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Anticancer and antiviral potential of folic acid conjugated with silver and gold Nanoparticles produced by Thermophilic *Bacillus alcalophilus subsp halodurans*

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ABSTRACT

Fifteen bacterial isolates were purified from Wady El Natrun loam salty calcareous sand soil textures and subjected to screen for their ability to produce folic acid. Bacterial isolate number WN12 was highly producer of folic acid (Vitamin 9) and it was selected tentatively to be identified physiologically and phylogeny. The strain grows at 15-50°C, pH 6.0-12.5 and at salinities of 3.0-27.5 % NaCl (w/v). It was Gram positive, translucent brownish smooth color colony, long rods; non spore forming, motile by peritrichous numerous flagella. WN12 isolate identify as *Bacillus alcalophilus subsp halodurans*. The TLC R_f values for crude folic acid was 0.7 by using solvent system n-butanol, glacial acetic acid, water, methanol (20:10:20:5 v/v). The UV analysis of the fractions to the purified bacterial folic acid peak at 270 nm. Also, TEM images estimated the average size nearly around 0.1µm. While HPLC chromatograms of free folic acid maximum absorption wavelength was selected at 280 nm. And the FTIR spectra show absorption bands for pure folic acid infrared spectrum showed several identical absorption bands including OH, band at 3500 cm⁻¹, C-H, band at 2900 cm⁻¹, C=O band at 1100 cm⁻¹. Comparison of the two synthesis conjugates Nano gold (AuNPs) and Nano silver (AgNPs) with folic acid (FA) characterization were done. Six treatment were tested for measuring infected dose 50 (IC₅₀) on four cancer cell lines. Cytotoxic effect of folic acid alone and Ag NP showed a variable pattern of reactivity to cell lines arranged in the order of MCF7 > HCT > CaCO₂ > HEPG2 (P < 0.05). AuNPs have no effect on HEPG2 and have highly effect on hct, MCF7₂ and Caco2 cell lines. Also, combination with FA showed the same pattern of antagonism. Preparation of trivalent mix of Ag-Au-FA showed antagonistic reaction to cell lines and toxicity of mix to cell lines was arranged in the order of MCF7 > CaCO₂ > HCT > HEPG2 (P < 0.05). Also conjugated treatment FA+AGNPs, (FA+AUNPs and of trivalent mix of Ag-Au-FA have highly effect only on breast cancer (MCF₇) IC₅₀. Finally Folic acid toxicity with the least concentration showed between 0.130 mg/ml to 0.180 mg/ml significant IC₅₀ high affecting on different cell line. Antiviral potential were tested. Conjugated FA+AGNPs, FA+AUNPs were tested against A virus (HAV) and Vesicular stomatitis virus (VSV). Au-FA sample and Ag-FA sample materials could induce viral load depletion in the order of 21% and 74% for HAV post cell treatment with two test materials respectively. In the mean time VSV virus infectivity titer was decreased recording depletion of infectivity titer in the order of 3.75(68%) and 4.13(75%) for AuNP-FA- and AgNP-FA respectively.

Keyword: Folic acid, *Bacillus alcalophilus subsp. halodurans* bacteria, antitumor's, Antivirus, nanosilver and nanogol

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INTRODUCTION

Folic acid or folate are forms of water-soluble B vitamin. Folate occurs naturally in food, and folic acid is the synthetic form of this vitamin. It is also referred to as vitamin M, vitamin B₉, pteroyl-L-glutamic acid, and pteroyl-L-glutamate. Folic acid molecular formula is C₁₉H₁₉O₆ and molecular weight is 441.4 . The federal government has mandated that grain-based products (bread, breakfast cereal, pasta, etc.) be supplemented with this vitamin. Other sources rich in folic acid are leafy vegetables, yeast and liver (Klaassen *et al.*, 1986)¹.

Folate deficiency has been implicated in a wide variety of disorders from Alzheimer's to coronary heart diseases; osteoporosis, increased risk of breast and colorectal cancer, {Luchsinger *et al.* 2007, Baines *etal.*2007, and Van Guelpen *etal.*2006}²⁻⁴.poor cognitive performance , hearing loss and of course, neural tube defects {Durga *et al.* 2007 a , Durga *et al* 2007b and Czeizel 1992}⁵⁻⁷.Common symptoms of folate deficiency include diarrhea, macrocytic anemia with weakness or shortness of breath, nerve damage with weakness and limb numbness peripheral neuropathy (Chudarkodi *et al.*, 2015).⁸ Annually, approximately 80,000 new cases of oral cancer are reported worldwide. Death will occur in more than 60% of these cases Han *et al.*(2012)⁹.

Human life can not exist without folate since this B-group vitamin is involved in essential functions of cell metabolism such as DNA replication, repair and methylation, and synthesis of nucleotides, vitamins and some amino acids. Folic acid is a vitamin which is essential for the biosynthesis of nucleotide bases and is very consumed by proliferating cells. It is stable during storage, compatible with many solvents and low cost. Because of these properties it has become a widely used molecule for targeting cancer cells. Vitamin B₉ (folic acid and folate) is essential for numerous bodily functions (Parket *al.*,2005; (Chudarkodi *et al.*,2015)^{10,8}.Folate is involved, as a cofactor in many certain metabolic reactions, including the biosynthesis of the building blocks of DNA and RNA, the ribonucleotides. It is especially important in aiding rapid cell division and growth, such as in infancy and pregnancy (Bailey and Ayling, 2009)¹¹. Healthy cells, in turn, depend on the continued, faultless replication of our DNA. DNA can be seriously damaged through attacks by free radicals so an adequate antioxidant status is essential to cell health. Folates possess antioxidant properties that protect the genome by inhibiting free radical attack of DNA in addition to their role in DNA repair and replication mechanisms (Duthie *etal.*2002)¹².

The aims of this study are to isolate some extreme haloalkalophilic bacteria and select the highly folic acid production isolate to study some Phenotypic taxonomical characteristics and identification. The production, extraction and purification of folic acid from the fermentation

medium and the spectroscopic analysis (UV, IR, HPLC spectrum and TEM) were also investigated. Application of folic acid conjugated nano silver and nano gold were done as antitumor and antiviral drugs or antioxidant. The work was investigate evaluation of anticancer against MCF7, CaCO₂, HCT and HEPG2 cancer cell line and antiviral potential against anti hepatic (A virus) and Vesicular stomatitis virus (VSV) in vitro using folic acid produced from Thermophilic *Bacillus alcalophilus subsp halodurans*

MATERIALS AND METHOD

Loam salty calcareous sand soil texture sample was collected from Wady El Natrun soil, Egypt. A total of 15 haloalkalophilic bacteria isolates were isolated by dilution plate method and modified Horikoshi medium (Horikoshi, 1999)¹³. Dilution plate method was used for the isolation of haloalkalophilic bacteria. One ml of the dilutions was plated on appropriate sterilized solid modified Horikoshi agar medium, which contains (g/l): Glucose, 5.0; polypeptone, 5.0; K₂HPO₄, 1.0; MgSO₄.7H₂O, 0.2; Na₂CO₃, 10.0; agar 25.0, all ingredients were dissolved in 900 ml tap water. Na₂CO₃ and glucose were sterilized separately each in 50 ml water and added to the medium before pouring (Horikoshi, 1999) and incubated for 7 days at 37°C for selected bacteria. The morphological differences of selected bacterial strain WN12 has been studied on Sato medium (Sato *et al.*, 1983)¹⁴ or Horikoshi medium (Horikoshi, 1999)¹⁵. Furthermore, microscopic observations after different chemical treatments that give detailed information on the cell morphology under different extreme conditions, and then they were examined by scan electron microscope. (Regional Center for Mycology and Biotechnology (RCMB lab.) .

Folate Production by Microbiological Assay

Fifteen strains were subjected to screen for their ability to produce folic acid by growing them in the chemically medium of folic acid in the folic acid casei medium incubated at 30°C. All haloalkalophilic isolates were screened for their ability to produce folate extracellularly, using *Lactobacillus casei* subsp. *rhamnosus* ATCC7469. Folate was determined by promoting zone diameter of microbiological assay method.

Principles of the Procedure and Formula of Casei assay media

Folic acid Casei medium is a folic acid-free dehydrated medium containing all other nutrients and vitamins essential for the cultivation of *L. casei* subsp. *rhamnosus* ATCC 7469. The addition of folic acid in specified increasing concentrations gives a growth response that can be measured by promoting zone diameter. (Water and Molin 1961)¹⁶.

Folic acid Casei medium consists of per liter, Charcoal Treated Pancreatic Digest of Casein., 10.0 g ,Dextrose. 40.0 g ,Sodium Acetate. 40.0 g ,Dipotassium Phosphate 1.0 g ,Monopotassium Phosphate. 1.0 g ,DL-Tryptophan.. 0.2 g,L-Asparagine. 0.6 g ,L-Cysteine Hydrochloride. 0.5 g , Adenine Sulfate. 10.0 mg ,Guanine Hydrochloride. 10.0 mg,Uracil.10.0 mg ,Xanthine. 20.0 mg ,Polysorbate 80. 0.1 g, Glutathione (reduced) 5.0 mg , Magnesium Sulfate (anhydrous). 0.2 g ,Sodium Chloride. 20.0 mg ,Ferrous Sulfate. 20.0 mg, Manganese Sulfate 15.0 mgRiboflavin. 1.0 mg ,*p*-Aminobenzoic Acid. 2.0 mg ,Pyridoxine Hydrochloride 4.0 mg, Thiamine Hydrochloride. 400.0 µg ,b Calcium Pantothenate. 800.0 µg, Nicotinic Acid. 800.0 µg , Biotin. 20.0 .

Extraction of folic acid by Thin-layer chromatography (TLC):

TLC was performed with plates covered with MN-cellulose 300. The following solvent systems were used for crude folic acid , n-butanol, glacial acetic acid, water and methanol (20:10:20:5 v/v). Rappold and Bacher (1974)¹⁷

Folic acid estimation

Analytical characterization of folic acid were done by, UV-visible absorption spectroscopic measurements, high pressure liquid chromatography (HPLC) ,transmission electron microscopy (TEM) images and Fourier transmission infrared (FT-IR).

UV-visible absorption spectroscopic measurements

Detection is performed by UV-absorbance measurement. Which has sufficient sensitivity only for fortified bacterial product containing high levels of folates[Hoppner and Lampi, 1982)¹⁸ .UV were recorded on a double beam UV-vis spectrometer,(Agilent 8453, using quartz cells of 1 cm path length and methanol as the reference solvent at room temperature). National Centre for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Cairo, Egypt.

Folate analysis by high pressure liquid chromatography (HPLC)

Preparation of crud sample for HPLC analysis

Curd sample of thermophilic *Bacillus alcalophilus* subsp .*halodurans* cells were analyzed for folate by HPLC method. To prepare cell free extract, Crud sample was centrifuged at 10,000 rpm for 10 minutes and the supernatant was collected and mixed with the mobile phase for HPLC analysis. HPLC detector GBC UV/Vis,GBC LC1110 pump and Win chrome chromatography ver.1.3 . HPLC condition were Kromasil- c18 150 x4.6mm,UV 280 nm and HPLC gradient elution with methanol-water (15-85) was used to separate the folates, according to (Herranen *et al.*, 2010)¹⁹ . (NCRRT), Atomic Energy Authority, Cairo, Egypt.

Fourier transmission infrared (FT-IR) spectra.

Vibrational spectroscopy is featured by the FTIR measuring the absorption bands of molecular vibrational modes in the infrared, the orientation of long molecule chains and the composition of thin films are analyzed. method and Fourier transmission infrared (FT-IR) spectra (Perkin Elmer FTIR system; Spectrum GX) in the range of 400–4000 cm^{-1} with a resolution of 0.2 cm^{-1}). (JA SCO FTIR 3600 (NCRRT), Atomic Energy Authority, Cairo, Egypt.

Determination of Phenotypic taxonomical Characteristics

The morphological differences of bacterial strain has been studied on Sato medium (Sato *et al.*, 1983) or Horikoshi medium (Horikoshi, 1999)¹⁵. Methods used to clarify the Gram classification of this bacterium included modified Gram's stain (Paik, 1980)²⁰; KOH test (Wallace and Gates, 1986)²¹; aminopeptidase activity test and spore examination (Cowan and Steel's, 1977; Cowan 1992)²²⁻²³.

The bacterial cells cultivation and phenotypic characterization were tested. The physiological conditions were carried out as growth at different pH ranges, temperatures range, different salt concentrations, susceptibility to lysis, nitrogen and carbon sources utilization, and different enzymes detections. Phenotypic methods also include biotyping, and antibiogram according to (Cowan and Steel's, 1977; Cowan, 1992; Horikoshi and Grant 1998; Horikoshi, 1999)^{22,23,15}. Depending on phenotypic and chemotaxonomic properties, eubacteria isolate shared almost the same broad range of taxonomical characteristics according to classification of (Sneath, 1986; Holt *et al.*, 1994; Vos *et al.*, 2009)²⁴⁻²⁶; Cluster Analyses for studying phylogenetic relationships of the investigated isolate with other similar reference strains viz. were evaluated by using statistical cluster analysis with joining tree clustering. (Sigma lab.).

Transmission Electron Microscopy (TEM) Examination:-

The isolate WN12 was studied during growth period, until 4 days old as exponential phase at 45⁰C. Also the isolate behavior on high salinity were examined, after 4 days under 28% NaCl., by using TEM (Hitach-H-7500) in (NCRRT), Atomic Energy Authority, Cairo, Egypt. According to Kessel and Klink. (1981)²⁷.

Silver and gold Nanoparticles were prepared for conjugation with folic acid

A) Preparation of Spherical Gold Nanoparticles AuNPs)

Spherical gold nanoparticles (AuNPs) were synthesized in an aqueous medium by the citrate reduction of HAuCl, sodium citrate serves also as a capping material prevents aggregation and further growth of the particles. 100ml of tetra chloroauric (III) acid ,1mM HAuCl₄ solution is heated to boiling while being stirred. Then 10 ml aqueous solution of a 38.8 mM tri-sodium citrate is added quickly. The color of the solution changed from yellow to colorless and finally to deep

red. The solution is then refluxed for an additional 15 min, then the heater is turned off and the solution is stirred until it reached room temperature to control the particle size and thus achieving a narrow particle size distribution. according to Zhaoyang *et.al.* (2010)²⁸.

B) Preparation of Spherical Silver Nanoparticles (AgNPs)

Spherical silver nanoparticles (AgNPs) were synthesized in an aqueous medium by the chemical reduction of AgNO₃ with a modification in the method, the reduction here occurred by using NaBH₄ as reducing agent while sodium citrate and Poly vinyl pyrrolidone (PVP) serves as a capping materials to prevent aggregation and further growth of the particles . A mixture of 0.3 mM of tri-sodium citrate and 0.2 g PVP has been dissolved in 10 ml distilled water and mixed well with stirring. 100 ml of 1mM AgNO₃ solution is added to stirred solution. Then 0.5 ml aqueous solution of 1 mM NaBH₄ is added drop by drop. The color of the solution changed from colorless to yellow according to **Karla Chaloupka (2010)**²⁹ .

(C) Preparation of folic acid-functionalized with Gold and Silver Nanoparticles .

(i) Preparation of FA- AuNPs Nanoparticles:

Dissolve 0.004414 gm(44.14 mg)of folic acid (0.001M) in 10ml of HAuCl +0.01 gm of tri-sodium citrate ,then sonicate for 2 hour at room temperature..

(ii) Preparation of FA- AgNPs Nanoparticles:

Dissolve 0.004414 gm(44.14 mg)of folic acid (0.001M) in 10ml silver nitrate (0.001M) + 0.01 gm of tri-sodium citrate then sonicate for 2 hour at room temperature..

(iii) Preparation of trivalent FA-AU-AG using the extracted folic acid:

One gram of the powder which contains 44 mg of folic acid has been dissolved in 0.01 M NaOH solution. Folic acid will react to give sodium folate, the un-reacted residual has been separated by centrifuge. The solution has been added into 10ml Au⁺³ (0.001M) /or 10 ml of 0.001M AgNO₃ solution pluse 0.01 gm of tri-sodium citrate then sonicate for 2 hour at room temperature. Preparation, surface modification and characterization of gold nano-particles(AuNPs) and (AgNPs) were carried out .Spherical AuNPs and (AgNPs) have been prepared by citrate reduction method using tetra chloroauric (III) acid (HAuCl₄) or HAgCl₄) as mentioned above. Tri sodium citrate initially acts as a reducing agent to reduce the Au(III) ions to Au (0) then acts as the stabilizing agent by forming a layer of citrate ions over the AuNPs or HAgCl₄) surface, inducing enough electrostatic repulsion between individual particles to keep them well dispersed in the medium and prevents aggregation or further growth of the particles (Yi-Cheun *et al.* 2012)³⁰ .

Comparison of the two synthesis conjugates AuNPs and AgNPs nanoparticles with FA characterization were done . Six treatment were tested for measuring infected dose 50 (IC₅₀) on

four cancer cell lines, colorectal (hct) ,liver cancer (HEPG₂), colon cancer(Caco2) and breast cancer(MCF₇) .The six treatments were(1)Nano gold particles (AuNPs). (2) Nano gold + folic acid(FA-AuNPs). (3)Nano silver particles(AgNPs) . (4) Nano silver + folicacid (FA-AgNPs).(5)Trivalent FA-AgNPs-AuNPs particles.(6) Purified folic acid (FA).

Antitumor activity assay In Vitro :-

Four cancer cell lines, (hct) ,(HEPG₂),(Caco2) and (MCF₇) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50µg/ml gentamycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO₂ and were subcultured two to three times a week.(Mosmann, 1983; Elaasseret *al.*, 2011)³¹⁻³² . For antitumor assays, the tumor cell lines were suspended in medium at concentration 5x10⁴ cell/well in Corning 96-well tissue culture plates, then incubated for 24 hr. The tested compounds were then added into 96-well plates (six replicates) to achieve eight concentrations for each compound. Six vehicle controls with media or 0.5 % DMSO were run for each 96 well plate as a control. After incubating for 24 h, the numbers of viable cells were determined by the MTT test. Briefly, the media was removed from the 96 well plate and replaced with 100 µl of fresh culture RPMI 1640 medium without phenol red then 10 µl of the 12 mM MTT stock solution (5 mg of MTT in 1 mL of PBS) to each well including the untreated controls. The 96 well plates were then incubated at 37°C and 5% CO₂ for 4 hours. An 85µl aliquot of the media was removed from the wells, and 50µl of DMSO was added to each well and mixed thoroughly with the pipette and incubated at 37°C for 10 min. Then, the optical density was measured at 590 nm with the microplate reader (SunRise, TECAN, Inc, USA) to determine the number of viable cells and the percentage of viability was calculated as [1-(ODt/ODc)]x100% where ODt is the mean of optical density of wells treated with the tested sample according to Gomha *et al* (2015)³³ and ODc is the mean optical density of untreated cells. The relation between surviving cells and drug concentration is plotted to get the survival curve of each tumor cell line after treatment with the specified compound.The 50% inhibitory concentration (IC₅₀), which is the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots of the dose response curve for each conc. using Graphpad Prism software (San Diego, CA. USA) (Mosmann, 1983; Elaasseret *al.*, 2011 and Gomha *et al.*2015)³¹⁻³³ .

Statistical analysis:

Results were analyzed statistically using student's t-test. Values of p<0.05 were considered statically significant. All data in the text and tables are expressed as a percentage of dark control ±

standard error (SEM) of at least three samples; experiments were repeated 3 times. The statistical analysis was carried out by Graphpad prism software (USA).

Antiviral Activity assay In Vitro:

Antiviral activity of Anti-hepatic (Avirus) and Vesicular stomatitis virus(VSV) virus were determined to evaluate the infectivity titer in virus cells according to Aoki and Messiha, (1999)³⁴

RESULTS AND DISCUSSION

Mammalian cells cannot synthesize folate; therefore, an exogenous supply of this vitamin is necessary to prevent nutritional deficiency. Recently, many reviews focus on folate production by lactic acid bacteria and levels of folate present in foods fermented by/or containing these valuable microorganisms. The proper selection and use of folate producing microorganisms is an interesting strategy to increase “natural” folate levels in foods so they can carry out important functions within the cell (Jean *et al.*,2007)³⁵. Fifteen isolates haloalkalophilic bacteria were cultured into folic acid casei medium for screening their ability to produce folate extracellularly was determined by microbiological assay (Table 1) . A significant differences in vitamin accumulation were found among the species tested (Table1).The highest folate level was detected in an aerobically grown strain

Table 1: Determination of biosynthesis of folic acid by different isolates.

Isolates No.	Protein Content (mg/g)	Vitamin Biosynthesis (mm)
WN 1	0.23	20.8
WN2	0.24	22.5
WN3	0.25	25.5
WN4	0.27	28.2
WN5	0.2	12.3
WN6	0.3	27.5
WN7	0.3	27.8
WN8	0.3	14.8
WN9	0.21	25.1
WN10	0.21	26.7
WN11	0.25	29.3
WN12	0.32	35.9
WN13	0.26	30.6
WN14	0.27	30.3
WN15	0.29	29.8

Number (WN12) with (0.32 mg/gm and 35.9mm) protein content and vitamin biosynthesis respectively. Folic acid production in the highest strains was analyzed by TLC (Table 2). The TLC R_f values for crude folic acid was 0.7 by using solvent system n-butanol, glacial acetic acid, water, methanol (20:10:20:5 v/v).

Table 2: Thin layer chromatography vitamin B₉ produced by isolate No. WN12

Compound	R _f values
Produced vitamin B ₉	0.7
Standard vitamin B ₉	0.65

The spectroscopic analysis UV, HPLC, FTIR spectrum and TME were used for comparative studies between vitamin B₉ produced by culture, isolate No. WN12 and authentic vitamin B₉. Typical TEM images for folic acid template shown in figure (1A). It can be estimated that the average size of folic acid lies nearly around 0.1µm.

The UV analysis of the fractions indicated that the first peak corresponded to the standard folate at peak 232 nm and the second one to the purified bacterial folic acid peak at 270 nm figure (1B).

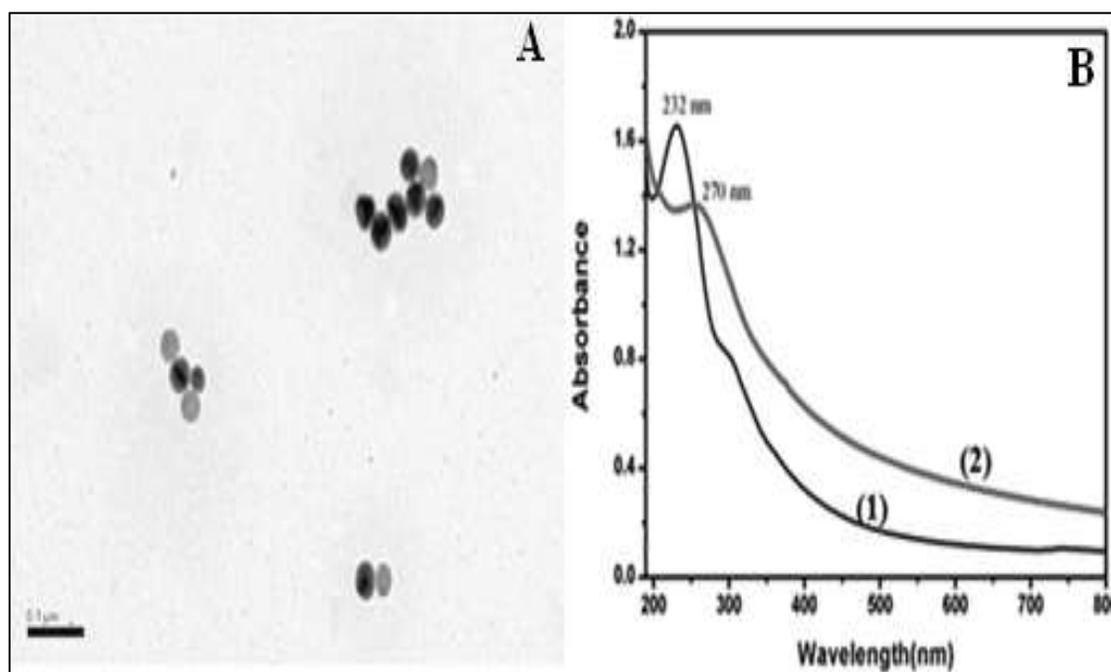


Figure 1: A- Morphology of folic acid by TEM picture; B-Folic acid UV spectra of FA stander molecules (1) and aqueous solution extracted FA (2).

The retention times and spectral characteristics of specific folate calibrators was used to identify the folate derivatives purified from the cells (Figure 2) show the HPLC chromatograms of free folic acid (FA). The detection wavelength was selected at 280 nm, which is the characteristic maximum absorption wavelength of FA .

Folic acid had been determined qualitatively and quantitatively through applying FTIR technique as shown in Figure (3). Pure folic acid infrared spectrum showed several identical absorption bands including OH, band at 3500 cm⁻¹, C-H, band at 2900 cm⁻¹ C=O band at 1100 cm⁻¹, which corresponding to the residual functional groups of folic acid template as shown in table (3).

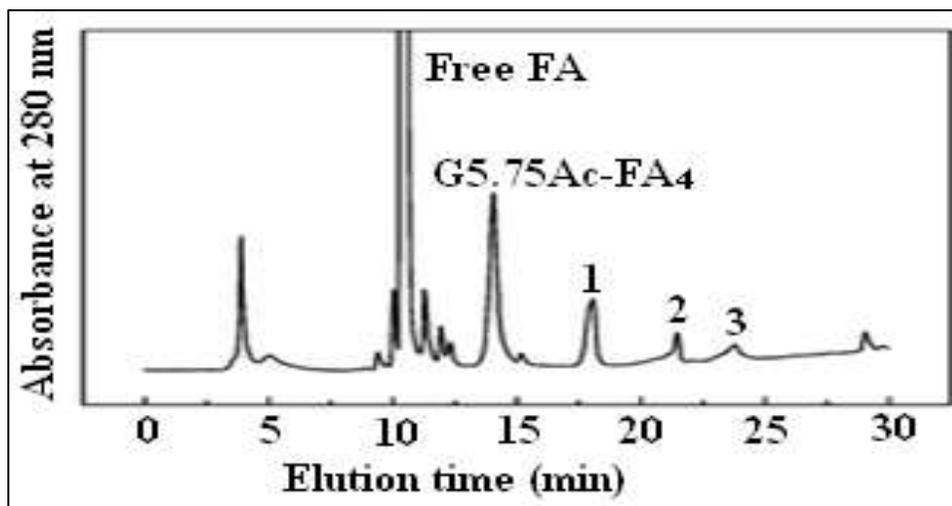


Figure 2: HPLC of free Folic acid

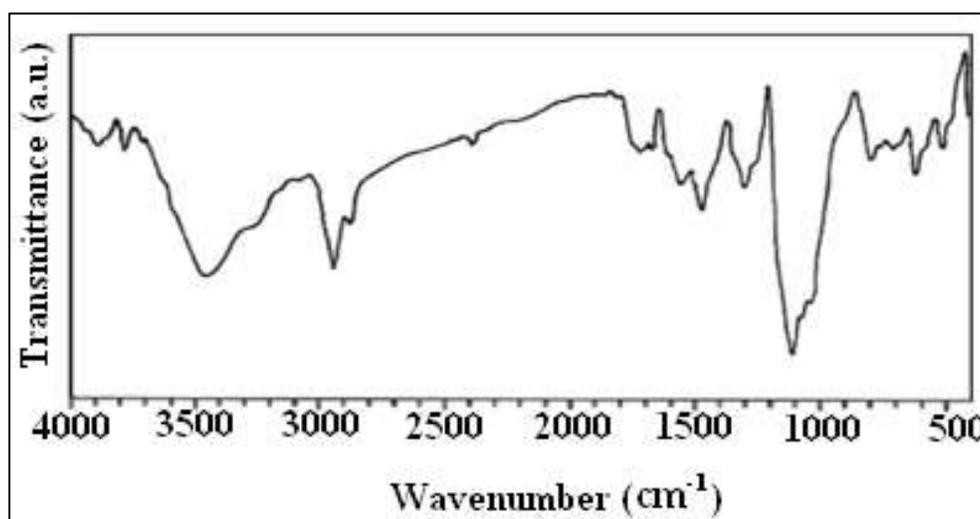


Figure 3: FTIR spectrum of folic acid (0.5 mg)

Table 3: FTIR absorption frequencies for residual groups of folic acid

Wave number (cm ⁻¹)	Absorption band
~ 3500	O-H mode
~ 2900	C-H mode
~ 1500	Symmetric C=O stretching mode
~ 1100	asymmetric C=O stretching mode

In another studies ,Herranen *etal.*(2010)¹⁹ isolated and identify 66 endogenous bacteria in commercial oat bran and rye flake products in order to study their folate production . The most common bacterial *Acinetobacter*, *Bacillus*, and *Staphylococcus*. For comparison, folate production was also examined in a number of common lactic acid bacteria. The best producers belonged to the genera *Bacillus*, *Janthino bacterium*, *Pantoea*, , *Pseudomonas Chryseobacterium*, *Erwinia*, and

Plantibacter . Compared to the endogenous bacteria, lactic acid bacteria were poor folate producers.

Wilbert *et.al* (2003)³⁶ investigated that , Several species and strains from the lactic acid bacterial genera *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc* were screened for folate production. The lactic acid bacteria *L. lactis* MG1363 and *S. thermophilus* B119 were further analyzed for folate production under different growth conditions. *L. lactis*, *S. thermophilus*, and *Leuconostoc* spp. produced folate in the range of 5 to 291 μ g/liter. *Lactobacillus* strains, with the exception of *Lactobacillus plantarum*, did not produce folate. In several strains, folate analysis performed after deconjugation resulted in detection of higher folate levels. Meanwhile, Rossi *et al.* (2011)⁴⁸ found that, *Lactobacillus plantarum* constitutes an exception among lactobacilli, since it is capable of folate production in presence of para-amino benzoic acid (pABA) and deserves to be used in animal trials to validate its ability to produce the vitamin *in vivo*. In addition, Mostafa *et al.*(2013)⁴⁷ investigated that, The ability of some bacterial isolates specially the selected isolates showed great ability to consume the agricultural wastes as substrates to produce vitamin B12 and folic acid . The isolates *Klebsiella pneumoniae* and *Citrobacter freundii* showed the highest yields of 38.74 and 45.21 μ g/l for vitamin B12 and folic acid, respectively, from 200 g/l of the studied wastes. The optimum fermentation temperature for this production was 30°C for 3 days.

Chudarkodi *et al.* (2015)⁸ investigated that, isolation of many microbes as folate producing and probiotic property, it mainly consist of lactic acid bacteria such as *Lactobacilli* , *Streptococci*, *Enterococci*, *Lactococcus*, *Bifidobacteria* and also some *Bacillus* sp. aswell as some yeast like *Saccharomyces* spp and mold like *Aspergillus* spp. While, Norfarina *et al.*(2016)³⁷ stated that, interesting research has been focused on production of folic acid using lactic acid bacteria currently stimulated by various techniques. In this study, several lactic acid bacteria such as *Lactococcus lactis* NZ9000, *L. lactis* MG 1363, *Streptococcus thermophilus* BAA 250 and *Lactobacillus plantarum* UL4, were screened for folic acid production. The production of folic acid was examined using microbiologically assay and *S. thermophilus* BAA250 was shown as the highest producer of folic acid (10.12 μ g/L) as compared to other strains. However, comparable folic acid concentration (9.90 μ g/L) was also obtained by local strain of *L. plantarum* 1_UL4 which was isolated from ‘TapaiUbi’

3 Phenotypic and taxonomical analyses

Out of a total of 15 microbial isolates, only one (No.WN12) highly folic acid (Vitamin 9) producer bacteria was selected from loam salty calcareous sand soil of Wady El Natrun, Egypt, and

tentatively identified physiologically and phylogenetically by sequence analysis of the 16S rRNA genes.

The bacterial cells cultivation and phenotypic characterization were tested. The physiological conditions as the growing of bacteria at different pH ranges, temperature ranges, different salt concentrations, susceptibility to lysis, nitrogen and carbon sources utilization, and different enzymes were detection. Phenotypic methods and also biochemical test include antibiogram according to (Cowan and Steel's, 1977; Cowan, 1992; Horikoshi and Grant, 1998 and Horikoshi, 1999)^{22,23,13,15} were carried out .The selected isolate as folic acid producer was extremetolerant (PH 10 and 22% NaCl).

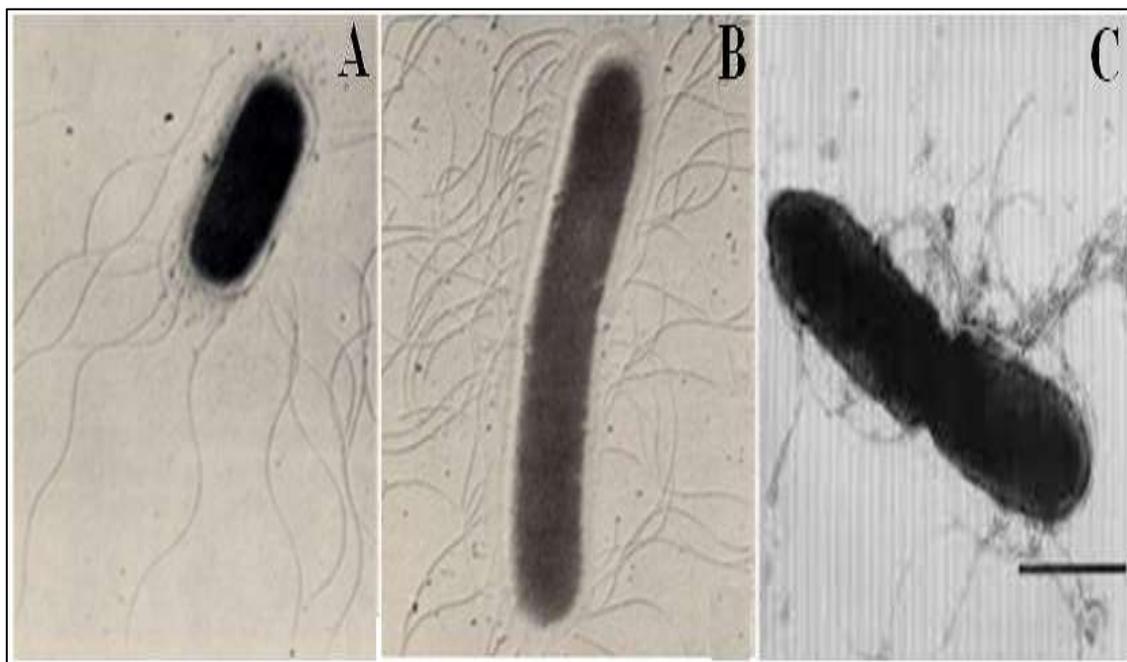


Figure 4: A and B- Transmission electron micrographs (negative stain) for isolate WN12, 96hrs. old (1 μ m - 2 μ m); C- isolate WN12 under 28% NaCl (2 μ m)

Table 4: Phenotypic characteristics of Haloalkalophiles Isolate Number WN12.

No	General characteristics	Reference Isolate <i>Bacillus alcalophilus</i> subsp <i>halodurans</i>	Identified Isolate (WN12)
1-	Morphology of colony	White	Translucent Brownish Smooth, flat, opaque and circular with entire margins: Colony color change under sever condition to pale colony color(0.2-0.7ml)
(2)	Cell morphology	Rod with round ends	Long rods
a)	At early stage		
b)	Habitat		Sand-loam salty calcareous soil textures
c)	Cell dimension length	0.7-0.9um 3-4um	0.4-0.5um 2.5-8.5um
3)	Gram classification :		
a)	Gram stain	Gram positive	Gram variable
b)	KOH test	ND	G+ve
4)	Aminopeptidase activity test	DN	G+ve
5)	Spore forming	Non spore forming	Non spore forming
6-	Flagellation (position)	Peritrichous numerous flagellate	Peritrichous numerous flagellate
7-	Motility	Motile	Motile
8-	Pleomorphism	ND	-ve
9-	O ₂ requirement	Aerobic	Aerobic
10-	- NaCl range at 35°C - At 55°C - Optimum	0-7% -ve ND	3-27.5% 3-20% 8-20%
11-	pH range6-7.5	+	+
12	- At 35°C - At 50°C - pH range in broth - Optimum	7-10 -ve 7-10 9.5	6.5-12.5 7.5-9.5 6-12.5 8 -9.5
13-	a)Temperature range	33-45 °C	15-50 °C
	b) -Optimum temp.	33-37°C	28-42°C
14-	Temperature tolerance at 65°C	ND	survive

No	General characteristics	Reference Isolate <i>Bacillus alcalophilus</i> subsp <i>halodurans</i>	Identified Isolate (WN12)
15-	Growth period (at pH 10 and 20% NaCl At 4 °C At 35 °C	-48hr -ve 72- 120h	36-144hr -ve 36-144hr
Biochemical tests			
16-	Oxidase	-/+	-
17-	Nitrate reductions	+	+
18-	Catalase	+	++
19-	Indole	-	-
20-	H ₂ S production	-	-
21-	Urease	-	-
22-	Gelatin liquefaction	+	+
23-	Starch hydrolysis	+	+
24-	Casein hydrolysis	+	+
25-	Hydrolysis of tributarne	ND	+
26-	Hydrolysis of Tween 40	ND	+
27-	Hydrolysis of Tween 80	ND	+
Enzymes production			
28-	-Neutral amylase - Alkaline amylase	ND	+ve
29-	-Neutral lipase -Alkaline lipase	ND	-ve
30-	-Neutral phosphatase -Alkaline phosphatase	ND	-ve
31-	-Neutral protease -Alkaline protease	ND	+ +
32-	-Neutral cellulase -Alkaline cellulase	ND	-ve -ve
33-	-Growth at pH6- pH 7.5 (in broth or agar) on 15% NaCl	ND	+ve (with change pH to 9.5)

No	General characteristics	Reference Isolate <i>Bacillus alcalophilus</i> subsp <i>halodurans</i>	Identified Isolate (WN12)
34-	Cell lysis in H ₂ O - SDS		- -
35-	Carbohydrate oxidation	non oxidized carbohydrate	Approximately non oxidized carbohydrate except glucose and fructose
36	Carbohydrate utilization	Acid no gas Production	Acid no gas Production
	Xylose	+	+
	-Arabinose	+	+
	-Rhamnose	-	-
	-Manitole	+	+
	-Glucose	+	+
	-Fructose	-	+
	-Mannose	-	-
	-Lactose	+	+
	-Sucrose	+	+
	-Maltose	+	+
	-Raffinose	ND	-
	-Starch	-ND	-
	-Cellulose	-ND	-
	-Salicin	-ND	-
	-Glycerol	-ND	-
	-Mannitol	-	-
	-Cholesterol	-	-
Antibiotic sensitivity			
37-	Rifampicin	ND	+++
	Vibramycin	ND	+
	puromycin	ND	++
	Tobramycin	ND	-
	Sulfamethin	ND	+
	Triple sulfa	ND	+
	Sulphonamides	ND	-
	Nitrofurantion	ND	-

No	General characteristics	Reference Isolate <i>Bacillus alcalophilus</i> subsp <i>halodurans</i>	Identified Isolate (WN12)
	Bactracin	ND	-
	Chloramphincol	ND	-
	Erythromycin	ND	++
	Novobiocin	ND	++
	Pencillin	ND	+
	cephalosporin	ND	+W
Antifungal effect			
38-	Furamazone	ND	-
	Nizarol	ND	-
	Lamyzol	ND	-
39-	Nitrogen sources requirement	utilize amino acid	utilize amino acid
40	Alanine	ND	+w
41	Arginine	ND	++
42	Asparagine	ND	++
43	Cysteine	ND	+
44	Cystine	ND	+++
45	Glutamic acid	ND	+
46	Glutamine	ND	+w
47	Histidine	ND	+
48	Isoleucine	ND	++
49	Lysine	ND	+
50	Methionine	ND	+
51	Phenyl – alanine	ND	++
52	serine	ND	+
53	Threonine	ND	+
54	Tryptophan	ND	+w
55	Tyrosine	ND	++
56	Glycine	ND	+
57	Peptone	ND	++
58	Urea	ND	+

No	General characteristics	Reference Isolate <i>Bacillus alcalophilus</i> subsp <i>halodurans</i>	Identified Isolate (WN12)
59	NaNO ₃	ND	+
60	(NH ₄) ₂ SO ₄	ND	+

ND= not detected .

Isolate No. WN12 grows at temperatures range 15-50°C, pH range 7.0-12.5 and salinities range 3.0-30 % NaCl (w/v). The isolate was Gram positive, translucent brownish smooth color colony, long rods. Non spore forming, with peritrichous numerous flagellate (Fig 4), motile, aerobic and non oxidized carbohydrate except glucose and fructose (Table 4) *Bacillus alcalophilus subsp halodurnce* reference shared isolate WN12 almost the same broad range of taxonomical characteristic (Table 4). This lead to consider WN12 is a different, variety under genus *Bacillus alcalophilus*. Cluster Analyses for studying phylogenetic relationships of isolate No. WN12 with other *Bacillus* strains by 16S ribosomes was carried out (Figure 5). Euclidean distance was 97% between isolate WN12 and *Bacillus alcalophilus* strain NBCR 15653 and also *Bacillus alcalophilus* 116s strain. Also euclidean distance was 96% between isolate WN12 and with *Bacillus halodurans* strain DSM497. From the previous data the identification of isolate WN12 is haloalkalophles thermoduric highly folic acid production, named *Bacillus alcalophilus subsp halodurans* by 16S ribosome and physiological characterization. Domain Eubacteria, Gram positive according to Bergey's manual of systematic bacteriology (Sneath, 1986; Holt *et al.*, 1994; Vos *et al.*, 2009)²⁴⁻²⁶. Concerning the extreme haloalkalophilic bacteria Anne (2011)³⁸ investigated many studies on alkaliphilic bacteria; isolation, characterization and identification, have been done on Kenyan soda lakes (a hyper saline lake with up to 30% salinity levels). 55 isolates were isolated from the lake. The bacteria were Gram positive and Gram negative, and they grew well at pH ranging from 5 – 10, temperature range of 25 – 50°C and sodium chloride range of 0- 30 %. Analysis of partial sequences using blast showed that 80 % of the isolates were affiliated to the genus *Bacillus* while 20 % were affiliated to members of *Gamma proteobacteria*. The isolates produced various extracellular enzymes such as amylases, lipases, proteases, cellulases and esterases. Antimicrobial assays done to determine the isolates range of *in vitro* activity against test organisms exhibited a range of inhibitory effects.



Figure 5: Dendrogram of isolate No WN12 and reference strains based on the similarity matrix of phonetic data

Also, Noha and Juergen (2012)³⁹ revealed the presence of an extensive diversity of aerobic and anaerobic bacteria and archaea that survive and grow under halophilic alkali thermophiles, a novel group of poly extremophiles existent in nature as order *Natranaerobiales* that require for growth and proliferation the multiple extremes of high salinity, alkaline pH, and elevated temperature. Meanwhile Shi *et al.*(2012)⁴⁰ analyzed environmental microbial samples from this extreme saline-alkali soil region from northeast China. The isolation and characterization of bacterial resources produced, the 20 novel strains were classified into obligate alkaliphilic and halophilic bacteria, obligate alkaliphilic halotolerant bacteria, and facultative alkaliphilic salt-tolerant bacteria. Through selective culture conditions, 8 bacterial strains were isolated from medium with no less than 1.5 M NaCl, 12 were isolated in the medium with a pH value of no less than 11.0, and 8 were isolated in the medium with of no less than 200 mMNa(2) CO(3).Eight strains belong to *Bacillus*, eight strains belong to the genera *Halomonas*, *Stenotrophomonas* and *Alkalimonas*.

Characterization of Gold and Silver Nanoparticles

(A)Characterization of Gold Nanoparticles (AuNPs)

Spherical AuNPs have been prepared by citrate reduction method using tetra chloroauric (III) acid (HAuCl₄) as mentioned above in the experimental section. Tri sodium citrate initially acts as a

reducing agent to reduce the Au (III) ions to Au (0) ,then acts as the stabilizing agent by forming a layer of citrate ions over the AuNPs surface, inducing enough electrostatic repulsion between individual particles to keep them well dispersed in the medium and prevents aggregation or further growth of the particles.

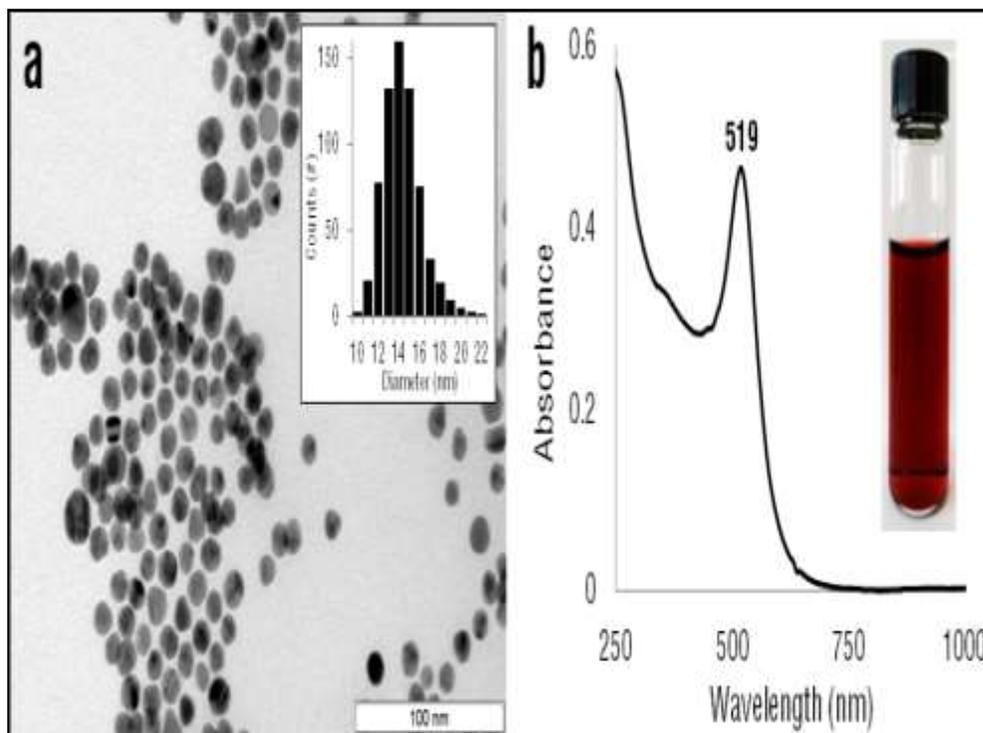


Figure 7: The absorption spectrum of spherical AuNPs capped with citrate. A) TEM image of FA-AuNPs, and their size. B)UV–Vis spectra of AUNPs and gold particles size

The absorption spectrum for the solution of AuNPs(**Figure (7)**) is characterized by the excitation of the plasmon resonance in the neighborhood of 519 nm, which gives rise to the bright red (pink) color of the colloidal solutions; the λ_{max} of the SPR band of the spherical AuNPs is slightly dependent on the size. AuNPs capped with citrate are negatively charged

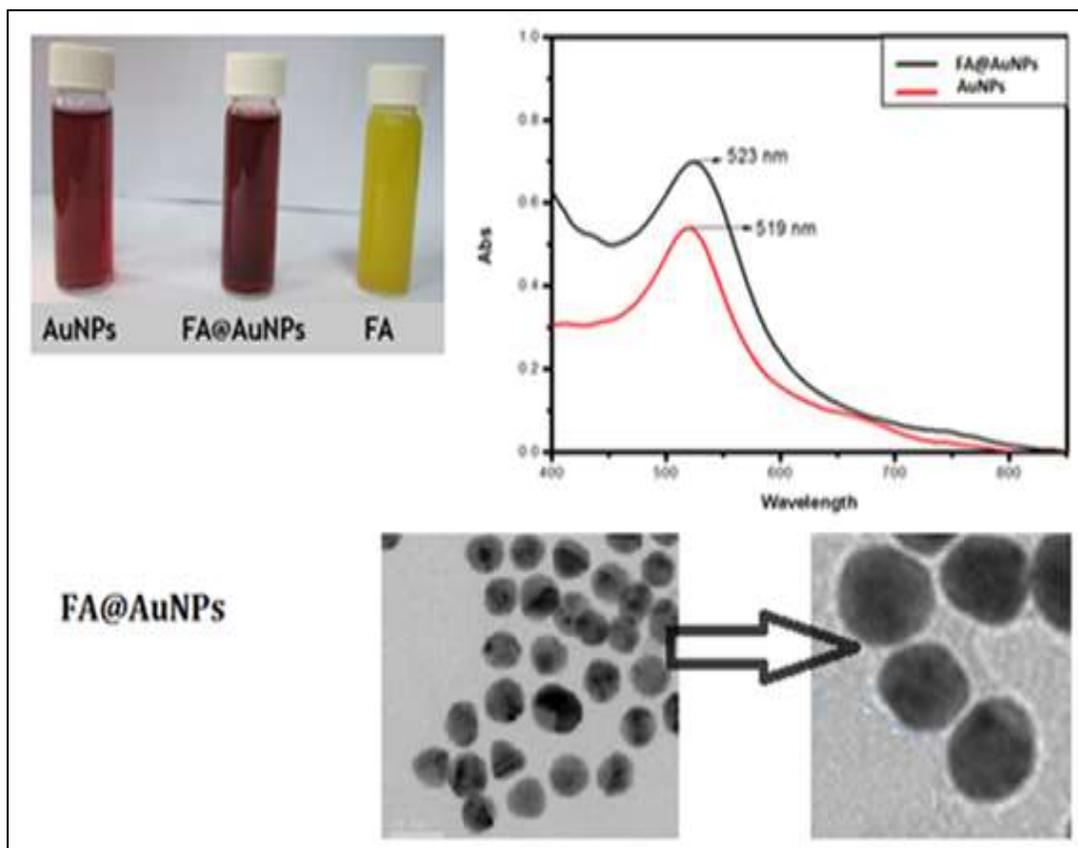


Figure 8: TEM image of FA-AuNPs, and UV-Vis spectra of AuNPs and FA-AuNPs (nanogold) and conjugation of Folic acid (FA) and AuNPs (FA@AuNPs)

Composites. TEM and histogram of prepared AuNPs, the size and shape of the AuNPs have been measured by TEM imaging. The particles show mostly spherical or elliptical shapes with highly uniform in size and shape. The histogram chart provides the size distribution of synthesized AuNPs is plotted by recording the sizes of a great number of particles on the TEM images, it shows that an average size (diameter) of the prepared AuNPs is around 13 ± 2 nm. It was clear, gold nanoparticles surrounded by folic acid.

Figure (8) shows the UV-Vis spectra and TEM of folic acid (FA) loaded on gold nanoparticles FA+AuNPs. FA+AuNPs have been prepared and characterized via UV-Vis absorption spectroscopy 523 and TEM. FA+AuNP is water-soluble, stable, and had good biocompatibility. Loading of folic acid (FA) on AuNPs prepared as previously described; the color changed immediately from pink to dark pink. This results are in accordance with previous study (Zhaoyang *etal.* (2010)²⁸, where he found that the TEM micrograph of FA-GNPs indicates that the particles are uniformly dispersed with a significant narrow size range of 15–20nm The average particle size is 13nm. Our result clear, UV-Vis absorption spectra of AuNPs and FA-AuNPs are presented, the unmodified nanoparticles display characteristic surface plasmon absorption at 519nm, while FA-

conjugated AuNPs show a surface plasmon band around 523nm. This shift (8nm) in the position of the maximum absorption indicates that FA has interacted with AuNPs.

(B) Characterization of silver Nanoparticles (AgNPs) :

The absorption spectrum for the solution of AgNPs is characterized by the excitation of the plasmon resonance in the neighborhood of 530 nm, which gives rise to the dark grey color of the colloidal solutions. The colloidal silver solution show strong stability, this indicates electrostatic stabilization via citrate anions bonded on the AgNPs surface. Therefore, AgNPs capped with citrate are negatively charged composites. TEM and histogram of prepared AgNPs, Figure (9) the size and shape of the AgNPs have been measured by TEM imaging. The particles show mostly spherical shapes with highly uniform in size and shape. The histogram chart provides the size distribution of synthesized AgNPs is plotted by recording the sizes of a great number of particles on the TEM images, it shows that an average size (diameter) of the prepared AgNPs is around 15 ± 5 nm Figure (9) .

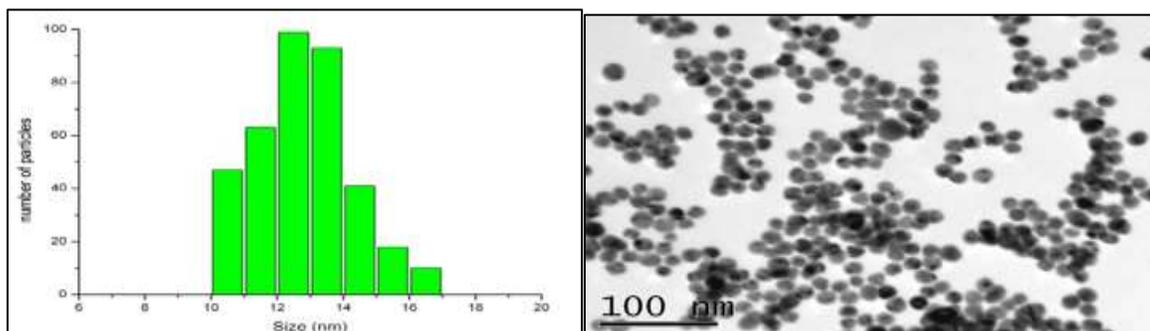


Figure 9: Silver particles TEM and AGNPs particles size

Conjugation of Folic acid (FA) and AGNPs (FA+AGNPs Figure (10)), TEM image of clear spherical AgNPs capped with citrate loading folic acid . And a representative spectrum UV in the range of 400–700 nm. In this section, we report the results of the comparative study utilizing the two nanoconjugates for the improvement of cellular internalization. It was clear , silver nanoparticles Surrounded by folic acid

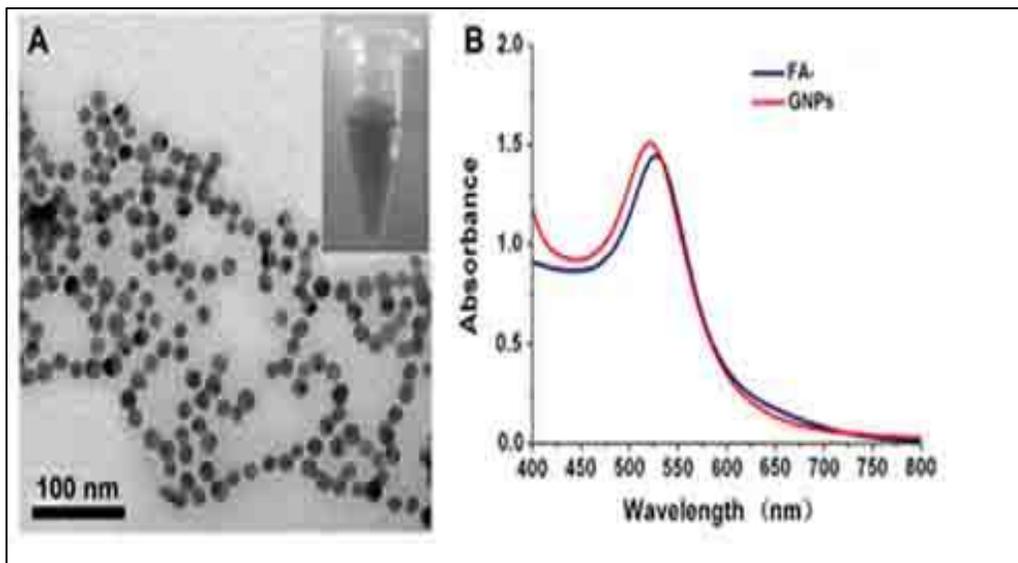


Figure 10: (A) TEM image of FA-AGNPs, and (B) UV-Vis spectra of AGNPs and FA-AGNPs (nano silver)

Table 5: Cytotoxicity Effect (IC₅₀) of folic acid, silver nanoparticles and gold nanoparticles separate and conjugated on different cancer cell lines

NO	Cell line	HCT-116	CaCo2	HEPG2	MCF-7
	Treatment	IC ₅₀ (mg/ ml)			
1	Nano gold particles(AuNPs)	0.427	0.060	37.223	0.785
2	Nano gold + folic acid(FA-AuNPs)	2.148	12.855	5.106	6.542
3	Nano silver particles (AgNPs)	0.237	0.459	0.559	0.146
4	Nano silver + folic acid (FA-AgNPs)	1.380	2.302	1.513	0.480
5	Nano silver-gold -folic acid(FA- AgNPs-AuNPs)	28.553	6.729	41.804	0.834
6	Purified folic acid (FA)	0.160	0.180	0.130	0.150

Six treatment were tested for measuring IC₅₀ on four cancer cell lines HCT, HEPG2, Caco2 and MCF7. Data recorded in [Table 5] revealed that different cell lines showed a variable reactivity to test materials either in a single or combined forms. Regarding Au NP toxicity to cancer cell lines was arranged in the order of Caco2 > HCT > MCF7 > HEPG2 (P < 0.05). Also, combination of AuNP with FA showed a clear antagonistic potential against cell death. While cytotoxic effect of Ag NP showed a variable pattern of reactivity to cell lines arranged in the order of MCF7 > HCT > CaCO₂ > HEPG2 (P < 0.05). At the same time, AgNP combination with FA showed the same pattern of antagonism. Preparation of trivalent mix of Ag-Au-FA showed antagonistic reaction to cell lines and toxicity of mix to cell lines was arranged in the order of MCF7 > CaCO₂ > HCT > HEPG2 (P < 0.05). Finally Folic acid toxicity showed the least concentration significant IC₅₀ with highly effecting on different cell line.

Folic acid and silver nanoparticles treatments have highly effect IC₅₀ on (hct) 0.160 mg/ml and 0.237 mg/ml, (HEPG2) 0.130 mg/ml and 0.559. mg/ml (CaCo2) 0.180 mg/ml and 0.459 mg/ml and (MCF7) 0.150

mg/ml and 0.146. mg/ml. Nano gold alone have no effect IC50 on HEPG2 and have highly effect on hct , MCF7 and Caco2 cell lines . While conjugated treatment FAGuNP, FA AgNP and FA-Au-Ag have highly effected only (MCF7).

In the present investigation separate treatment folic acid , nanogold and nanosilver gave high inhibitory concentration IC50 effect in colon cancer and breast cancer . This results agree with Ali Mansoori *et al* (2010)⁴⁶.They report a comparative study of two folate-conjugated gold nanoparticles (AuNP) differing in linkers and AuNP sizes for selective targeting of folate-receptor positive cancerous cells. In addition to the analytical characterization of the nanoconjugates, the cell lethality was measured in HeLa (high level of folate receptor expression) and MCF-7 (low level of folate receptor expression) cells. The nanoconjugates themselves, as well as the intense pulsed light (IPL) were not harmful to cell viability. However, upon stimulation of the folate targeted nanoconjugates with the IPL, ~98% cell killing was found in HeLa cells and only ~9% in MCF-7 cells after four hours incubation with the nanoconjugate. This demonstrates that folate targeting is effective in selecting for specific cell populations. Also Steenweg *etal.*(2012)⁴¹ found that, folic acid in the diet seems to protect against the development of some forms of cancer, including: Colon cancer , Breast cancer, Cervical cancer, Pancreatic cancer and Stomach cancer . Some think that folic acid keeps DNA healthy and prevents mutations that can lead to cancer Several studies are in progress to test this new drug delivery system in vivo. Among the possible low molecular weight (MW) targeting agents, folic acid could be exploited to actively target cancer cells. Indeed, folic acid is a vitamin whose receptor is frequently over expressed on the surface of human cancer cells. Therefore, this receptor has been identified as a tumor marker, especially in ovarian carcinomas, Garin Chesa *et al.* (1993)⁴² Franklin *etal.*(1994)⁴⁴.

Van Guelpen *et.al.*(2006)⁴³ found that, dietary folate is believed to protect against colorectal cancer (hct). However, few studies have addressed the role of circulating levels of folate. Their findings suggest a decreased hct risk in subjects with low folate status. This possibility of a detrimental component to the role of folate in carcinogenesis could have implications in the ongoing debate in Europe concerning mandatory folate fortification of foods.

Antiviral activity using conjugated AuNP-FA- and AgNP-FA

Antiviral activity of folic - AuNP and FA- and AgNP-FA,(Figure 11)against VSV

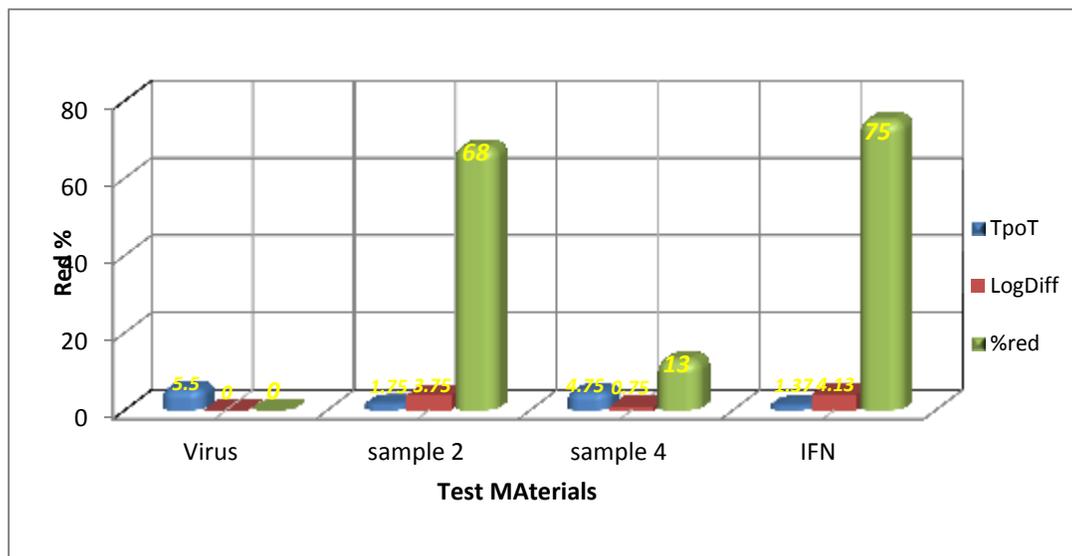


Figure 11: Evaluation of antiviral activity against VSV post treatment with test materials

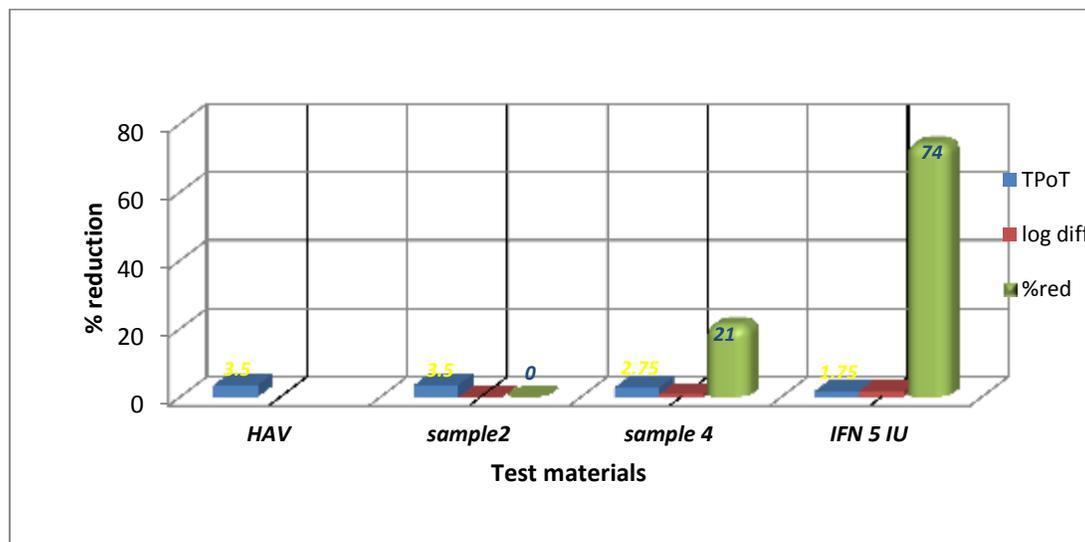


Figure 12: Evaluation of antiviral activity against HAV post treatment with test materials

Model was determined in cell culture and it was found that antiviral potential of test sample namely F-AuNP was significantly effective compared with F-Ag nanoparticles ($P < 0.05$). In the mean time the antiviral potential of F -AuNP was insignificantly effective compared with IFN used as standard antiviral drug. Also, the antiviral potential can be arranged in the order of IFN > F-AUNP > F-AgNP recording reduction % in the order of 75%, 68% and 13% respectively. A virus (HAV) was tested (Figure 12). Data revealed that test AuNP and FA sample and AgNP-FA materials could induce viral load depletion in the order of 0.75 log (10)/ ml [21 %] and 1.75 log (10) / ml [74%] for HAV post cell treatment with test materials AuNP- FA- and AgNP-FA respectively.

Our results agree with, Ramendra *et al.*(2010)⁴⁵ who found that, synthesis ,bioconjugates bearing dipeptide, fatty acids and folic acid have antibacterial activity with MIC ,ranging between 0.09and 0.67 mM against Gram-positive cocci and Gram-negative bacilli. Synthesis, bioconjugates have been screened for their antiviral activities against HSV, HAV,VSV, FIPV, FHV, which shown good results with EC50 0.011m M and 0.029 mM against VSV and FIPV/FHV, respectively .

CONCLUSION:

Isolation and identification of haloalkalophles thermophilic highly folic acid production of isolate No.WN12 have taken the name *Bacillus alcalophilus subsp halodurans* according to 16S ribosome and physiological and phylogenetic characterization. Then determination and purification of folic acid from WN12 strain by TEM , UV, FTIR and HPLC were carried out . Practical application for folic acid , silver nanoparticles and gold nanoparticles treated separately and conjugated have highly effect IC50 on (hct),(HEPG₂), (CaCo2) and(MCF7) cell lines and antivirs hepatic A and antivir VSV highly effect of conjugated folic acid plus nano gold sample and folic acid plus nano silver samples .

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