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Synthesis of Bio-Nano Composite Film and Their Application in Filtration and Sterilization of Heat Labile Materials

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ABSTRACT:Nanotechnology is the fast-growing science with a variety of applications in every sphere of life. Nano-materials have been widely investigated for their unique physicochemical properties such as anti-microbial, due to their unusual interfacial effects.It is well known that the silver in its nano form has a broad range of application in the field of nanotechnology since silver nano particles (AgNPs) is biocompatible metal and has low toxicity towards mammalian cells and tissues. AgNPs have an extremely large relative surface area to volume, and hence increasing their contact with bacteria or fungi which vastly improves their bactericidal and fungicidal effectiveness. This project attempts to provide an alternative way to the expensive techniques such as ultrafiltration and microfiltration used for filtration and sterilization of heat labile substances by exploring the field of nanotechnology. The bio-nano composite film fabricated aims to serve a better way of filtration and sterilization as compared to their conventional filter counterparts and base polymer. Such film would be economical at industrial scale and could be used more readily for commercial applications. A novel method was implemented to impregnate AgNPs into Microcrystalline cellulose (MCC) by curry leaf (Murrayakoenigii) extract. The active reduction of silver ions by curry leaf extract was explored for the *in situ* impregnation ofAgNPs into MCC. Curry leaf extract caused the reduction of silver ions into silver, which nucleated in to silver nanoparticles on the micro-crystalline fibrils. The bioreduction of silver nanoparticles and its impregnation was investigated through various characterization techniques. The characterization of AgNP impregnated MCC powder confirms the formation of Ag structure on microcrystalline cellulose. The MCC impregnated with AgNPs was used to prepare a film after testing for its anti-microbial activity. The film was characterized for its pore size. Lastly the film was assessed for anti-microbial activity for it to be used as a filter.AgNPs were successfully synthesized which was confirmed through UV-visible analysis. The AgNPs were impregnated into MCC as revealed by analysis. The size of the nanoparticles was determined to be in the range of 5-20nm.The film was fabricated through solvent casting method using sodium alginate as the polymer. The anti-microbial analysis gave positive results and thus could be used as an alternate method for the sterilization of heat labile substances.

KEYWORDS:Silver nanoparticles, microcrystalline cellulose, bio-reduction&curry leaf.

I. INTRODUCTION

Nanotechnology is rapidly growing by producing nanoproducts and nanoparticles (NPs) that can have novel and size-related physicochemical properties differing significantly from larger matter. The novel properties of NPs have been exploited in a wide range of potential applications in medicine, cosmetics, renewable energies, environmental remediation and biomedical devices. Among them, silver nanoparticles (AgNPs or nanosilver) have attracted increasing interest due to their unique physical, chemical and biological properties compared to their macro-scaled counterparts [1].Nanoparticles due to their small size have a much increased surface area per unit mass compared to bigger particles.

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In addition, quantum effects become more dominant at the nanoscale. All properties, including electrical, optical and magnetic ones are altered [2].

Many disease causing culprits such as bacteria and viruses are nanosize. The ancient Greeks used silver to promote healing and prevent infection, but the treatment took backseat when antibiotics came on the scene. The old cure is improved by coating a burn and wound bandage with nanosize silver particles that are more reactive than the bulk form of metal. They penetrate into skin and work steadily. As a result, burn victims can have their dressings changed just once a week. Historically, silver (Ag) compounds have been used in numerous fields to prevent microbial growth. Like many nonessential heavy metals, Ag is a natural biocide, but compared with titanium, zinc, and copper, Ag nanoparticles (AgNPs) show the highest antimicrobial efficacy against bacteria, viruses, and other eukaryotic microorganisms. Silver naoparticles is chosen to be impregnated in the film because first it has a powerful anti-microbial property i.e. germ killing ability. Secondly, the smaller form has a larger ratio of surface area to volume, which dramatically increases the potential for silver ions to be released—the primary mode of silver and nanosilver toxicity. In addition, nanosilver can go places in the body that larger silver particles can't, and it may be small enough to enter cell or cross the blood–brain barrier. Lastly, is biocompatible metal and has low toxicity towards mammalian cells and tissues.

Steam sterilization requires a continuous source of heat (wood fuel, kerosene or electricity), requires equipment (steam sterilizer), which must be expertly maintained to keep it in working condition and strict adherence to time, temperature and pressure settings. Gas sterilization requires special handling because of flammability, toxicity; Long sterilization and decontamination time; Potential health hazard; fumes must be monitored.

Ultrafiltration only removes suspended matter and bacteria; Sensitive to oxidative chemicals (e.g. nitric acid, sulphuric acid, peroxide and persulphate in high concentrations); and pH dependent. Microfiltration systems will not remove dissolved contaminants, such as nitrates, fluoride, dissolved metals, sodium and VOCs. Colors, tastes and odors are also untreated.Membrane fouling is a serious phenomenon affecting performance and service of membrane filters. Fouling can arise from several mechanisms: Surface associative materials, such as colloids and other partially charged macromolecules; biofilm growth resulting from microbiological activity of the accumulated pathogens; suspended solids; and precipitation of insoluble salts experiencing a concentration gradient across the membrane device.

Most of these methods are expensive and unaffordable.Hence, these are not feasible at commercial levels. There is a requirement of an effective method which can be readily employed in laboratories as well as industries.Cost-effective filter materials coated with silver nanoparticles is an alternative technology that could help to solve these problems up to a certain extent.

Literature Review

Our goal is to find a way to deal with the problems mentioned above up to a certain extent. With the literature review we carried out we were able to find a way to fabricate film using silver nanoparticles and Micro crystalline cellulose (polymer) as main components .This film combines microbe-killing capacity with the ability to remove chemical contaminants.

Silver nanoparticles (AgNP) deposited or impregnated materials (metal, polymer, metal oxide, carbon, cellulose) have been widely investigated for their unique physicochemical properties such as optical, electrical, catalytic and anti-microbial, due to their unusual interfacial effects.

Along with antimicrobial activity additional properties such as thermal, mechanical, barrier, moisture resistance and linear thermal expansion coefficient also necessary, that will widen the opportunities for numerous commercial products. Coatings are usually effective for short-term applications. There is always need for research to design better antibacterial biomaterials for long-term use. This project shows a method for the preparation of nanocomposite (NC) to achieve long term antibacterial functionality [3].

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Inclusion of AgNP into natural fibres (cellulose as well as cotton) has been performed using various techniques including sonochemical, microwave, chemical reduction (using sodium borohydrate) as well as bioreduction (using fungus, plant leaf extract). Among them, plant leaf extract mediated biological process is found to be simple as well as cost effective that also utilize ambient conditions for the reduction reaction [4].

Hence, we use tree leaf extracts based bioreduction process for the fabrication of antimicrobial cotton fibers with AgNP. The effective reduction of silver ions into AgNP by curry leaf extract has motivated to fabricate AgNP impregnated microcrystalline cellulose (MCC) as functional fillers in order to form antimicrobial bio-nano composite films.

Characterization methods like Transmission electron microscopy (TEM) analysis and Scanning electron microscopy of MCC coated with AgNP can show the formation of silver nanoparticles. Further the, EDS analysis of MCC/Ag nanocomposite can confirm the formation of Ag structure on microcrystalline cellulose. Energy Dispersive X-Ray Spectroscopy (EDS) analysis of MCC/Ag nanocomposite confirms the formation of Ag structure on microcrystalline cellulose. The crystalline nature of the in situ impregnated AgNP is investigated using an X-ray powder diffractometer. The thermal behavior of the film can be observed by Thermo gravimetric analyzer.

Coatings are usually effective for short-term applications. There is always need for research to design better antibacterial biomaterials for long-term use. This project shows a method for the preparation of nanocomposite (NC) to achieve long term antibacterial functionality [4].

II. MATERIALS AND METHODOLOGY

Materials

The implementation of the project involves the use of various organic as well as inorganic chemicals for wide range of applications. The nano-particles are produced into a film with the help of polymers and plasticizers. The AgNP impregnated MCC as well as the film are tested for their antimicrobial activity using nutrient broth and nutrient agar medium. The characterization of the film and the prepared MCC powder involves the use of various solvents as well. Some of the important materials and their role are presented in the **Table 1**.

Table 1: List of materials used during the implementation of project along with their function

Material	Function
Curry Leaves	Reduction of silver nitrate to silver
Silver Nitrate ($AgNO_3$)	Production of silver nano-particles (AgNPs)
Micro Crystalline Cellulose (MCC)	Preparation of MCC powder impregnated with AgNP
Sodium Alginate	Polymer for the fabrication of film
Glycerol	Used as plasticizer for the fabrication of film
Calcium Chloride	Insolubilization of the film
Bacillus culture	Culture for testing the antimicrobial activity of the film and powder
Nutrient Agar and Broth	Medium to test the antimicrobial activity
Chloroform	Solvent used for the characterization of the prepared MCC powder

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Methodology

The methodology involves the preparation of curry leaves extract and is used for the preparation of silver nanoparticles (AgNP). These AgNPs are impregnated in Micro Crystalline Cellulose (MCC) which is characterized for various physical properties and is then used to prepare film using solvent casting method. The prepared film is then characterized for pore size and is studied for anti-microbial activity.

Preparation of Curry Leaf Extract

The curry leaf extract was prepared using 10 g of fresh curry leaves, which were rinsed with deionized water and cut into small pieces. The chopped leaves were boiled in 75 ml of deionized water for 10 minutes and allowed to cool. Further the cooled leaf broth was filtered, which yielded 50 ml of broth and it was stored in a refrigerator at 4°C[5].

Impregnation of AgNPs into MCC

First, 10 g of MCC was suspended in 1000 ml of 10-3 M silver nitrateandsonicated for 10 minutes [6]. 50 ml of curry leaf broth was added to the mixture and stirred for 6 hours [7]. Curry leaf extract caused the reduction of silver ions into silver, which nucleate in to silver nanoparticles on the micro-crystalline fibrils. After 6 hours the mixture was allowed to settle down and the excess reaction mixture was de-canted off. The AgNP impregnated MCC was washed with deionized water and dried in an oven at 55°C over night[8].

Antimicrobial Test of AgNP impregnated MCC

One day prior to test, a sterilized Nutrient Broth medium was prepared and was inoculated with Bacillus sp.. The inoculated medium was incubated for 24 hours. The next day, fresh nutrient agar plates were prepared and were seeded with 24 hour old Bacillus culture [9]. Sterile discs were placed on the medium and were loaded with 20µl of positive control (1mM Ampicilin) [10] and samples. One disc indicating negative control was also placed and was not loaded with anything. The plate was incubated at 37°C for 24 hours.

Characterization of the Silver Impregnated Microcrystalline Cellulose

The bioreduction of silver nanoparticles was investigated through a Varian UV-Vis spectrophotometer (300 UV-Vis) operated between 300 and 800 nm with 2 nm resolution [11]. Transmission electron microscopy (TEM) analysis of the as received as well as AgNP impregnated microcrystalline cellulose was performed at an accelerating voltage of 100 kV [12]. A drop of MCC suspension was placed on carbon coated copper grids and allowed the solvent to evaporate prior to analysis. Scanning electron microscope-EDS analysis of the AgNPs impregnated MCC was performed at an accelerating voltage of 30 kV [12]. The crystalline nature of the in situ impregnated AgNPs was investigated using a X-ray powder diffractometer employed with Cu Kα radiation. The XRD pattern was recorded between 10° and 80° at the scanning rate of 2° per minute. The dried MCC powders were loaded on the glass subtrate and subjected to X-ray and the diffracted pattern was recorded.

Solvent casting of Sodium Alginate/MCC composite

A batch was prepared from 100 grams of the film mixture solution containing 7.5% Sodium alginate as the film forming material. The sample (AgNP impregnated MCC) and plasticizer (glycerol) both at 40% w/w were added based on the dry weight of the polymer [13].

Table 2: The basic formula for preparation of film

Material	Quantity (in g)
Sodium alginate	7.5
Glycerol	3.0
Sample	3.0
Water	86.5
Total	100

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Weighed amount of Sodium alginate was hydrated by dispersing it in weighed quantity of water. The sample was dissolved in glycerin and the mixture was uniformly dispersed in the alginate solution**Table 2**.From the above mixture a weighed quantity (3g) was taken and cast on a glass perti-plate. The plate was kept undisturbed for 16 hours. The film was then insolubilized in a bath of 10% solutions of Calcium chloride for 5 hours[14]. After insolubilization period, the film was washed in a glycerin bath (50%) and allowed to dry at room temperature [15].

Characterization of film

The film was characterized for pore size. The pore size was determined using Scanning Electron Microscopy analysis. This also helps to ensure the effective deposition of AgNP impregnated MCC inside the film to have antimicrobial activity.

Antimicrobial test of the film
One day prior to experiment, sterilized nutrient broth medium was prepared and was inoculated with Bacillus sp.. The inoculated medium was incubated for 24 hours.

For antimicrobial test, 4 conical flasks containing nutrient broth were prepared. The flasks were labeled as nutrient broth (NB), nutrient broth + Bacillus (NB + Bac), autoclave and filtrate respectively. The conical flask labeled as autoclave was inoculated with 50µl of 24 hour old culture and was sterilized using autoclave after 2 hours of incubation.

10ml of Bacillus culture was filtered through the prepared film [16]. 50µl of the filtrate and culture was inoculated in the conical flasks labeled as filtrate and NB + Bac respectively. The flasks were incubated at 37°C for 24 hours in an orbital shaker. After 24 hours incubation, absorbance of the samples was recorded at 620nm using nutrient broth as reference [17].

III. EXPERIMENTAL RESULTS

Results and Discussion
The characterization and antimicrobial test of AgNP impregnated MCC powder and the prepared film was done. The results are discussed below.

Antimicrobial Test of AgNP impregnated MCC
The antimicrobial test of AgNP impregnated MCC was carried out using the zone inhibition test. After incubation period of 24 hours, the plates were observed for the formation of zone**Figure 1**. The following results were obtained:
The diameter of zones obtained is as follows:

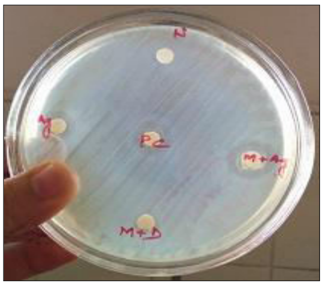

Figure 1: Formation of zones around sterile discs loaded with positive control and samples

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Table 3: Results of zone of inhibition

Sample	Zone Diameter (mm)
Positive control(1mM)	12
$AgNO_3$(1mM)	11
$AgNO_3$(1ml of 1mM) + MCC(0.01g)	14
DDW (1ml) + MCC(0.01g)	9

Formation of Zone of Inhibition around the disc loaded with MCC dissolved in Double Distilled Water (DDW) indicates the anti-microbial activity of the prepared MCC powder.Furthermore, the difference in the zones formed around $AgNO_3$ and MCC + $AgNO_3$, with latter being larger conforms the anti-microbial activity of MCC.

Characterization of AgNP impregnated MCC powder

UV-Visible Spectroscopy

The UV-visible spectra of the as received initial reaction mixture and the final reaction mixture (after 2 hours of incubation) was recorded between 300nm to 800nm. The spectrum obtained is depicted **Table 4**.

Table 4: Absorbance values for Initial and Final reaction mixture ($AgNO_3$ + MCC + Curry leaves)

Absorbance	Initial Reaction Mixture	Final Reaction Mixture
300	0.88	1.34
350	0.89	1.21
400	0.91	1.38
450	0.93	1.42
500	0.94	1.41
550	0.96	1.31
600	0.97	1.23
650	0.98	1.2
700	0.99	1.14
750	0.99	1.1
800	1.00	1.08

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The graph for the above readings is represented in **Figure 2** as follows:

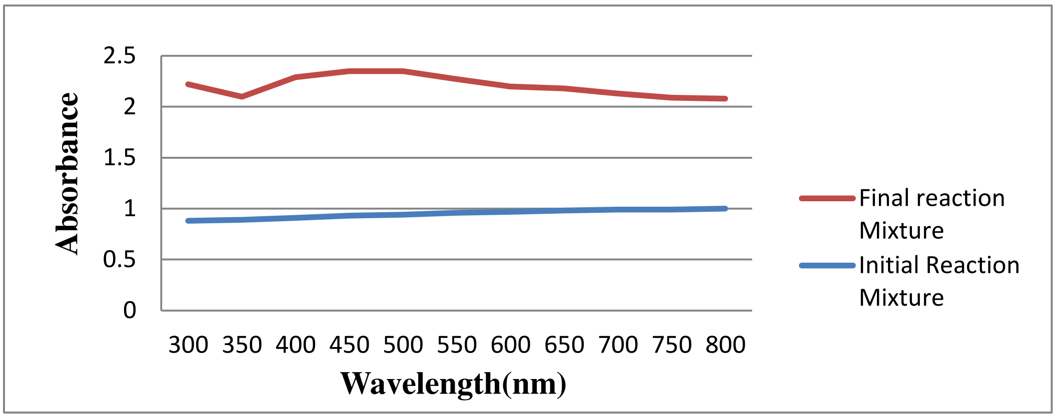

Figure 2: UV-Visible spectra of initial and final reaction mixture ($AgNO_3$ + MCC + Curry Leaf Extract)

The UV-visible spectroscopy was done to confirm the formation of silver nano-particles. From the above graph it is observed that the peak was obtained at 450nm. The peak at 450nm is attributed to the plasmonic vibration of AgNP and confirms the formation of AgNPs [18].

TEM and Visual Analysis

Visual analysis is done to further confirm the formation of AgNPs and their impregnation into MCC. The initial and final MCC powder after incubation with curry leaf extract and $AgNO_3$ had colordifference and is depicted in **Figure 3**.

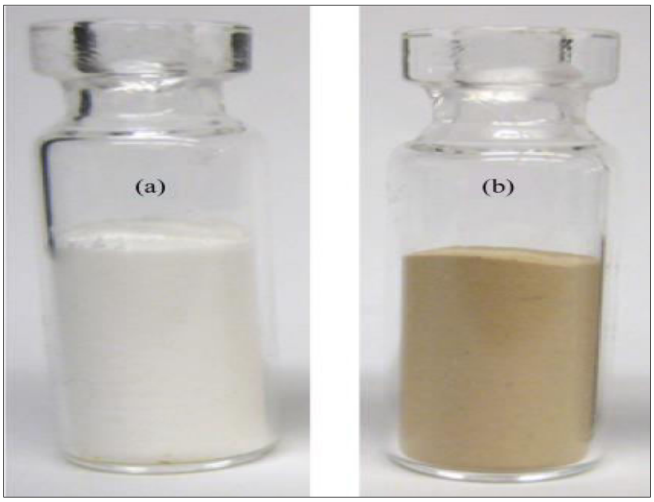

Figure 3: Photograph of
- **a) As received MCC;**
- **b) MCC impregnated with AgNP**

The change in color of MCC from white to yellowish brown indicates the impregnation of AgNPs in MCC [19].

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TEM images of the as received MCC powder (control) and the MCC powder impregnated with AgNP (sample) at different magnifications was recorded.

The TEMimage confirms the effective impregnation of Ag nanoparticles into microcrystalline cellulose by curry leaf extract based bio reduction process. The impregnated AgNPs are polydisperse and ranges approximately from 10 - 25 nm with spherical shape. AgNPs were distributed among the MCC fibrils and the high tendency of particle agglomeration was observed. From this analysis it is observed that the AgNP content in some MCC fibrils are greater than others. This may be explained as the nanoparticles adsorption on MCC fibrils is strongly dependent on the physicochemical characteristics of the fibers, which indicate the necessary of physical/chemical pretreatment to the MCC fibrils [20].

Scanning electron microscope-Energy Dispersive X-Ray spectroscopy (SEM-EDS)

The SEM-EDS was recorded in order to provide further confirmation on the formation of AgNPs on cellulose fibrils and also identify their approximate bulk atomic composition.

The obtained EDS spectrum of Ag nanoparticles impregnated MCC confirms the existence of silver in the MCC and it is quantified as ~1 wt%. The formation of peak at 0.3 keV confirms the formation of AgNPs [21].

X-Ray Diffraction (XRD)

Power X-ray diffraction patterns of pristine and Ag nanoparticles coated MCC fibrils were recorded. The diffraction pattern for pristine MCC shows intense peak at 15°, 16°, 22°, 23° and 26° are the characteristic peaks of cellulose fibres, which coincides with reported literature values [22].

XRD pattern for AgNPs impregnated MCC fibrils shows additional peaks at 38°, 44°, 64° and 77° which are assigned to fcc crystalline structure of silver nanocrystals. It was observed that the characteristic peaks of AgNPs have significant width, which indicates the formation of smaller Ag particles.

Crystallite size of the silver nanoparticles was calculated usingScherrer's formula:

$$d = \frac{K.\lambda}{\beta.\cos\theta}$$

Where
λ: Wavelength of X-rays = 0.154nm,
K: Constant, generally assumed as 0.9,
β: Full Width at Half Maximum (FWHM) of the diffraction line,
θ: Diffraction angle
The value of d at additional peaks is presented in **Table 5**.

Table 7: Crystallite size evaluated at the additional peaks obtained using Scherrer's Formula

K	λ (nm)	2θ (°)	cosθ	β (°)	β (rad)	d (nm)
0.9	0.154	38	0.95	1.5	0.0262	5.57
0.9	0.154	44	0.93	0.5	0.0087	17.13
0.9	0.154	64	0.85	1	0.0175	9.32
0.9	0.154	77	0.78	1	0.0175	10.15

The value of crystallite size ranges from 5nm to 20nm which in in accordance with the results obtained through TEM analysis.

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Characterization of film

The SEM image of the prepared composite film was obtained. SEM image shows different levels of a multi-layered AgNP impregnated MCC – Sodium alginate porous thin film. The pores having an average diameter of 30nm are hexagonally arranged and are disjointed between layers. The AgNP impregnated MCC with an average size of 12nm have been deposited via the solvent casting method and are present both in the pores and on the walls of the matrix. Thus the film incorporates the silver-nanoparticles impregnated on MCC.

Antimicrobial test of film

After 24 hours of incubation conical flasks were observed for microbial growth. It was observed that the flask label as NB + Bac had a significant microbial growth as indicated by the turbidity present in the culture.

The flasks which were inoculated with autoclaved and filtered samples had no microbial growth as there was no turbidity observed. These results are depicted in **Figure 4**.

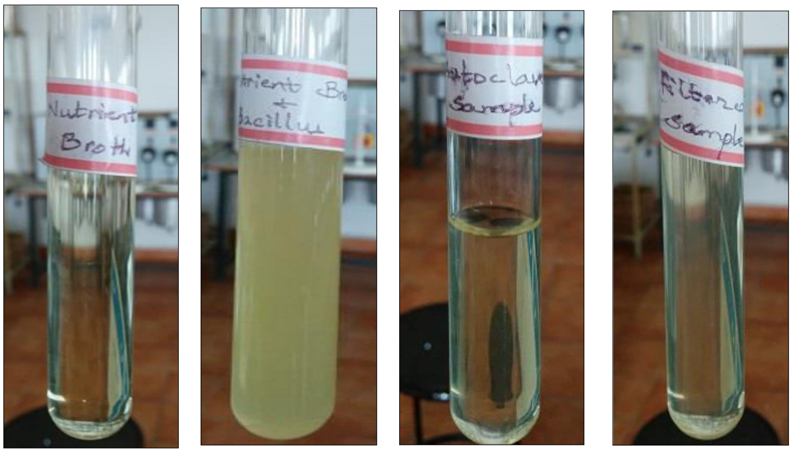

Figure 4: (Starting from left) Nutrient Broth; Nutrient broth inoculated with Bacillus, Autoclaved sample; Filtered sample

The above results were conformed using the absorbance values recorded for samples and NB + Bac using NB as the reference. The spectrophotometer results are as presented in **Table 8**.

Table 8: Spectrophotometer results for samples and Nutrient broth + Bacillus using Nutrient broth as reference

	NB + Bacillus	Autoclave	Filtration
Wavelength (nm)	620	620	620
Absorbance	1.325	0.037	0.066
Transmittance	4.7	91.8	85.9

The comparative graph for the above reading was plotted. It was inferred from the graph that the absorbance for autoclave and filtrate samples were similar as compared to nutrient broth + Bacillus. Since the absorbance readings for the autoclaved sample and the filtrate were similar thus it can be inferred that the film can be effectively used for sterilization application. The graph obtained is represented in **Figure 5**.

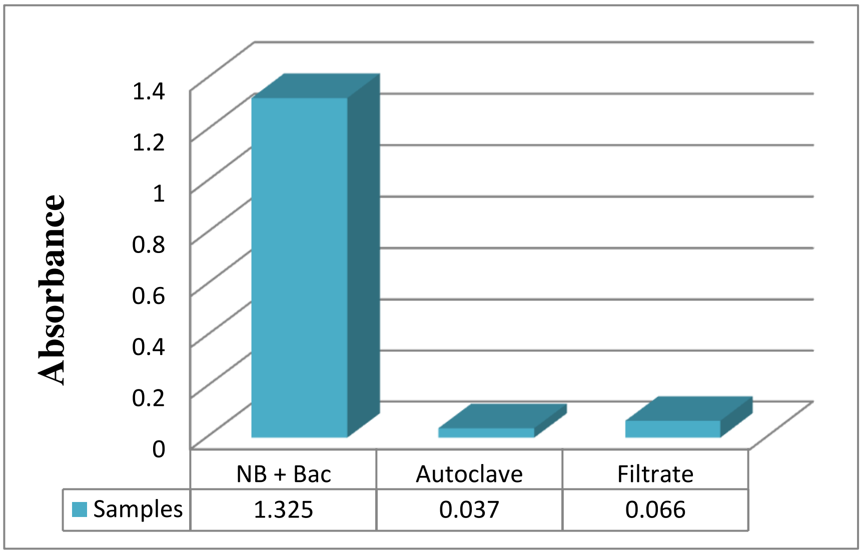

Figure5: Absorbance values of the samples using Nutrient broth as reference at 620nm

IV. CONCLUSION

A plant leaf extract using curry leaves was successfully prepared in order to carry out the bio-reduction of silver ions into silver nanoparticles. The antioxidant potential of the curry leaf extract effectively reduced silver nitrate to silver nanoparticles. The *in situ* impregnation of silver nanoparticles into microcrystalline cellulose was successfully carried out by the curry leaf extract mediated bio-reduction process. This was confirmed by XRD and SEM-EDS analysis.UV-visual spectra of reaction mixture with MCC dispersion and silver nitrate solution was compared with the spectrum of reaction mixture with curry leaf extract (after 2 hours of addition) confirmed the formation of AgNPs.The size of AgNPs was found to be in the range 5-20 nm which was confirmed by XRD and TEM analysis.Silver impregnated MCC fibrils were further utilized for the fabrication of biodegradable composite film with sodium alginate as polymer matrix by employing solvent casting process. The silver impregnated MCC antimicrobial bio-nanocomposite film was fabricated by using the plasticizer glycerol. The preliminary analysis showed that these films exhibit significant antimicrobial properties.The antimicrobial activity of the prepared bio-nanocomposite film was tested using spectrophotometric analysis which confirmed antibiotic resistance of the film.The results of filtration of Bacillus containing medium through AgNP impregnated on MCC membrane and through tradition method of sterilization i.e. autoclave revealed the reduction in cell concentration which was found to be approximately 95.38% while through autoclave it was found to be approximately 99%.

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