate and negative control (sea salt) were showing no cytotoxic effect, while 100% mortality was observed for etoposide drug.

The brine shrimp cytotoxicity is direct representation of the cytotoxic and antitumor properties of experimental sample [57]. HeLa cell line was enormously susceptible to the size of the AgNPs and the rate of viability drastically declined with increase in concentration (25–300 $\mu\text{g/ml}$) with LC_{50} (51 $\mu\text{g/ml}$). The % cell death obtained predicted that these AgNPs were also antitumor in nature beside cytotoxic effect [54]. It has been found that 25 $\mu\text{g/ml}$ AgNPs showed 75% inhibition and complete inhibition was observed from the concentration of 50–100 $\mu\text{g/ml}$. The intensity cytotoxicity of AgNPs against the brine shrimp caused 100% mortality after 24 hrs of exposure. The LC_{50} value for cytotoxic assay was found to be 0.3 mg/ml [57]. The mortality (*A. salina*) was directly proportional to the concentration of AgNPs. At 100 nM concentration maximum mortality was calculated while LD_{50} value was 10 nM/ml [184]. The CC_{50} values (cytotoxic activity) for vero cell line were 15, 11.7 and 10.0 mg/ml for AgNPs (*Ricinus communis* fruit extract), AgNPs (*Salvia officinalis* leaf extract) and AgNPs (*Ricinus communilis* leaf extract) respectively [59]. *Artemia* was able to live for days in silver nitrate, potassium bichromate and potassium permanganate solutions [185].

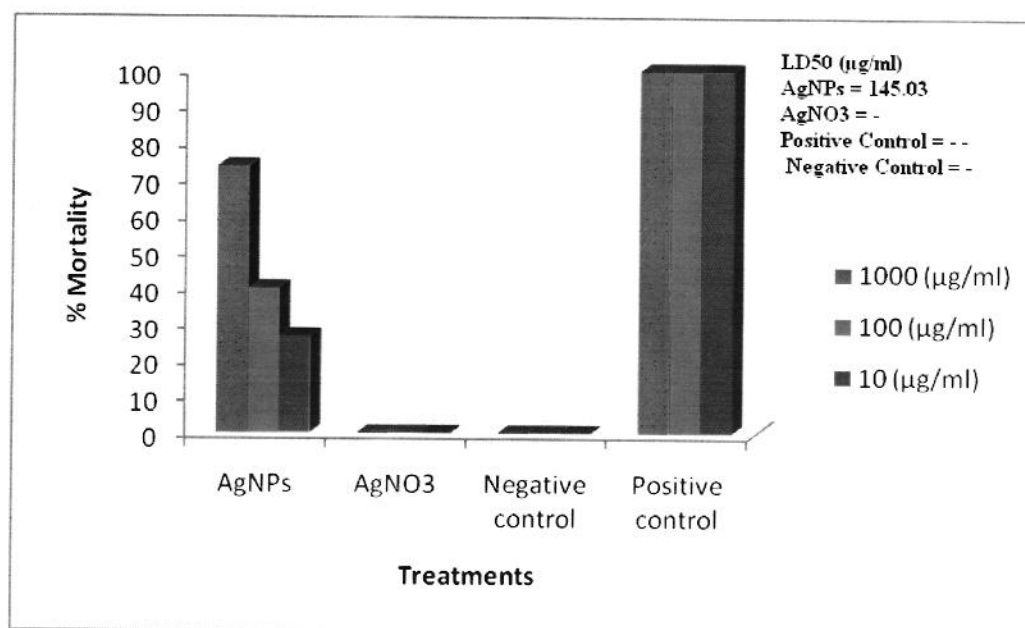


Fig. 52. Cytotoxic Activity of AgNPs against *Artemia salina*.

3.17.7. Phytotoxic Activity of AgNPs

The phytotoxic activity of AgNPs is shown in Fig.53. The results showed that growth regulation (%) was 36.67 and 16.67 at 500 $\mu\text{g/ml}$ and 50 $\mu\text{g/ml}$, while at 5 $\mu\text{g/ml}$ no phytotoxicity was observed. Our results concluded that the prepared AgNPs were having low phytotoxic activity against *Lemna minor*. The AgNO_3 and E-medium (negative control) have no effect on *Lemna minor*, while 100% mortality was calculated in a positive control (Paraquat). The LD_{50} of AgNPs was 1050.48 $\mu\text{g/ml}$.

The growth inhibition of *L. paucicostata* treated with AgNPs and TiO_2NPs were found EC_{50} (ppm) values 13.8 and 538.5 respectively. When *Lemna* was treated at ≥ 100 ppm concentration of AgNPs, the complete growth inhibition occurs. The AgNPs and TiO_2NPs caused additional observable damages to *Lemna* fronds i.e. chlorosis [61]. The AgNPs were inhibited the *L. minor* growth at low concentrations (5 $\mu\text{g/l}$) and this result became more severe with a longer period exposure. There was a linear dose-response correlation after 14 days exposure [186]. It was summarized that 40 mg Ag/l silver nitrate improved the germination rate of five studied wetland plants [64], while 40 mg Ag/l gum arabic coated silver nanoparticles (GA-AgNPs) exposure considerably enhanced the germination rate of one specie and the germination rate of three species were reduced. While polyvinylpyrrolidone-coated silver nanoparticles (PVP-AgNPs) had no effect on germination as showed on 11 common wetland plants species. It was concluded [63] that the seeds placed in MS medium containing AgNPs exhibited 90% germination, whereas 70% germination was found in control seeds. The reason could be that the AgNPs can enter into seed coat and make the embryo active. It was [187] observed that higher concentration

(1000 µg/ml) of chemically synthesized silver nano-particles were toxic to the seedlings of *Oryza sativa* in Hoagland's nutrient solution. Nanoparticles toxicity has been shown to be connected with particle size, coating and surface charge [64]. Plants cell walls act as likely sieves and NPs may perhaps have to enter in plasma membranes and then layers of epidermal within roots to penetrate in xylem. Finally this may leads to translocate and uptake in the course of stems to leaves [188]. The plant cell walls pore dimension is typically in few nanometers range [189], which was much smaller than our prepared AgNPs. This might to some extent make clear why our bioinspired synthesized AgNPs was less phytotoxic. The effects of metal oxide nanoparticles [190] on the growth of *Arabidopsis thaliana* showed a considerable positive effect on root elongation for all tested concentrations of aluminium oxide and for silicon oxide 400 mg/L. At all concentrations zinc and magnetite oxide exerted inhibitory effects, but silicon oxide exerted inhibitory effects at 2,000 mg/L and 4,000 mg/L. The ionic form of silver in the form of nitrate, like silver nitrate plays prominent role in influencing somatic embryogenesis, synthesis of shoot and root [191]. Silver nitrate was utilized in tissues culture research to inhibit ethylene activity for the reason that it was soluble in H₂O and no phytotoxicity on valuable concentrations [192]. In favor of the majority NPs, comparatively elevated concentrations were required for observable toxicity causes in plants and the toxicity threshold was depend upon species to species [193, 194].

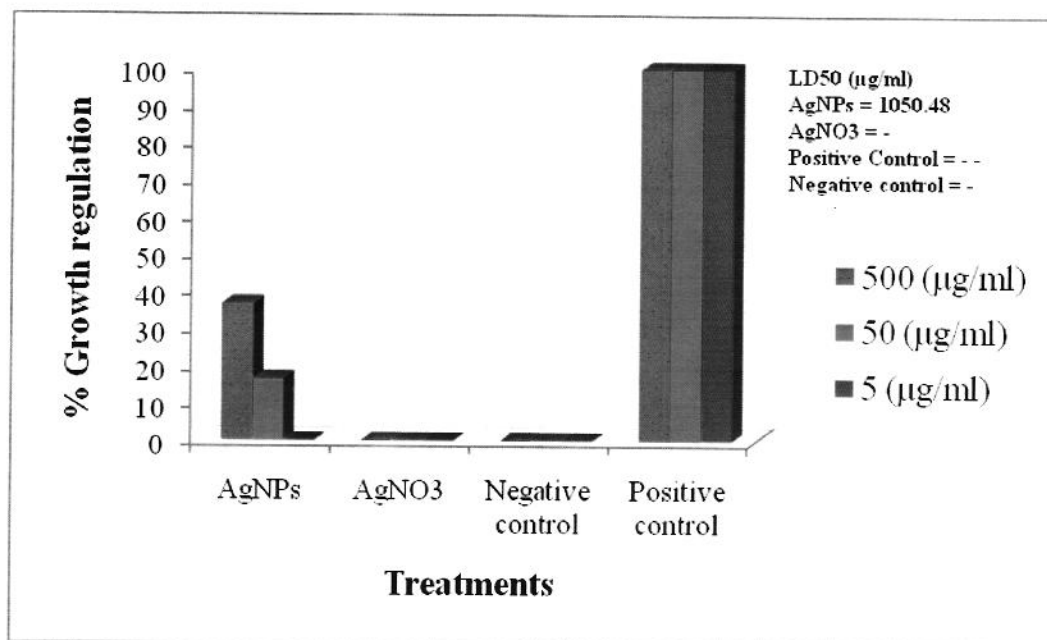


Fig. 53. Phytotoxic Activity of AgNPs against *Lemna minor*.

3.17.8. Larvicidal Activity of AgNPs

Percent larvicidal activity of AgNps against vector mosquitoes (*A. aegypti*) is shown in figure 54. The AgNPs percent larval mortality against *A. aegypti* at 24 hrs, 48 hrs and 72 hrs with 200 ppm was 27 ± 0.50 , 30 ± 0.58 and 33 ± 1.25 respectively, while at 400 ppm the larval mortality (%) was 41 ± 0.96 (24 hrs), 54 ± 1.73 (48 hrs) and 67 ± 0.50 (72 hrs). With 600 ppm at 24 hrs, 48 hrs and 72 hrs incubation time the larval mortality (%) was 74 ± 1.30 , 86 ± 0.56 and 93 ± 0.96 respectively. Larvicidal activity (*A. stephensi*) of AgNPs is presented in figure 55. The *A. stephensi* larval mortality (%) was 31 ± 0.50 for 24 hrs, 35 ± 1.7 for 48 hrs, 47 ± 1.7 for 72 hrs at 200 ppm, 55 ± 0.96 (24 hrs), 61 ± 0.96 (48 hrs), 70 ± 1.3 (72 hrs) at 400 ppm, 79 ± 0.96 for 24 hrs, 88 ± 0.82 and 95 ± 0.56 for 72 hrs at 600 ppm. Silver nitrate and control (distilled water) were found to have no mortality. The results concluded that larval mortality (%) increases when the concentration of AgNPs were increased.

The probit analysis of bioinspired synthesized AgNPs is noted and presented in Table 15. The LC_{50} (24 hrs), LC_{90} (24 hrs), LC_{50} (48 hrs), LC_{90} (48 hrs), LC_{50} (72 hrs) and LC_{90} (72 hrs) of AgNPs against *A. aegypti* were 394.85, 1200.00, 312.86, 853.33, 267.50 and 605.89 ppm respectively. While LC_{50} (24 hrs), LC_{90} (24 hrs), LC_{50} (48 hrs), LC_{90} (48 hrs), LC_{50} (72 hrs) and LC_{90} (72 hrs) of AgNPs against *A. stephensi* were 322.69, 988.10, 281.71, 743.15, 221.88 and 656.65 ppm respectively.

Larvicidal activity of biosynthesized (*Pedilanthus tithymaloides* L. Stem aqueous extract) AgNPs against IV instars of *A. aegypti* at 0.25% concentration observed percent mortality was 72 along with LC_{50} value was 0.167% and $AgNO_3$ solution (0.25%) concentration was found 100 percent mortality [70]. The LC_{50} values of

AgNPs against *Anopheles subpictus* and *Cx. quinquefasciatus* were 5.14 mg/l and 4.56 mg/l respectively [65]. Larvicidal activity of biosynthesized AgNPs (*Euphorbia hirta*) against *Anopheles stephensi* at 50 ppm percent larval mortality were 71.6, with LC₅₀ 27.89 ppm and LC₉₀ 69.94 ppm [69]. Hundred percent mortality of the larvae was calculated in 5.0 mg/l NPs at three hours incubation [72]. Percent mortality of AgNPs synthesized from *Rhizophora mucronata* [71] against *Aedes aegypti* and *Culex quinquefasciatus* larvae at 10.0 and 20.0 concentration (mg/l) were 100. The biosynthesized AgNPs [73] showed 100% mortality against the larvae of *A. stephensi* and *C. quinquefasciatus* at 72 hrs. Control (distilled water) showed nil mortality and Silver nitrate also showed no mortality. It was calculated that larvicidal activity of AgNPs against *A. subpictus*, *C. quinquefasciatus* and *Pediculus humanus capitis* observed 100 percent mortality at 20, 20 and 25 mg/ml concentration. The silver nitrate and distilled water have no mortality [198].

The AgNPs may penetrate through the larval membrane and rupture the midgut epithelium due to interaction with cell molecules of some organelles resulting in death of larvae [70]. Evidenced showed that AgNPs caused loss of cellular functions as well as the proton motive force essential for ATP construction [195]. The correct mechanism of NPs has been documented that from blood circulation the NPs come out, instantly intermingle with the intestinal fluid and extracellular matrix and finally make interactions with cells, lymphatic system and peripheral tissues leading injure them by blocking the systems [196]. The biologically reduced silver ion primary interact with cytoplasm in the interior of the cell wall and denature the ribosome, finally suppressed the expression of enzymes and proteins essential to ATP

production leading to disruption of the cell [197]. The reasons by which the mortality of larvae could occur are the NPs ability to penetrate in the course of membrane larvae [72]. In the intracellular space AgNPs be able to attach to phosphorus containing molecules (DNA) or S-containing proteins, leading to the deterioration of some enzymes and organelles. After, disorder in proton drive force and reduction in membrane permeability leads the failure of organelles function and lastly cell death.

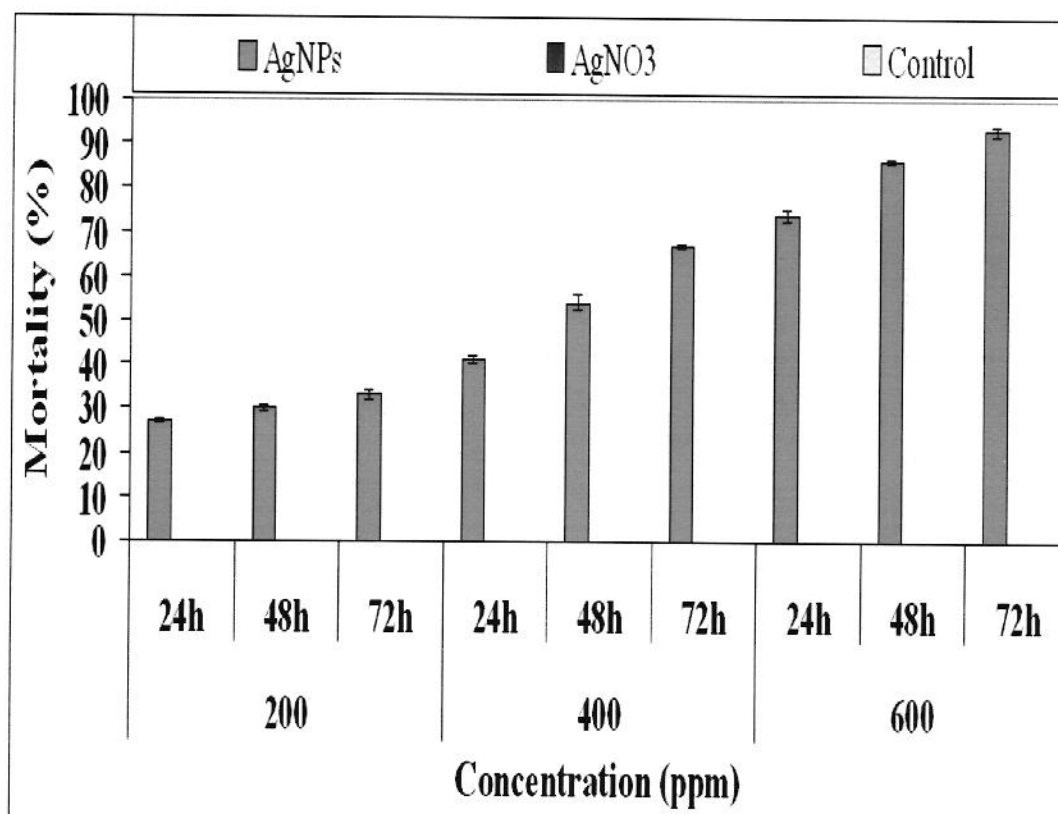


Fig. 54. Larval Mortality (%) of AgNPs against *A. aegypti*. h = hours.

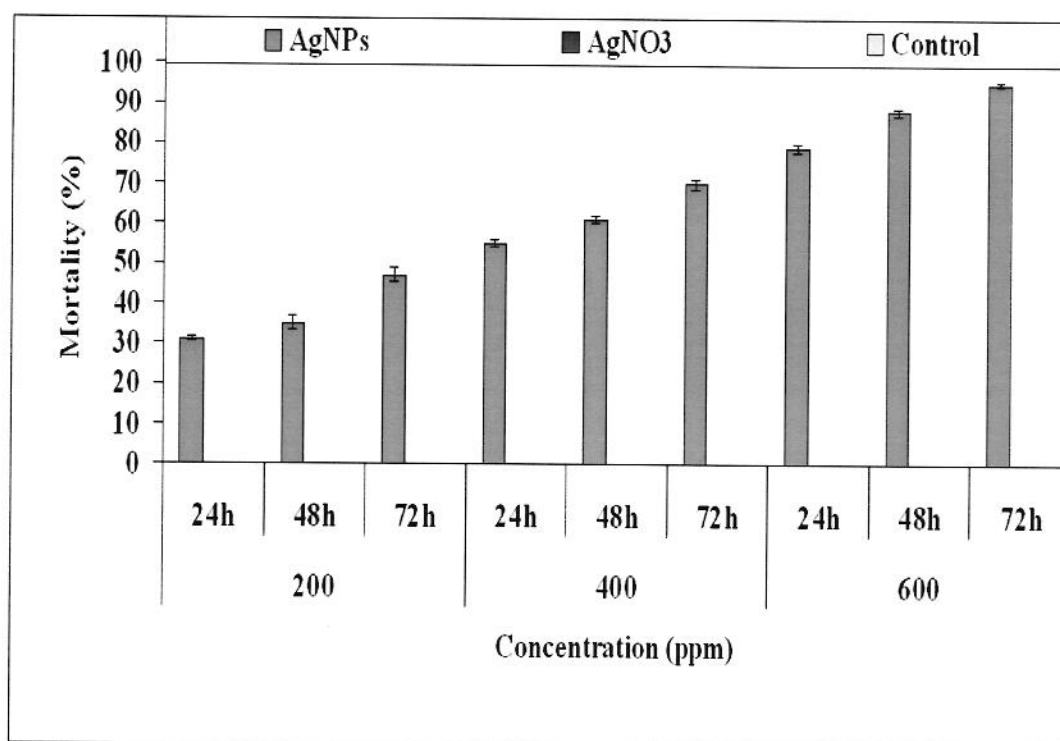


Fig. 55. Larval Mortality (%) of AgNPs against *A. stephensi*. h = hours.

Table.15. LC₅₀ and LC₉₀ of AgNPs against Vector Mosquitoes Larvae.

LC ₅₀ and LC ₉₀ at different time	<i>A. aegypti</i>	<i>A. stephensi</i>
LC ₅₀ (24 hrs)	394.85	322.69
LC ₉₀ (24 hrs)	1200.00	988.10
Regression equation	$Y = -1.92 + 0.40X$	$Y = -1.60 + 0.39X$
Chi-square value (X)	1.70	0.588
LC ₅₀ (48 hrs)	312.86	281.71
LC ₉₀ (48 hrs)	853.33	743.15
Regression equation	$Y = -2.33 + 0.40X$	$Y = -2.46 + 0.41X$
Chi-square value (X)	1.33	1.06
LC ₅₀ (72 hrs)	267.50	221.88
LC ₉₀ (72 hrs)	605.89	656.65
Regression equation	$Y = -4.034 + 0.43X$	$Y = -1.31 + 0.41X$
Chi-square value (X)	0.779	0.584

LC₅₀: Lethal concentration required to kill 50 per cent of the population exposed.

LC₉₀: Lethal concentration required to kill 90 per cent of the population exposed.

CONCLUSION

Our findings revealed that *H. rhamnoides* L. leaves are a rich source of proteins and mineral which are beneficial for human being, while the extracts inhibited many pathogenic bacterial and fungi strains. The qualitative phytochemicals examination showed that tannins, phenols and flavonoids were present in large quantity in all leaves extracts, except *n*-hexane in which phenols and flavonoids were absent. Further leaves extracts could be a strong accepted source of antioxidant and used as a curative agent to prevent or slow oxidative stress linked with degenerative diseases. The leaves can be used in health foods for therapeutic and additive purposes therefore it can be used as antioxidant additives or as nutritional supplements. The extracts showed low phytotoxic and cytotoxic activity. As the *H. rhamnoides* L. leaves are still in use as a traditional herbal medicine, containing a number of useful phytochemicals which provide a scientific base for further primary health care system. In budding countries mosquito borne diseases are the alarming issue in the public health. These diseases can be controlled by preventing the mosquito bite using plant extract having larvicidal activity and showed good repellent and killing properties against mosquito. The rich sources of novel bioactive compounds are from higher plants and can be used to control insect by developing environmentally safe methods. It can be concluded from the present study that the larvicidal activity is due to the phytoconstituents present in the extracts of *H. rhamnoides* L. leaves. The extracts of this plant can be used to control vector born diseases. Moreover, further studies are needed for the isolation and identification of the principal constituents responsible for antimosquito activity and their mode of action by different trails to

recommend its use as a useful agent. The FTIR spectra of all extracts revealed the presence of different functional groups ranging from OH stretching for hydroxyl group, alkanes (C-H), alkenes (C=C), aromatic rings (C=O), carboxylic (C=O) and amides (aromatic). All the above experimental research leads that *H. rhamnoides* L. leaves was a very suitable source for the bioinspired synthesis of AgNPs.

To the best of our knowledge, this was the 1st report to utilize *H. rhamnoides* L. aqueous leaves extract for the bioinspired synthesis of AgNPs. It was concluded that using aqueous extract solution of *H. rhamnoides* L. leaves has been documented a new beneficiary method using non-expensive raw materials for the biosynthesis of AgNPs. This low cost, non-complicated, less time consuming and environment friendly biosynthetic preparation gives potent applications in various human benefits related fields. This approach will enhance AgNPs sustainable management and economic viability. Utilization of plant materials for the synthesis of NPs has given great importance to biological approach of NPs synthesis. The prepared AgNPs have strong antibacterial activities against human pathogenic bacteria and hence has a great ability in the drug formulation against microbial diseases. The strong antifungal activity represents a prominent approach in the nanomaterials with realistic implications. This novel approach would be effective to develop the nanosized biopesticide using natural resources as eco-friendly approach against target dreadful vector and able to reduce/avoid the development of resistant in the responsible vector. Our results demonstrated that the prepared AgNPs were more effective against all the tested fungal strains due to its low concentration, while *H. rhamnoides* L. leaves extract affective in high concentration. Although the standard drug fluconazole have

shown high zone of inhibition, but synthetic in nature, side effect and cost effective, but our prepared AgNPs raw materials abundantly available, no toxic chemicals required and environment friendly process.

The findings of the current research work concluded that AgNPs were effective antibacterial agents against a group of bacterial pathogens that are concerned in gastrointestinal disorders, diarrhea, dysentery, typhoid fever and other infectious diseases. AgNPs demonstrated strong antifungal action and could be applied like an antifungal source to control different human and plant diseases originated by these fungi. Additionally field applications research and to know the correct entrance means of AgNPs into the cell wall of fungi are desired.

The free radical scavenging characteristic of synthesized AgNPs showed that inhibition percentage increases in a dosage dependent mode. Therefore the prepared AgNPs may perhaps play the neoadjuvant antioxidant function contribution to useful defense as of free radicals in a broad variety of circumstances. Cytotoxic activity of AgNPs was shown against brine shrimp, whereas lethality assay of brine shrimp make clear their significance in pharmaceutical field. The prepared NPs also exhibited low phytotoxic effect on the *Lemna minor*, however more investigation is necessary to find out the toxicity and uptake mechanistic approach of NPs within intracellular injuries. The efficiency here killing of mosquito larvae (*A. aegypti* and *A. stephensi*) were promising. Additionally, to consider protection point of view and mosquito resistance appearance to traditional insecticides create natural origin larvicide which give superiority over non-natural larvicide. Non-synthetic larvicides,

particularly plant origin with the purpose of extra choose, degradable and more talented within this characteristic.

Overall, our current findings grow the efficient utilization of the bioinspired fabricated NPs from aqueous leaves extracts of *H. rhamnoides* L. in numerous human benefits related fields especially health care system and to develop new drugs in favor of human being benefits in near future.

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