Phytochemical, antimicrobial, antioxidant and anticancer properties of Allium sativum L. extract

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Abstract : The Garlic (Allium sativum) bulb extracts showed the presence of phytochemical constituent's namely alkaloids, carbohydrates, spaonins, phenols, tannins, flavonoids and terpenoids and the absence of reducing sugars. In, Garlic (Allium sativum) bulb extracts, the combined effect of DMSO and water added erythromycin possess higher antimicrobial activity against commercial antibiotic Erythromycin and Garlic extract. DPPH radical scavenging tends to increase the inhibition with increased the concentration of A. sativum extract; on that evidence check the Garlic (Allium sativum) extract anticancer properties against A498 (Human Kidney Carcinoma) cell lines. The cytotoxicity of A498 (human kidney carcinoma cell lines shows anticancer properties against Garlic (Allium sativum) extract hold a huge Pharmaceutical and Nutraceutical potential.

Keywords: Phytochemical; DPPH assay; Human Kidney Carcinoma cell lines; Allium sativum L; Garlic.

I Introduction

Garlic (*Allium sativum*) has been used medicinally since before the time of the Sumerian civilization (2600–2100 BC), by when it was already widely cultivated in India and China [1]. Traditionally used as an antimicrobial agent, garlic has been reported also to modulate cardiovascular and immune functions as well as having antioxidant and anticancer properties. The bioactive effects of garlic are attributed to the sulphur-containing molecules; other smaller metabolic breakdown products of these molecules have received increasing attention for their antimicrobial efficacy [2,3].

Early literature, the Garlic has been focused detailed antimicrobial activity. Lemar *et al*., (2005) have reported that growth and respiration of *Candida albicans* were sensitive to extracts of *Allium sativum* [4]. Garlic extract tested against bacteria and fungus, *Staphylococcus aureus*, *Salmomella enteritidis*, and three fungi, *Aspergillus niger*, *Penicillium cyclopium* and *Fusarium oxysporum*, was investigated[5]. Ethanolic extracts of garlic (*Allium sativum* Linn.) treated against *Staphylococcus aureus*; *Bacillus spp*., *Escherichia coli and Salmonella spp*. studied [6].

Bioactive secondary metabolites of medicinal plants such as flavanoids, Alkaloids and Terpinoids are used to cure many types of diseases including disorder and infectious diseases. Overall, despite some progress in cancer control, incidence and death rates are increasing for cancer types. Recently, the applications of phyto-componet have revealed novel strategies for the treatment and diagnosis of cancer [7].

In the present work, *A. sativum* crude extract is prepared by methanol and cured characterizations done by phytochemical analysis, antimicrobial, antioxidant and anticancer studies were carried out.

II Materials and Methods

2.1 Phytochemical Screening

Phytochemical analysis of the methanol extracts was performed using the methods described by Trease and Evans, (1983) [8]. Approximately 0.2 g of the extract was dissolved in 2 ml of methanol and heated in flame for a minute. A chip of magnesium metal was added to the mixture, followed by the addition of few drops of concentrated hydrochloric acid. The formation of red color was indicative of the presence of flavonoids. To 0.5 ml of extract, few drops of Mayer's reagent was added by the side of the test tube. The creamy white precipitate indicates the presence of alkaloid. Approximately 0.5 ml of extract was added with 0.5 ml of Benedict's reagent. The mixture is heated on a boiling water bath for 2 minutes. A formation of red precipitate indicates the presence of sugar. To 0.5 ml of garlic extract equal amount of concentrated sulphuric acid was added and the appearance of red color indicates the presence of Quinone. Few drops of 10% ammonia solution were added to 0.5 ml of garlic extract. Appearance of pink color precipitates indicates the presence of phlobatannins.

To the extract, few drops of 5% ferric chloride reagent were added. A dark green color indicates the presence of phenolics. To 0.5 g of the extract 3 ml of chloroform was dissolved, and few drops of filtered concentrated sulphuric acid were carefully added to the filtrate forms a reddish-brown color at the interface, which was a positive indicator for the presence of steroids. Addition of 1 ml of solvent extracts with 20 ml of diluted distilled water and shaken in a graduated cylinder for 15 minutes. The formation of 1 cm layer of foam indicates the presence of saponins. 2 ml of trichloroacetic acid was added to 1 ml of extracts and the formation of a yellow-to- red precipitate shows the presence of terpenoids. About 0.5 ml of the extract was added with few drops of 0.1% ferric chloride and boiled. The formation of brown green or blue-black color indicates the presence of tannins [9].

2.2 Determination of total phenolic content (TP)

Total phenolic content was determined by using Foline-Ciocalteu method, based on the reduction of phosphor wolframate to phosphomolybdate complex by phenolics to a blue color product [10]. Briefly, an aliquot of the crude extract was made up to 3 ml with distilled water and allowed to react with 0.5 ml of Folin-ciocalteu reagent. After 3 minutes of reaction, 2 ml of 20% sodium carbonate was added to their action mixture and incubated for 30 minutes at room temperature. The absorbance was read at755 nm using UV-Visible spectrophotometer (Shimadzu UV-1601, Columbia, MD, USA). The total phenolic content was expressed as mg of gallic acid equivalents (GAE) / gram of the extract. The estimation of total phenolics in the extract was carried out in triplicate and the results were averaged.

2.3 Determination of total flavonoid content (TF)

A modified aluminum chloride method was used for determining the total flavonoid content in the plant extract [11]. A total of 100 µl of sample (1 mg/ml) was mixed with 600 µl of methanol, 40 µl of 10% aluminum chloride, 40 µl of 1 M potassium acetate and made up to 2 ml with distilled water. The tubes were kept at room temperature for 30 min and the absorbance was measured at 420 nm using UV-Visible spectrophotometer. The total flavonoid content was obtained from extrapolation of the calibration curve, which was made by preparing various concentrations of a quercetin solution in methanol. The total flavonoid content was expressed as mg of quercetin equivalents (QE) / gram of the extract.

2.4 Antimicrobial Activity

The antimicrobial activity of the DMSO dissolved *A. sativum* (D), Water dissolved *A. sativum* (W), Erythromycin mixed DMSO dissolved *A. sativum* (D+W+E) and Erythromycin mixed water dissolved *A. sativum* (D+W) were investigated by the disc diffusion method against the test Gram positive bacteria (*S. aureus* and *S. pneumoniae*), Gram-negative bacteria (*K. pneumonia, E. coli, S. dysenteriae, P. vulgaris,* and *P. aeruginosa*) and antifungal (*Candida albicans*) on Mueller Hinton agar and Potato dextrose agar according to the Clinical and Laboratory Standards Institute (CLSI). The media plates (MHA and PDA) were streaked with bacteria 2-3 times by rotating the plate at 60˚ angles for each streak to ensure the homogeneous distribution of the inoculums. After inoculation, disc loaded with 25 µl/mL of the test samples were placed on the bacteria-seeded plates. The plates were incubated at 37 ˚C for a day. The inhibition zone around the disc was measured and recorded. Erythromycin (15 µg/mL)(Hi-Media) was used as the positive controls.

2.5 2, 2-Diphenyl- 1-picrylhydrazyl (DPPH) scavenging activity

DPPH radical scavenging activity was adopted from Singh *et al*., (2002) with slight modifications [12]. DPPH is a violet colored stable free radical solution which decolorizes in to colorless solution by the addition of a substrate that can donate a hydrogen atom. Briefly, a total of 1 ml of 0.135 mM DPPH prepared in methanol was mixed with 1.0 ml of aqueous extract ranging from 20 to 50 µg /ml. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. Changes in the absorbance of the samples were measured at 517 nm. The inhibition percentage was calculated using the following formula:

% Radical scavenging activity = [(Abs con. – Abs sample)]/ (Abs con.)] × 100

where, Abs con is the absorbance of control;

Abs sample is the absorbance of test sample /standard.

Butyl hydroxytoulene (BHT) was taken as reference standard. Mean values were obtained from triplicate experiments. The percentage inhibition versus concentration was plotted and the concentration required for 50% inhibition of radicals was expressed as IC_{50} value.

2.6 Anticancer activity

2.6.1 Cell culture

A498 (Human Kidney Carcinoma) cell line were cultured in liquid medium (DMEM) supplemented 10% Fetal Bovine Serum (FBS), 100 u/ml penicillin and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO_2 at 37^{o}C.

2.6.2 MTT assay

The *A. sativum* extract was tested for *in vitro* cytotoxicity, using A498 (Human Kidney Carcinoma) cell line by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cultured A549 cells were harvested by trypsinization, pooled in a 15 ml tube. Then, the cells were plated at a density of 1×10^5 cells/ml cells/well (200 µL) into 96-well tissue culture plate in DMEM medium containing 10 % FBS and 1% antibiotic solution for 24-48 h at 37°C. The wells were washed with sterile PBS and treated with various concentrations of the *A. sativum* extract in a serum free DMEM medium. Each sample was replicated three times and the cells were incubated at 37°C in a humidified 5% CO_2 incubator for 24 h. After the incubation period, MTT (20 µL of 5 mg/ml) was added into each well and the cells incubated for another 2-4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium together with MTT (220 µL) were aspirated off the wells and washed with 1_X PBS (200 µl). Furthermore, to dissolve formazan crystals, DMSO (100 µL) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a micro plate reader (Thermo Fisher Scientific, USA) and the percentage of *A. sativum* extract cell viability and IC_{50} value was calculated using GraphPad Prism 6.0 software (USA).The optical density (OD) value was used to calculate the percentage of viability using the following formula.

$$= \frac{mean\ OD\ of\ untreated\ cells\ (control) - mean\ OD\ of\ treated\ cells}{mean\ OD\ of untreated\ cells\ (control)} \text{ x } 100$$

All the *in vitro* experiments were done in triplicate, and the experiments were repeated at least thrice. The statistical software SPSS version 17.0 was used for the analysis. *P* value <0.01 was considered significant.

III Results and discussion

3.1 Preliminary phytochemical screening

The phytochemical characters of the *A. sativum* bulb extract investigated are presented in Tables 1. Table 1 shows the behavior of *A. sativum* bulb extract with various chemical reagents. In the present investigation, preliminary phytochemical screening done in the extracts methonalic of *A. sativum* bulb extract showed the presence of phytochemical constituent's namely alkaloids, carbohydrates, spaonins, phenols, tannins, flavonoids and terpenoids and the absence of Reducing sugars are shown in Table 1.

Table 1 Behavior of *A. sativum* bulb extract with various chemical reagents

S. no	Phyto-constituents	Tests	GSE
1	Alkaloids	Mayer's test	++

2	Carbohydrates	Molisch's	++
3	Reducing sugars	Fehling's test	+
4	Saponins	Foam's test	-
5	Phenols	Ferric chloride test	++
		Lead acetate test	++
6	Tannins	Ferric chloride test	+
7	Flavonoids	Lead acetate test	+
		Alkaline reagent test	++
8	Terpenoids	Salkowski's test	+

3.2 Antimicrobial activity

Antimicrobial activity of DMSO dissolved *A. sativum* (D), Water dissolved *A. sativum* (W), Erythromycin mixed DMSO dissolved *A. sativum* (D+E) and Erythromycin mixed water dissolved *A. sativum* (D+E) tested against Gram-positive (*S. aureus* and *S. pneumoniae*), Gram-negative (*K. pneumonia, E. coli, P. vulgaris, S. dysenteriae* and *P. aeruginosa*) and antifungal (*candida albicans*) are studied by disc diffusion method as shown in Fig. 1.

A. sativum has been known to have anti-bacterial, anti-fungal, and anti-viral activity [13]. Reuter *et al*., (1996) reported that Garlic extract to inhibit the growth of *Staphylococcus* [14]. Crude juice of Garlic has been found to be high active against *E. coli* and *Salmonella typhi* investigated by Abdou *et al*., (1972)[15]. Sasaki *et al*., (1999) have investigated that the Garlic activity against *methicillin-resistant Staphylococcus aureus* and *candida albicans* [16]. Garlic extract possesses anti-bacterial activity against H. pylori at moderate concentration, thus it has protective effect against stomach ulcer [17]. This observation needs many studies and investigations. Allicin is biologically active compound responsible for the anti-microbial properties of Garlic. The inhibitory effect of Garlic on the growth of both G+ and G- bacterial, this is due to the important allicin compound in the Garlic extract.

In present work, synergistic antimicrobial effect were studied the DMSO dissolved *A. sativum* (D), Water dissolved *A. sativum* (W), Erythromycin (15µg/ml) mixed DMSO dissolved *A. sativum* (D+E) and Erythromycin (15µg/ml) mixed water dissolved *A. sativum* (W+E) tested against Gram-positive (*S. aureus* and *S. pneumonia*) and Gram-negative (*K. pneumonia, E. coli, P. vulgaris, S. dysenteriae* and *P. aeruginosa*) and antifungal (*candida albicans*)using disc diffusion method to determine their ability to serve as a potential antimicrobial agent.

Figure.1. shows the size of the zone of inhibition and antimicrobial activity formed around each D, W, E, D+E and W+E loaded with test samples. The DMSO dissolved *A. sativum* (D), Water dissolved *A. sativum* shows most significant effect on zone of inhibition of 6 to 7 mm for all microbial activity. The combined effect of Erythromycin (15µg/ml) mixed DMSO dissolved *A. sativum* (D+E) and Erythromycin (15µg/ml) mixed water dissolved *A. sativum* (W+E) possess higher than that commercial antibiotic Erythromycin and garlic extract.

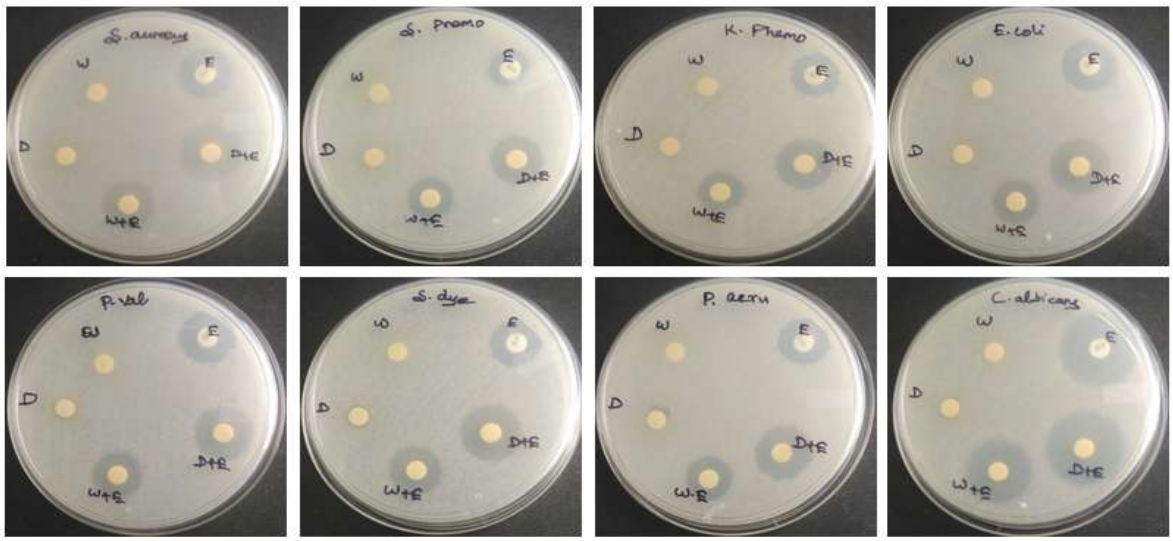

Figure 1 The antimicrobial activity of the D, W, E, D+E and W+E tested against Gram-positive (*S. aureus* and *S. pneumoniae*), Gram-negative (*K. pneumonia, E. coli, P. vulgaris, S. dysenteriae* and *P. aeruginosa*) and antifungal (*candida albicans*).

3.3 In *vitro* antioxidant properties

The balance between antioxidation and oxidation is believed to be a critical factor in maintaining a healthy biological system [18]. In recent years, many studies have indicated the important role of free radicals and reactive oxygen species (ROS) in the aetiology and progress of many human diseases [19-23].ROS are probably best known in biology for their ability to cause ROS. They can damage DNA, cell membranes, and cellular proteins, and may lead to cell death. Hydroxyl radical ($^{\bullet}OH$) is the most reactive oxygen radical known, and reacts very quickly with almost every type of molecule found in living cells [24].Such reactions will probably dominate the recombination of two $^{\bullet}OH$ radicals to form hydrogen peroxide (H_2O_2). Superoxide anion radical ($^{\bullet}O_2^-$), on the other hand, poorly permeates to cell membranes and is less toxic. Hydrogen peroxide is also considered to be a weaker oxidizer, but it can cause cell damage via hydroxyl radicals produced by the Fenton reaction [25, 26].In addition, hydrogen peroxide in the presence of ($^{\bullet}O_2^-$) can generate singlet oxygen, which is very toxic, are of the greatest biological significance. ROS are produced continuously in all cells, as metabolic byproducts of a number of intracellular systems.

$$^{\bullet}O_2^- + H_2O_2 \rightarrow OH^- + OH^{\bullet} + {}^1O_2$$

However, this process requires a metal or other catalyst [26].

The results on the consequence of different concentrations (10-50µg/ml) *A. sativum* extract on DPPH radical and hydrogen peroxide radical scavenging activity is shown in Fig. 2 and values is given in Table 2. The DPPH radical scavenging solution contains both π system and an unpaired electron in nitrogen atom. Substitution on aromatic ring increases the molar absorptivity and the effect becomes important when the substituent increase conjugation length take place. The comprehensive conjugation regularly cause shifted in the benzene absorption bands from shorter to longer wavelength. The peak at 517 nm is responsible for $n \rightarrow \pi^*$ energy transition. DPPH radical scavenging solution gradually the color changes from deep violet to pale yellow was presented in Fig.2. DPPH radical scavenging tends to increase the inhibition with increasing the concentration of *A. sativum* extract; this is the evidence toward the free radical scavenging of *A. sativum* extract. At concentrations 10 to 50µg/mL, *A. sativum* extract showed an IC_{50} concentration at 137.11 µg/ml. The above observed activity was lower than that of the average BHT, IC_{50} concentration at 39.41µg/ml (in Table2). The antioxidant activity of *A. sativum* extract may be due to the transfer of electron pressure located at oxygen to the odd electron located at nitrogen atom in DPPH resulting the increase inhibition of $n \rightarrow \pi^*$ transition at 517 nm.

Table 2DPPH radical scavenging of *A. sativum* extract

S.no	**Assay model**	**IC_{50} of BHT (µg/ml)**	**IC_{50} of *GSE*(µg/ml)**
1	DPPH radical scavenging	39.41	137.11

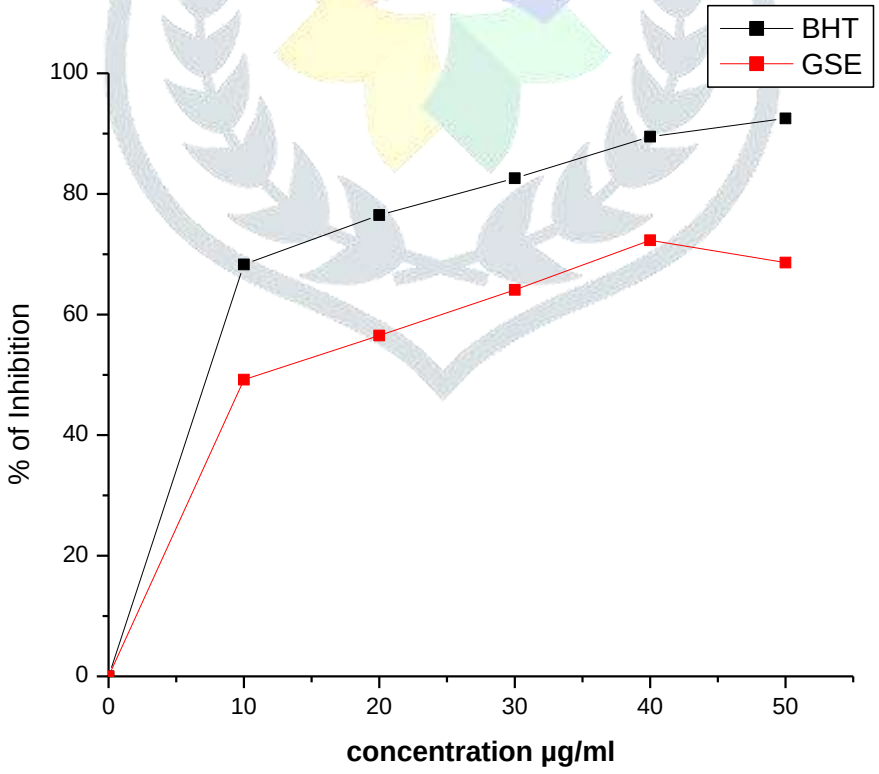

Figure 2 Antioxidant activities of *A. sativum for* scavenging activity to DPPH-radical.

3.4 Cell viability test

The C. viscosa synthesizedAgNPsweretestedfor theiranticanceractivityagainsthumanA549andPA1 celllines.ThegreensynthesizedAgNPsshowedthe substantialanticanceractivitiesonlungandovarian cancercelllineswiththelowestIC 50 concentrationat 28and30 mg/mLresp ectively.Theabilityofgreen synthesizedsilvernanoparticlestoinhibitcancercells

The Garlic extract was tested for *in vitro* cytotoxicity, using A498 human kidney cancer cell line and cells were incubated with different (10-100 µg/ml) concentrations of garlic extract as shown in Fig. 3. In MTT assay, the IC_{50} value of 73.34µg/mL(evaluated after 24h) of Garlic extract against A549 cells was ($p \leq 0.05$ P value <0.01). Cell morphological changes were observed for light microscope with different concentration 10, 50 and 100 µg/ml (in Fig 4 (a-d)). The results revealed that the occurrence of morphologically altered cells observed garlic extract treated group as compared to control group.

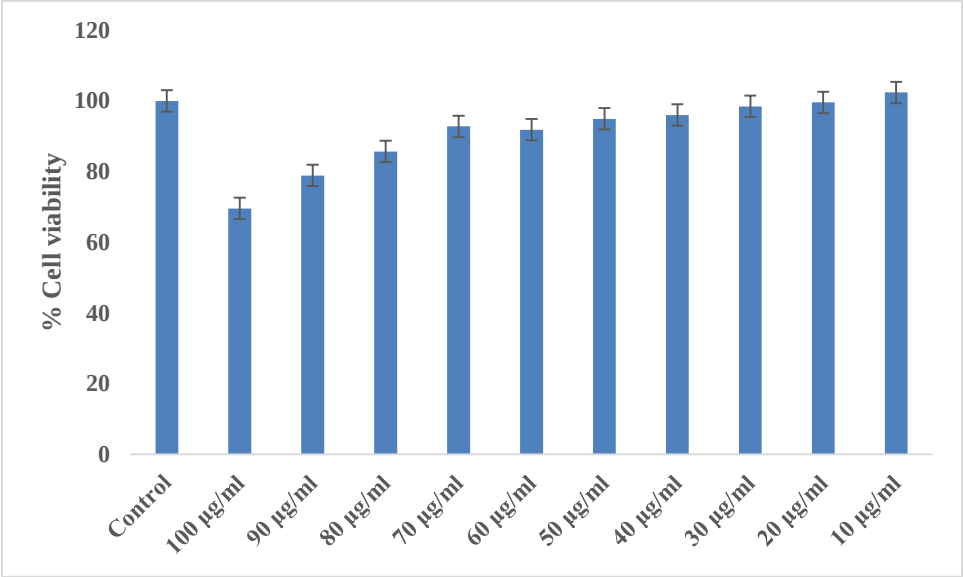

Figure 3 the progressive anticancer activity of the garlic extract tested against human kidney cancer cell line.

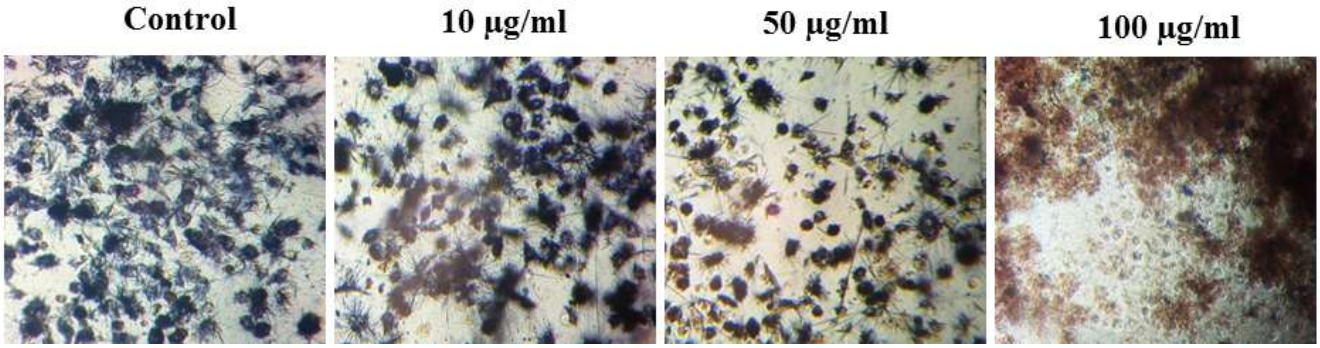

Figure 4 Formation of formazan crystals in control cells and garlic extract treated cells

IV Conclusions

In summary, the preliminary phytochemical screening were done in the extracts methonalic of *A. sativum* bulb extract showed the presence of phytochemical constituent's namely alkaloids, carbohydrates, spaonins, phenols, Tannins, Flavonoids and terpenoids and the absence of Reducing sugars components. From the antimicrobial activity, the combined effect of Erythromycin (15µg/ml) mixed DMSO dissolved *A. sativum* (D+E) and Erythromycin (15µg/ml) mixed water dissolved *A. sativum* (W+E) possess higher than that commercial antibiotic Erythromycin and garlic extract. DPPH radical scavenging tends to increase the inhibition with increased the concentration of *A. sativum* extract; this was the evidence toward the free radical scavenging of *A. sativum* extract. Based on the scavenging activity of *A. sativum* extract have anticancer properties, they greatly induced apoptosis and led to the consequent anticancer effect against human kidney cancer cell lines. *A. sativum extract* containing antimicrobial and antioxidant substances for the prevention and reduction of food pathogenic microorganism. Finally, the present study suggests that the *A. sativum extract* possess superior antibacterial activity and its can be used for antimicrobial food packaging and biomedical applications.

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