e-ISSN 2320 –7876 www.ijfans.org
Vol.11, Iss 7, 2022
  UGC CARE Listed (Group -I) Journal

Antibacterial Activity of Silver Nanoparticles extracted from Betanin Pigments of *Beta vulgaris* L. And *Amaranthus tricolor* L.

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ABSTRACT

Plant pigments with phyto-compounds are used for synthetizing nanoparticles. These pigments were used to compare the efficacy of silver nanoparticles synthesized from betanin pigment extracted from *Beta vulgaris* L. and *Amaranthus tricolor* L. The silver nanoparticles and betanin related derivatives were synthesized from the pigment and analysed using UV-Vis spectrophotometry. The SEM images of nanoparticles showed the appearance of spherical shape with definite size. The FTIR analysis was carried out to identify possible biomolecules responsible in bio-reduction of silver ions and compare their compounds. FTIR and UV-Vis spectra showed that biomolecules, proteins and peptides, are mainly responsible for the formation and stabilization of AgNPs. These molecules interact with Phyto-compounds and form a stabilized complex. The antibacterial activity using resazurin dye showed 6.25±0.03 µg/ µl inhibition of *Bacillus* sp. and *E.coli* strains by the silver nanoparticles synthesized from *B. vulgaris*. Both gram positive and gram negative bacteria, *Bacillus* sp. and *E.coli* strains were used to measure the MICs. Resazurin dye was used to for the detection of the inhibitory level of nanoparticles synthesized pigments from *Beta vulgaris* L. and *Amaranthus tricolor* L.The study confirms abundant silver nanoparticles seen on *B. vulgaris* when compared to *A. tricolor*. Also, the Betanin pigment extracted from natural source showed antimicrobial property could be a promising molecule for new innovation in pharmaceutical sciences. The crude and silver nanoparticle pigment derived from plants could be used in textile industry for making the antibacterial garments. Further, it can be applied as a coating agent for capsule preparation in medicinal field.

Keywords: Nanotechnology, Silver nanoparticles, Antibacterial, Biomolecules, Phyto-compounds

INTRODUCTION

Nanotechnology is the study of manipulating matter on an atomic and molecular scale. Buzzing of nanotechnology in each and every aspect of science and technology has been booming at a tremendous rate now a day. Carrying foreword the success of nanotechnology in field of physical, chemical and medical sciences, it has now started revolutionizing the drug delivery sciences (Chakraborty *et al*., 2011). Recent researches proposed the use of nanoparticles in a range of materials, as some metal nanoparticles are known to possess antibacterial property. They are very easy and economical routes for their synthesis have been discovered. These do not require the technical expertise of well-equipped laboratory professionals (Malik *et al*., 2014). To avoid the toxicity of nanoparticles, green synthesis of nanoparticles has been proposed as a cost effective and environment/eco- friendly alternative. These alternative forms are emerging as an reliable innovations.

The effectiveness of NPs enhanced due to the increase in specific surface area and surface energy released during reduction of silver ions (Nair and Pradeep, 2002). Silver has long been documented in having an inhibitory effect on many microorganisms commonly present in medical and industrial habitats (Merin *et al*., 2010). AgNPs proved to be effective as an antimicrobial agent even at a very low concentration and thereby it inhibits the growth of antibiotic resistant bacteria. AgNPs interact with membrane protein surface and genetic material of bacteria. These have sulphur and phosphorous complex on the surface area that have high affinity towards AgNPs (Arokiyaraj *et al*., 2014). The biological methods for the synthesis of NPs using bacteria (Prasad *et al*., 2011), fungi (Vahabi *et al*., 2011; Mukherjee *et al*., 2001), plants (Geethalakshmi and Sarada, 2012; Sharma *et al*., 2015; Park *et al*., 2011 ; MubarakAli *et al*., 2011), algae (Sharma *et al*., 2010, Chen *et al*., 2019, Castro *et al*., 2013), sea weed (Thakkar *et al*., 2010), and lichen (Mie *et al*., 2014) have been recommended as eco-friendly product to chemical and physical process. Biological synthesis of NPs are free of hazardous material on their surface and are more skin friendly. They have wider application in medicinal field to treat diseases. Thus, NPs can be used as a new tool for targeting in cancer therapy.

Although the exact mechanism of NPs biosynthesis by various plant extracts is ambiguous, it has been revealed that the biomolecules in plant extract such as protein, phenol and flavonoids play a significant role in the reduction of metals ions and capping the biosynthesized nanoparticles (Krishnaraj *et al*., 2010).

Vegetables are considered to be the cheapest and most available resources of important proteins, carbohydrates, vitamins, dietary minerals and essential amino acids (Awah *et al*., 2012). Previous studies showed that the consumption of vegetables are closely related with the decrease risks of diseases that resulted from oxidative stress, including cancer, diabetes and various infectious diseases (Doll, 1990; Liu, 2004). While previous reports suggested that amaranthine and its epimeric model act as major betalains in *A. spinosus* seeds (Cai *et al*., 2005). The analysis of betanins pigment and its bological applications have so far not been investigated much in literature surveyed. Many medicinal plant crude extracts are subjected to NPs synthesis and had got good pharmacological activity. But extraction of betanin pigment and its ability to cap the silver compounds are yet to be investigated. The main purpose of this work was to consider the potential of betanin pigment of *Beta vulgaris* and *Amaranthus tricolor* L. for the biosynthesis of Ag NPs and investigation up on their antibacterial activities against both Gram-positive and Gram-negative species of bacteria. This work can place an evidence that betalin pigment coupled silver can act as antibacterial agents against multidrug resistant strains of bacteria.

MATERIALS AND METHODS

Plant material

The stem of *Amaranthus* and *Beta* (Amaranthaceae) was collected and the barks were removed from the stems with a knife, dried in the laboratory at 30 ^{o}C for 36 h, and pulverized in a laboratory mortar. The obtained ground materials are then stored out of light and dampness. The pigments are obtained from red pigment seen on the sides of the stem part of plant material.

Extraction and purification of extracts

About 5.00 g of ground plant material especially stem portion of the plant are placed in sea sand before homogenization in a mortar to ease extraction with 25 ml purified water. The betanins and phenolics obtained are separated from the material by passing the slurry through a funnel with a Whatmann filter paper. The pH of the resulting solutions was 5.4 for *Amaranthus*, respectively. From an aliquote of these crude extracts, slightly concentrated in vacuo at room temperature. Using these aliquote, betanin quantifications and colour determinations was done (Cai *et al*., 2001).

Betacyanin determination

Betacyanin content was determined by using a UV- Vis spectrophotometer (Thermo-evolution 220) at a wavelength of 538 nm. Phosphate buffer was prepared and it was used to replace with distilled water for betacyanin determination (Cai *et al*., 2001). The quantification of betacyanin was described (Song *et al*., 2015). The betacyanin content (mg/100 g of fresh weight) was calculated using A_{538} (MW) x V x (DF) / εLWx100 with respective wavelength.

Quantification of betanins

Quantification of betacyanins without the removal of phenolic compounds was carried out using a UV-Vis spectrophotometer (Thermo-evolution 220). Samples was then diluted in a 0.05 m phosphate buffer (pH 6.5) as previously described (Stack et al. 2003) using the extinction coefficients of betanin (ε = 60000 l/ mol · cm; λ = 538 nm; molecular weight = 550;and neobetanin (ε = 18200 l/mol · cm; λ = 476 nm; molecular weight = 548; amaranthine (ε = 56600 l/mol · cm; λ = 538 nm; molecular weight = 726; respectively (Castellanos-Santiago and Yahia, 2008; Gins *et al*., 2002). From these molecular extinction coefficient values the different class of pigments was calculated.

Biosynthesis of Silver nanoparticles (Mortazavi *et al*., 2021)

Aqueous solution of silver nitrate (1 mM) was prepared and mixed with betalin pigment of *Beta vulgaris* and *Amaranthus tricolor* L. at a ratio of 9:1. This solution was placed on a shaker with constant rotation in the room temperature at 27±2°C for 6 h. Color variation were noted at the incubation time. All stages of the experiment were implemented in three replicates (Mortazavi *et al*., 2021).

Characterization of the silver nanoparticles

The reduction in Ag+ ions was monitored by UV-VIS spectra of the silver nanoparticle solution after diluting a small aliquot of the sample into de-ionized water and the UV-VIS spectra were recorded by Shimadzu UV-1800 Spectrophotometer from 200-800 nm. The respective peaks were analyzed for each sample administered (Mortazavi *et al*., 2021).

Scanning Electron Microscopy

The particle size and surface morphology of synthesized Ag NPs are measured using the Scanning Electron Microscope (SEM) JSM-6390 LA coated with gold, examined and photographed using JSM-6390 LA. Image pattern of the crystals were visualized and interpreted (Mortazavi *et al*., 2021).

FTIR analysis (Fourier transform infrared)

FTIR technique was used to study the presence of phytochemical constituents. FTIR spectrum of Silver nanoparticle was recorded using FTIR spectrometer Thermo Nicolet; Avatar 370.The instrument is equipped with a deuterated triglycinesulphate (DTGS) detector. The Alpha-P ATR accessory is equipped with a single-reflection diamond ATR hemisphere and a spring loaded mechanical press for compacting solid samples at the ATR waveguide surface with uniform and reproducible pressure. The data were recorded in the MIR spectral range from 4000–375 cm−1 at a spectral resolution of 2 cm−1. 200 scans were averaged for background and sample spectra, respectively (Mortazavi *et al*., 2021).

Antibacterial activity of biosynthesized Ag NPs

The antibacterial activity of the biosynthesized Ag NPs against Gram-positive and -negative bacteria species was done by disc diffusion method. Experimented bacteria were *Bacillus subtilis* (accession number: MTCC11985), and *Escherichia coli* (MTCC: 1921) that were procured from Jubilee Mission Hospital, Thrissur, Kerala.

Resazurin based microtitre assay

Under aseptic conditions, 96 well microtitre plates (Tarson) were used for Resazurin based Microtitre Dilution Assay. The first row of microtitre plate was filled with 100 µl of test materials in 10% (v/v) DMSO or sterile water. All the wells of microtitre plates were filled with 100 µl of nutrient broth. Two fold serial dilution (throughout the column) were achieved by transferring 100 µl test material from first row to the subsequent wells in the next row of the same column in the microtritre plate. 10 µl of resazurin solution as indicator was added in each well. Finally, a volume of 10 µl was taken from bacterial suspension and then added to each well to achieve a final concentration of 5×10^6 CFU/ml. Each microtitre plate had a set of 3 controls: (a) a column with Ampicillin and Gentamycin loaded as 0.1mg/ml as positive control (Sigma-Aldrich) as positive control, (b) a column with all solutions with the exception of the test extract and (c) a column with all solutions except bacterial solution replaced by 10 µl of nutrient broth. The plates were incubated in temperature controlled incubator at 37° C for 24 h. The lowest concentration of plant leaf extract at which color change occurred was recorded as the MIC value. All the experiments were performed in triplicates. The average values were calculated for the MIC of test material (Sarker *et al.*, 2007).

RESULTS

Spectophotometric analysis of betanin pigments

The present work evaluated the betacyanin pigment among the two plants of Amaranthaceae, *Beta vulgaris* and *Amaranthus tricolor* L. The result showed good betanin content among the plants and these pigments had potent antioxidant capacity from previous studies. Based on the observation using UV-VIS spectrophotometer, all samples showed absorbance at 536 nm indicates the presence of betacyanin compound. The highest content of betanin were reported in *Beta vulgaris* was found to be 106.8±0.09µg/µl, similarly for betanin content both plants showed a range of 78.23±0.05µg/µl to 99.13 ±0.62µg/µl concentration respectively as showed in Fig. 3.

UV- Vis analysis of Silver nanoparticles

It was noted that a change in color variation as the time passed, this was because of reduction of silver ions. The resulting colloidal solution of both *Beta vulgaris* and *Amaranthus tricolor* L. were scanned with UV-Vis spectroscopy and the observed spectrum are showed in Fig. 4 and 5. *Beta vulgaris* showed prominent bell shaped peak at 250-340 nm and followed by 450 nm where surface plasmon excitation had occurred.

SEM analysis of silver nanoparticles

The morphology of the Ag NPs was analysed through SEM images analysis. The majority of the particles were spherical in shape and the biosynthesized NPs are distributed throughout the solution. SEM micrograph of the synthesized Ag NPs from betanin pigment of *Beta vulgaris* stem showed the presence of mono-dispersed NPs with particle size ranging from 266.83- 480.83 nm with average particle size of 341.235 nm respectively (Fig. 6). It was observed that the particles are surrounded by a thin layer as capping organic material from the plant extract. This was exhibited by the synthesized Ag NPs from betanin pigment, which showed better antibacterial activity from the present study conducted as shown in Table 1.

FTIR

The functional group analysis study conducted, a prominent bands of absorbance was observed at around 3300–3500 cm^{-1} which indicates the presence of (O–H) functional group appears to be phenols and alcohol in *Beta vulgaris*. Other observed peaks for the red pigment include, 1635.00 cm^{-1} denote –C = O as the functional group. The peaks at $v \sim 1400\ cm^{-1}$ and $v \sim$ 1063.62 cm^{-1} are mainly due to C–C and C–N vibrations of the tetra-pyrrole ring of chlorophyll structure, which is associated with the UV–visible spectral range (Fig. 7). The bands at 595.11 cm−1, and also 829 and 550.88 cm−1 represent the presence of =CH as the aromatic compounds in the plant extract and the biosynthesized Ag NPs (Fig.8).

Antibacterial investigation of silver nanoparticles

The antibacterial activity of biosynthesized Ag NPs with betanin pigment and crude betanin pigment of two plants of Amaranthaceae are shown in Table 1 and Fig. 9. The measured MIC for the growth of bacteria was observed in each plate. Betanin pigment with biosynthesized Ag NPs are resistant to strains. *Beta vulgaris* showed a zone of inhibition in the range of 1.25± 0.05mg/ml-0.625± 0.23 mg/ml respectively. *Amaranthus tricolor* pigment also showed a moderate activity against gram positive and gram negative bacteria. Resazurin dye showed colour variation as the grown of the microbe will convert resorufin by oxidation reduction within viable cells. The antibacterial activity using resazurin dye showed 6.25±0.03 µg/ µl inhibition of *Bacillus* sp. and *E.coli* strains by the silver nanoparticles synthesized from *B. vulgaris*.

DISCUSSION

Plant pigments are used in different sectors of industry and pharmaceutical departments. Betanins are reported to exhibit preventive anti-carcinogenic properties (Cai, *et al.*, 2005, Stintzing and Carle, 2007). The red coloration of betanin of *B. vulgaris* and brown coloration after incubation time of $AgNO_3$ were visualized which showed the reacting mixture in the test tube. They constitute a prospective group of compounds (chromophore), not only for food, but also pharmaceutical or cosmetic industries (Khoo *et al.*, 2017). Betanins are effective group of class that found to be prominent in Amaranthaceae family and had wide range of application in industrial sector.

The purple color pigment (betanin) had showed a slight change in color after mixing with $AgNO_3$ solution. This transition to golden brown color indicated the formation of silver nanoparticles complexed with phyto-compounds present in the plants. While moving to the incubation time, the color intensity increased, which confirmed the Ag ion reduction and the formation of Ag NPs (Karthik *et al.*, 2016). Silver nanoparticle surface plasmon excitation on the samples causes color change in the solution (Bindhu and Umadevi, 2014), which is the primary and noticeable evidence for the formation of Ag NPs (Banerjee *et al.*, 2014). The number of peaks increases by increasing diversity of particles shapes (Raut *et al.*, 2009, Fatimah and Aftrid, 2019). For *Amaranthus tricolor*, a single peak was observed at 250-350nm as showed in Fig. 3. Then, it can be concluded that biosynthesized Ag NPs are unanimously spherical in nature, spread evenly in the solution.

This coupled silver on phyto-compounds on plants are appeared on the peaks detected. The capping organic material is useful to stabilize the nanoparticles in the solution. The crude leaf extract of *A.tricolor* showed lower uniform size of NPs with spheroid form from previous reports. The smallest size of Ag NPs demonstrates the highest antibacterial activity in the plant species (Sharma *et al.*, 2020).

The functional group analysis done on the plants clearly indicated the presence of valuable phytocompounds with therapeutic importance. The FTIR vibrational bands observed corresponding to the bonds such as C-C, C-N and C=O had being reported to be peculiar for proteins and phenols (Sengupta *et al.*, 2015). The identified absorption spectra of the betanin pigment are almost similar with the FTIR analysis reports from spinach extract (Fatimah and Indriani, 2018). Either the absence of 3182.75 cm^{-1} absorbance or the shift of 1387.4 cm^{-1} in AgNPs spectrum is the indication of the Ag^+ bio-reduction to Ag^0. The involvement of plant phyto-compounds in biosynthesizing of nanoparticles was confirmed with shifting of peaks as per obtained FTIR data. Thus, plant extract compounds including OH and CO groups have a vital role in reducing and stabilization of NPs synthesized (Franci *et al.*, 2015). This could signify that the extract has proteins and phenolic compounds. Silver had the property to couple with many phyto-compounds and function in diverse manner in order to stimulate the therapeutic property of the plant. These bands are basically due to the secondary metabolite compounds such as anthocyanin chlorophyll or flavonoid seen in the selected plants under study. These data are similar to other studies on AgNPs biosynthesis on different medicinal plant used in daily living (Sre *et al.*, 2015).

This potentiality of Ag NPs confirms the multifaceted strategy of Ag NPs in the bacteria exposure upon infection (Oves *et al.*, 2018). The mechanism of bactericidal activity of Ag NPs is most likely due to the attachment of the Ag NPs to the cell wall and the generation of free radicals during the process. This may also result in degradation of cell wall, genetic material and inhibit the metabolic pathways of pathogenic bacteria. Moreover, the presence of Ag NPs in the cell membrane of bacteria has been proven in earlier studies (Vazquez-Muñoz *et al.*, 2019). Silver ions' release from Ag NPs acting as reservoir causes antibacterial activity of Ag NPs on the bacterial surface (Pirtarighat *et al.*, 2019). Previous studies revealed that plant-mediated metal nanoparticles like copper and zinc play a crucial role in drug delivery and can be used against many microbes because of the efficiency antimicrobial properties they possess (Nisar *et al.*, 2019, Akbar *et al.*, 2020). This suggested that the synthesized silver nanoparticle has good efficacy against bacteria and could be used in treating bacteria related diseases.

e-ISSN 2320 –7876 www.ijfans.org
Vol.11, Iss 7, 2022

CONCLUSION

Betanin pigment in Amaranthaceae family are being compared first time for the tribe Amaranthinae (*Amaranthus tricolor*) and Gromphidea (*Beta vulgaris*). This data provides a significant idea regarding the silver nanoparticles produced from two plants of same family. Shift in the peak was observed for pigment and pigment synthesized silver nanoparticles from *Beta vulgaris*. In this study, possible functional groups and effective compounds responsible in reduction of silver ions were assigned. This study also confirms that *Beta vulgaris* has more betanin pigment and has got good antibacterial effect on selected strains of bacteria. The synthesized pigment along with nanoparticles can be used as a dyeing agent in textile industry producing antibacterial cloth. The study can provide an effective platform for industrial based application.

ACKNOWLEDGEMENT

The authors express the gratitude to STIC, CUSAT for providing support for instrumentation facility.

CONFLICT OF INTEREST

The authors declare no conflict of interest for the study conducted.

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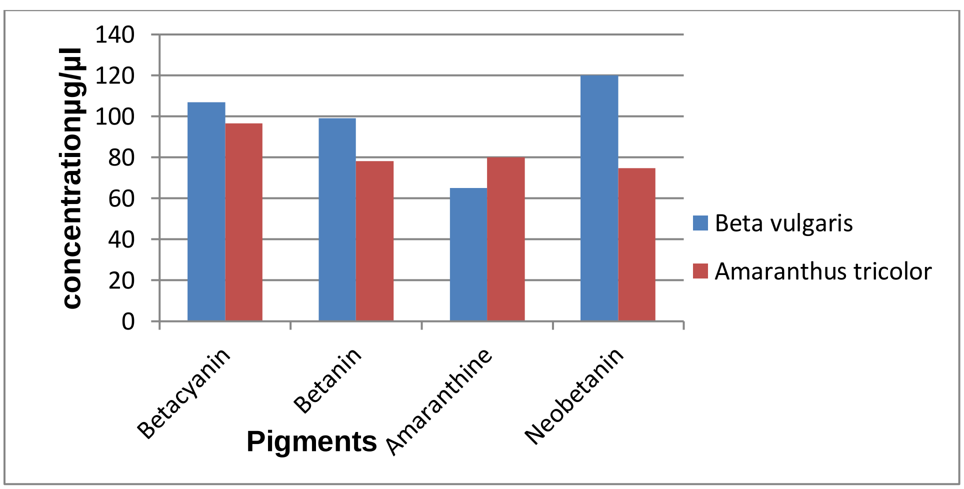
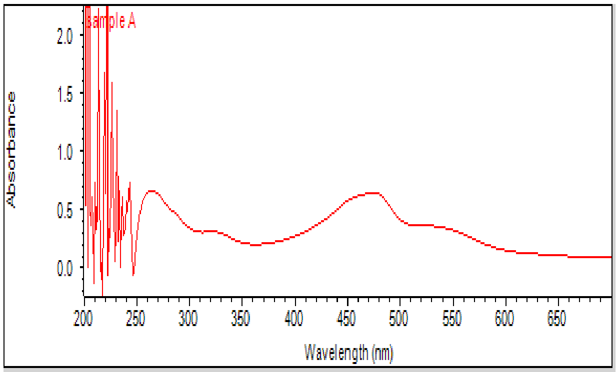
Fig 1. Red colored betanin pigment after adding AgNO3

Fig 2. Brown coloration of betanin (*Beta vulgaris*) after incubation time

Fig 3. Comparison of Pigments in *B. vulgaris* and *A. tricolor*.





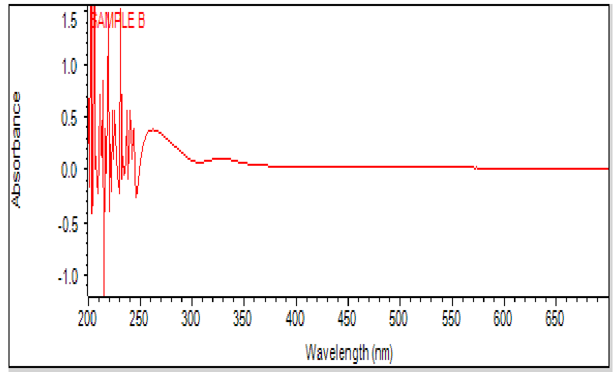


Fig. 4- UV Vis absorption spectra of silver nanoparticles by *Beta vulgaris*

Fig .5-UV Vis absorption spectra of silver nanoparticles by *Amaranthus tricolor*

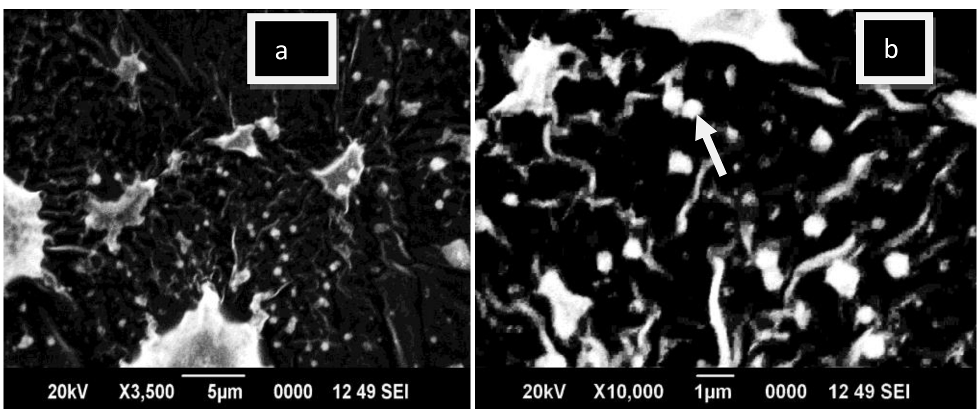


Fig.6- SEM images scale a) 5µm and b) 1µm. Nanoparticles coated with organic materials of plant pigment have been indicated in arrow in the picture.

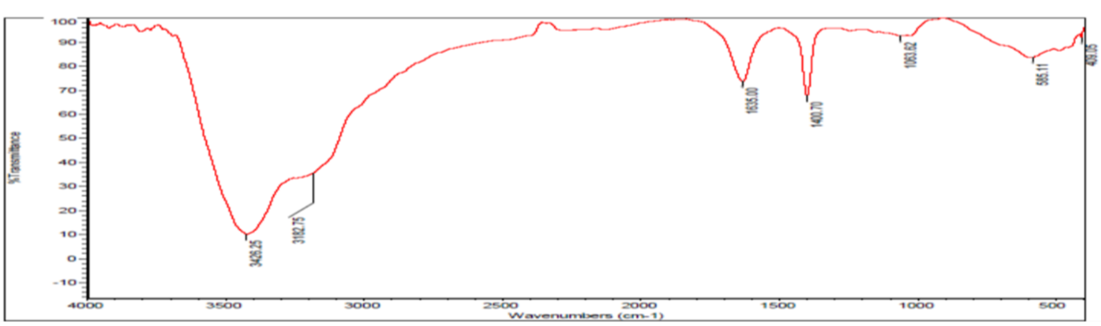


Fig.7 -FTIR of Betanin extract of Beta vulgaris

e-ISSN 2320 –7876 www.ijfans.org
Vol.11, Iss 7, 2022
Research Paper

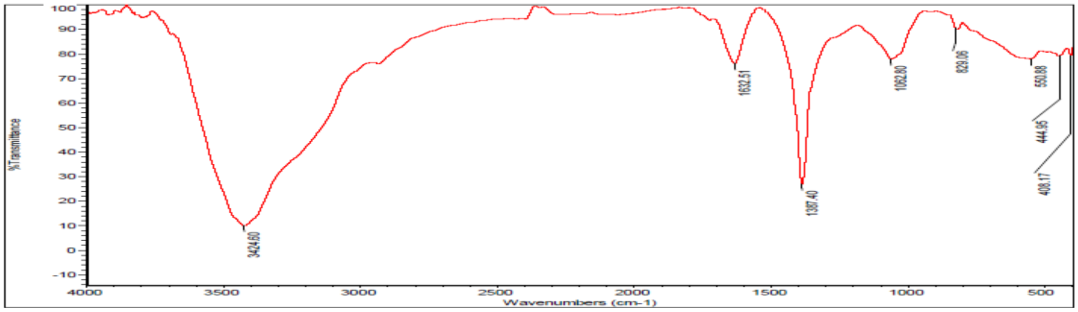


Fig. 8- FTIR of silver nanoparticles from Betanin in B. vulgaris

Table 1 . Antibacterial activity of silver nanoparticle of crude extract of betanin pigment

Bacteria	**MIC(mg/ml) *B.vulgaris***	**AgNPs(*B.vulgaris*) MIC(mg/ml)**	***A.tricolor* (mg/ml)**	**AgNPs(*A.tricolor*) MIC(mg/ml)**	**Control MIC(mg/ml)**
Bacillus substilis	0.625±0.23	6.25±0.23	1.25±0.23	1.25±0.02	1.25±0.02
E.coli	1.25±0.05	1.25±0.05	0.5±0.05	1.25±0.02	6.25±0.12

Mean±SD

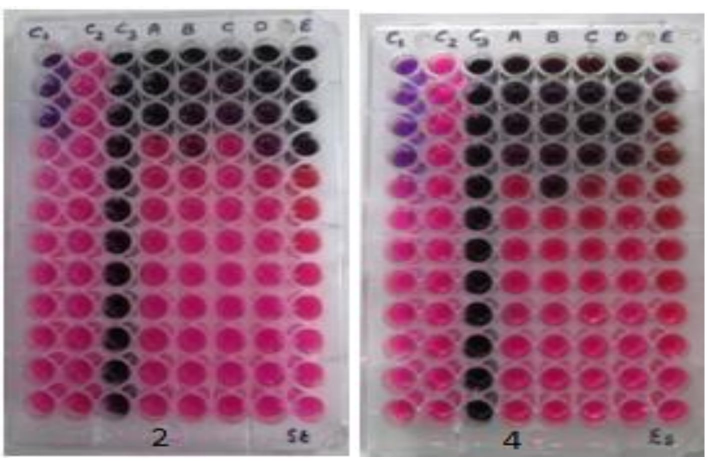


Fig. 9. C1- Positive control, C2- Negative control, C3-Broth control, A-B.vugaris, B-AgNPs of B. vulgaris, C-A.tricolor, D-AgNPsA.tricolor,E-AgNPs