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Contents

Magnetic Nanoparticles for Targeted Cancer Therapy

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Abstract

In cancer chemotherapy, the chemotherapeutic agent exerts anticancer action through cytotoxic mechanisms, where prolonged exposure of tumor to high concentrations is a prerequisite for therapeutic efficacy. Sub therapeutic exposure may not only fail to eradicate the tumor, but it may even stimulate overgrowth of resistant malignant cells. To improve the anticancer potential of chemotherapeutic agents, specific targeting seems to be crucial. This could be achieved by using nanoparticles as proved by various experimental-preclinical studies. Magnetic nanoparticles complexed with the therapeutic molecule could be targeted and retained at tumor site using a locally applied external magnetic field. This strategy helps to selectively target drug molecules to the tumor site, increasing the therapeutic efficacy and lower the concentration in other tissues, thereby reducing its systemic toxic effects.

Key words: Magnetic nanoparticles, Cancer, Superparamagnetic iron oxide, Chemotherapeutic agent

1. Introduction

Cancer is a complex family of diseases, and carcinogenesis, events that turn a normal cell into a cancer cell, is a complex multistep process (from a molecular and cell biological point of view). Cancer is a number of diseases caused by similar molecular defects in cell function resulting from common types of alterations to a cell's genes, it is a disease of abnormal gene expression which happens by a number of mechanisms such as via a direct insult to DNA, such as a gene mutation,

translocation, amplification, deletion, loss of heterozygosity, or via a mechanism resulting from abnormal gene transcription or translation. The overall result is an imbalance of cell replication and cell death in cells ultimately evolving into a population of abnormal cells that can invade tissues and metastasize to distant sites, causing significant morbidity of cells and, if untreated, death of the host. In normal tissues, cell proliferation and cell loss are in a state of equilibrium.

Cancer is a genetic disease because it originates due to alterations within specific genes, but in most cases, it is not an inherited disease. Because of these genetic changes, mainly in proto-oncogenes and tumor suppressor genes, cancer cells proliferate uncontrollably, producing malignant tumors that invade surrounding healthy tissue. Deregulation of the pathways that govern the normal developmental processes of cellular proliferation, apoptosis, differentiation, migration and invasion, and even metabolism during pre- and postnatal development, contribute to the cancer phenotype. As long as the growth of the tumor remains localized, the disease can usually be treated and cured by surgical removal of the tumor. But malignant tumors tend to metastasize, that is, to spawn cells that break away from the parent mass, enter the lymphatic or vascular circulation, and spread to distant sites in the body where they establish lethal secondary tumors (metastases) that are no longer amenable to surgical removal. In that case, the major modalities of treatment include radiotherapy where ionizing radiation or radioisotopes are used and chemotherapy where cytotoxic chemical molecules are used to eliminate the primary and metastasized tumor cells.

In cancer chemo therapy, the chemotherapeutic agent exerts anticancer action through cytotoxic mechanisms where prolonged exposure of tumour to high drug concentrations is a prerequisite for therapeutic efficacy (Gutman *et al.,* 2000). The biodistribution of a systemically administered drug depends on the physical-

chemical properties like molecular weight, lipophilicity, etc and often results in sub-therapeutic drug levels at the tumour site (Motl *et al.*, 2006). This sub-therapeutic exposure may not only fail to eradicate the tumour, but can even stimulate overgrowth of resistant malignant cells (Heimberger *et al.*, 2000). Most chemotherapeutic agents possess poor selectivity toward the target tissue and can harm normal cells as well as cancer cells. To improve the anticancer potential of chemotherapeutic agents, specific targeting to tumour tissue is crucial (Langer, 2001). A wide variety of targeting molecules such as humanized antibodies, peptides (Weiner and Adams, 2000), nucleic acid ligands called aptamers (Ellington and Szostak, 1990), colloidal drug delivery modalities such as liposomes, micelles or nanoparticles have been intensively investigated for their use in cancer therapy.

Nanotechnology is a multidisciplinary field that involves the design and engineering of materials smaller than 100 nm in diameter level to attain unique properties, which can be suitably manipulated for the desired applications. Functionalities can be added to nanomaterials by interfacing them with biological molecules or structures so as to confer desirable biological activities. Since most of the natural processes also take place in the nanometre scale regime, a confluence of nanotechnology and biology can address several biomedical problems and can revolutionize the field of health and medicine. Nanosized organic and inorganic particles are amenable to biological functionalization and are currently employed as a tool to explore certain avenues of medicine in several ways including imaging, sensing (Cheng and Van Dyk, 2004), targeted drug/gene delivery systems (Berry *et al*., 2003), artificial implants (Sachlos *et al*., 2006) etc. Based on enhanced effectiveness, the new age drugs are nanoparticles of polymers, metals or ceramics, which can combat situations like cancer (Brigger *et al*., 2002) and fight human pathogens (Stoimenov *et al*., 2002). Thus far, the integration of nanomaterials with biology has led to the development of diagnostic devices, contrast agents, analytical tools, physical therapy

applications, and drug delivery vehicles. Nanotechnology also has the potential for use in cancer diagnosis, treatment, and as delivery vectors for biologic or pharmacologic agents (Kam *et al.,* 2006) because of its unique size and large surface area-to-volume ratio.

Nanoscale devices have impacted cancer biology at three levels: early detection, tumor imaging using radiocontrast nanoparticles or quantum dots; and drug delivery using nanovectors and hybrid nanoparticles. Nanocrystal labeling with immune cells act as a platform technology for nanoimmunotherapy (Prabhu *et al.,* 2011). Cancer nanotherapeutics are rapidly progressing and are being implemented to solve several limitations of conventional drug delivery systems such as nonspecific biodistribution and targeting, lack of water solubility, poor oral bioavailability, and low therapeutic indices. To improve the biodistribution of cancer drugs, nanoparticles have been designed for optimal size and surface characteristics to increase their circulation time in the bloodstream. They are also able to carry their loaded active drugs to cancer cells by selectively using the unique pathophysiology of tumors, such as their enhanced permeability and retention effect and the tumor microenvironment. In addition to this passive targeting mechanism, active targeting strategies using ligands or antibodies directed against selected tumor targets amplify the specificity of these therapeutic nanoparticles. Drug resistance, another obstacle that impedes the efficacy of both molecularly targeted and conventional chemotherapeutic agents, might also be overcome or be reduced using nanoparticles. Nanoparticles have the ability to accumulate in cells without being recognized by P-glycoprotein, one of the main mediators of multidrug resistance, resulting in the increased intracellular concentration of drugs (Cho *et al.,* 2008).

2. Passive versus active cancer targeting

Strategies on delivering drug-encapsulated nanoparticles to cancerous tissue have been focused on passive and active targeting.

The passive approach uses the unique properties of the tumor microenvironment, such as

i. Leaky tumor vasculature, which is highly permeable to macromolecules relative to normal tissue and

ii. A dysfunctional lymphatic drainage system, which results in enhanced fluid retention in the tumor interstitial space (Maeda and Matsumura 1989).

As a result of these characteristics, the concentration of polymeric nanoparticles can be up to 100x higher in tumor tissues than normal tissue (Maeda and Matsumura, 1989). The tumor-specific deposition known as the enhanced permeability and retention (EPR) effect occurs as nanoparticles extravasate out of tumor microvasculature, leading to accumulation of drugs in the tumor interstitium. The extent of nanoparticle extravasation depends on the pore size of open inter-endothelial gap junctions and trans-endothelial channels which are between 400-600 nm (Yuan *et al.*, 1995). Particles with diameters less than 200 nm are most effective for extravasating tumor microvasculature (Langer and Tirrell, 2004).

Active tumor targeting is achieved by local or systemic administration of nanoparticles conjugated with targeting molecules that can recognize and bind to specific ligands that are unique to cancer cells. In the case of local drug delivery, particularly useful for primary tumors without metastasis, the cytotoxic drug encapsulated in the nanoparticles can be delivered directly to cancer cells while minimizing harmful toxicity to non-cancerous adjacent cells. For example, suicide

targeted gene delivery has been demonstrated to be effective in killing prostate cancer but not healthy muscle cells in xenograft mouse models of prostate cancer (Anderson *et al.,* 2004). In the case of metastatic cancers, the location, abundance and size of tumor metastasis limits its visualization or accessibility, and thus the drug delivery vehicle would be administered systemically.

Magnetic drug targeting is another novel field of research. Gilchrist injected 20–100 nm sized maghemite particles and selectively inducted heating of lymph nodes near surgically removed cancer (Gilchrist *et al.,* 1957). In 1963, Meyers described the accumulation of small iron particles intravenously injected into the leg veins of dogs, through an externally applied magnetic field (Meyers *et al.,* 1963). The use of magnetic particles for the embolization therapy of liver cancer followed (Jones and Winter, 2001). More defined spherical magnetic microspheres were made at the end of the 1970s (Widder *et al.,* 1979).

3. Applications of magnetic nanoparticle systems in diagnosis and treatment

For molecular imaging, four classes of nanoparticles are of primary interest: (1) magnetic nanoparticles, (2) magnetofluorescent nanoparticles, (3) fluorescent nanoparticles (e.g., fluorochrome doped nanoparticles, quantum dots), and (4) isotope tagged nanoparticles.

The most important diagnostic application of magnetic nanospheres is as contrast agents for magnetic resonance imaging (MRI). Superparamagnetic iron oxides (SPIOs) are approved and used for the imaging of liver metastases or to distinguish bowel loops from other abdominal structures since 1994.

3.1. Magnetic delivery of chemotherapeutic drugs to tumors

Molecular targeted therapeutics is superior to nonspecific cytotoxins due to a larger therapeutic index (Kim, 2003). Nanoparticles of many different compositions and

purposes can be used (Brigger *et al.,* 2002). Size, charge, hydrophilicity, and composition of nanoparticles can be manipulated to achieve optimal uptake within a tumor and specific targeting ligands (i.e., peptides, monoclonal antibodies, small molecules) (Merisko-Liversidge *et al.,* 1996) can be attached to the surface to provide tissue specificity. Because of their fine size, they are often taken up by cells.

The ability to selectively target antineoplastic agents to tumor can be done by encapsulation carriers such as liposomes (Gregoriadis and Ryman, 1971) and albumin microspheres (Kramer, 1974). However, the unrestricted circulation of liposomes and other carriers results in the rapid clearance by reticuloendothelial system and also in off target site specificity. Magnetic nanoparticles are excellent candidates for developing drug delivery systems, as it is feasible to produce and characterize (Berry *et al.,* 2004) and an external localized magnetic field gradient may be used to target it.

Magnetic drug delivery can be used to achieve very high concentrations of chemotherapeutic or radiological agents near the target tumor site, without any toxic effects to the normal surrounding tissue or to the whole body. Here a drug or therapeutic radioisotope is bound to a magnetic compound, injected into a patient's blood stream, and then stopped with a powerful magnetic field in the target area. Depending on the type of drug, it is then slowly released from the magnetic carriers (e.g. release of chemotherapeutic drugs from magnetic microspheres) or confers a local effect (e.g. irradiation from radioactive microspheres; hyperthermia with magnetic nanoparticles). It is thus possible to replace large amounts of freely circulating drug with much lower amounts of drug localized in disease sites resulting in effective and several-fold increased localized drug levels (Hafeli *et al.,* 1997).

Magnetic nanoparticles can be injected intravenously and transported through blood stream to the desired area of treatment (Berry *et al.,* 2003). Super paramagnetic

particles do not retain any magnetism after removal of magnetic field and they are physiologically well tolerated *in vivo*. Magnetic nanoparticles can be deposited on tumour tissues and heated by an alternating magnetic field to destroy the tumour (Muller *et al.*, 2005). It has been shown that magnetic nanoparticles can be retained at tumour sites, after local administration combined with a locally applied external magnetic field, due to the magnetic responsiveness of the iron oxide core, thereby enabling magnetic targeting (Alexiou *et al.*, 2003). Magnetic nanoparticles that are specifically and differentially taken up by the targeted cells and release their payload over an extended period to achieve a clinical response is important (Ferrari, 2005).

Several anticancer chemotherapeutics including paclitaxel (Koziara *et al.*, 2006), doxorubicin (Yoo *et al.*, 2000), 5-fluorouracil (Bhadra *et al.*, 2003) and dexamethasone (Panyam and Labhasetwar, 2004) have been successfully formulated using nanomaterials.

3.2. Magnetic targeting of radioactivity

Magnetic targeting can also be used to deliver therapeutic radioisotopes (Hafeli, 2001). The advantage of this method over external beam therapy is that the dose can be increased, resulting in improved tumor cell eradication, without harm to nearby normal tissue.

3.3. Treatment of tumors with magnetically induced hyperthermia

Ongoing investigations in magnetic hyperthermia are focused on the development of magnetic particles that are able to self-regulate the temperature. The ideal temperature for hyperthermia is 43– 45^{o}C, and particles with a Curie temperature in this range have been described (Kuznetsov *et al.*, 2002). Developments by Jordan and Chan led to hyperthermia application of single domain dextran-coated magnetite

nanoparticles in tumors. The first clinical trial is ongoing in Germany (Jordan *et al.*, 2001).

3.4. Magnetic control of pharmacokinetic parameters and drug release

The magnetic component in microspheres can also be used for purposes other than targeting such as to activate or increase the release of the drug by moving a magnet over it or by applying an oscillating magnetic field (Edelman and Langer, 1993).

3.5. Other magnetic targeting applications

Similar to chemotherapeutic drugs, many other drugs including peptides and proteins can be adsorbed or encapsulated into magnetic microspheres or nanospheres. Magnetically enhanced gene therapy (Scherer *et al.,* 2002) is another novel approach. Embolization (clogging) of capillaries by magnetic particles or ferrofluids under the influence of a magnetic field (Flores and Liu, 2002) utilizes the mechanical-physical properties of nanoparticles for therapy. In this way, tumors could be specifically starved of their blood supply.

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An Investigation into Rate of Adsorption of Various Acids on Activated Charcoal

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Abstract

Adsorption is present in many natural physical, biological, and chemical systems, and is widely used in industrial applications. Activated charcoal, captures and use waste heat to provide cold water for air conditioning and other process requirements, synthetic resins, increase storage capacity of carbide-derived carbons for tunable nanoporous carbon, and water purification. In this experiment we investigate the rate of adsorption of three different acids, acetic acid, oxalic acid and salicylic acid on activated Charcoal. Oxalic acid shows higher rate of adsorption compared to other two acids, which can be explained by Freundlich Adsorption isotherm.

Key words: Adsorption, Activated charcoal,Freundlich Adsorption isotherm

1. Introduction

The term adsorption was first used by keyser in 1881 in connection with the concentration of gases on free surfaces. When two immiscible phases are brought into contact, it is observed that the concentration of one phase is greater at the interphase than in the bulk. The tendency of accumulation of molecules at a surface is called adsorption. The phase, on the surface of which a substance from other phase accumulates is termed as adsorbent and the adsorbed substance is called adsorbate. Adsorption is generally accompanied by release of energy, that is, most

adsorption processes are exothermic in nature. Adsorption is a spontaneous process (Donald et al., 2010).

Charcoal may be activated to increase its effectiveness as a filter. Activated charcoal readily adsorbs a wide range of organic compounds dissolved or suspended in gases and liquids. In certain industrial processes, such as the purification of sucrose from cane sugar, impurities cause an undesirable color, which can be removed with activated charcoal. It is also used to absorb odors and toxins in gases, such as air. Charcoal filters are also used in some types of gas masks. The medical use of activated charcoal is mainly the adsorption of poisons, especially in the case of suicide attempts in which the patient has ingested a large amount of a drug. Activated charcoal is available without a prescription, so it is used for a variety of health-related applications. For example, it is often used to reduce discomfort due to excessive gas in the digestive tract (KL kapoor 2011).

Activated charcoal is a porous carbonaceous material prepared by carbonizing and activating the organic substances of mainly biological origin. Carbonaceous materials are usually classified into two groups i.e. amorphous and crystalline. The amorphous group includes the activated carbon, carbon black and activated products of thermal decomposition of organic substances such as cokes, chars, wood char etc. The crystalline group of carbon includes graphite and diamond. The application of charcoal as an adsorption to remove the coloring material is one of the oldest technique. The technique was noted in the fifteen century and used by Lowitz in 1791 to purify the raw sugar solutions. The knowledge of activation of charcoal accelerates their use as an efficient adsorbent. During the process of activation the spaces between the elementary crystalline become cleared of various non-organized carbonaceous compounds (tarry substances) resulting in voids or pores. A suitable activation process causes an enormous number of pores to be formed so that internal surface of the activated charcoal becomes very large. Activation process usually

depends upon selective oxidation of carbonaceous material in the presence of impregnates at elevated temperature. The impregnates is then treated with an oxidizing agent like air, steam carbon dioxide or chlorine. Addition of impregnates such zinc chloride ,calcium chloride and magnesium chloride etc. before carbonization influence the pyrolytic process so that the formation of tar and distillate (methanol, acetic acid and other) is restricted to minimum . These impregnates during carbonization also prevent the formation of the adsorbed films on the surface of charcoal which lessens its adsorbing power. Furthermore, the yield of charcoal in the carbonized product is also increased. The gaseous activation agents modify the charcoal surfaces and remove the tarry products along with the formation of new pores by complete burning out of less perfect crystallites .The adsorption properties of activated charcoal are intimately associated with their porous and chemical composition(Puri et al, 1962,B.S Bahl et al 2004).

In heterogeneous catalysis, the molecules of reactants are adsorbed at the catalyst surface where they form an adsorption complex. This decomposes to form the product molecules which then take off from the surface. Animal charcoal removes colors of solutions by adsorbing coloured impurities. Thus in the manufacture of cane- sugar, the coloured solution is clarified by treating with activated charcoal Activated charcoal is used as a filter, catalyst or as an adsorbent. Use of Charcoal as adsorbent plays an important role in various fields. Chilton et al conducted a study on adsorption process using Freundlich isotherm on agricultural by product based powdered activated carbons in a geosmin water system (Chilton et al 2002). Lagreena et al conducted experiments on corrosion inhibition and propose adsorption is the mechanism behind the processs. A report of their study says that higher the value of K_{ads} ensures the spontaneity of the adsorption process and is the characteristics of strong interaction and stability of the adsorbed layer(Lagrenee et al 2002).

2. Materials and Methods

(i) Prepare 250 cm^3 0.25M acid solutions, (ii) Take cleaned and dried six stoppered reagent bottles and label them from 1 to 6. Weigh accurately 2g of activated charcoal into each of the bottle.

(iii) Add by means of a burette 50,40,30,20,10 and 5 cm3 of 0.25M acid solution and 0,10,20,30,40 and 45 cm^3 of distilled water in bottle no. 1,2,3,4,5 and 6 respectively. Stopper the bottles and shake them vigorously for about 20 minutes. Suspend the bottles in thermostat (or a large trough containing water at room temperature), and leave them for about one hour with intermittent shaking. (iv) Filter the supernatant liquid of each of the bottles through a dry filter paper (use different filter papers for different bottles) and collect the filtrates in properly labeled dry flasks. Reject first 5-10cm^3 of the filtrate as the concentrations in the first fraction are likely to be changed by adsorption in the filter paper during filtrations.

(v) Take an aliquot of each filtrate (5cm^3 of the first and 2^{nd}, 10 cm^3 of and 3^{rd} and 4^{th} and 20 cm^3 of 5^{th} and 6^{th}) and titrate with the standard alkali solution (V_1)Make duplicate titration of each solution (P.P Chaturvedi et al 1970) .

When charcoal is shaken with a solution of acid, is found that at a given time, the amount adsorbed is given by the following relation.

$$\log X/m = \log K + 1/n \log C,$$

where X is the weight of acid adsorbed by m grams of charcoal, C is the concentration of acid, K and n are constants. Taking logarithm of the above equation

$$\log X/m = \log K + 1/n \log C$$

In order to test the validity of freundlich adsorption isotherm plot log x/m values as ordinate against log Ce values as abscissae .The slope and the intercept of the plot

will give 1/n and log k respectively; hence n and k can be calculated. Validity of Langmuir's adsorption equation can be tested by plotting Ce/x/m values (ordinate) versus Ce (abscissa). A linear plot obtained shows the applicability of the isotherm.

3. Results and Discussion

3.1. Rate of Adsorption

In this experiment we determine the rate of adsorption of three different acids, oxalic acid, acetic acid and salicylic acid towards activated charcoal by means of volumetric analysis. Table 1 gives the results of the vol. of NaOH (V_1) used for titration of three acids .It is clear that oxalic acid show lower value due to higher adsorption compared to other two acids.

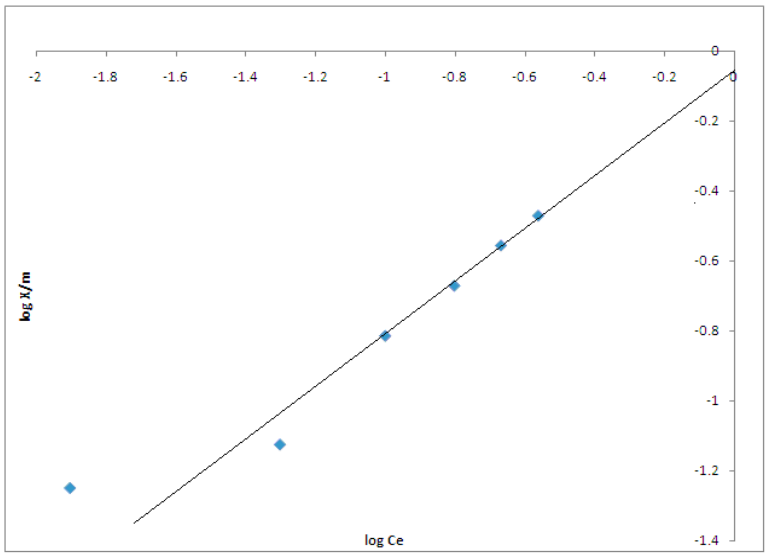
Table 1. Rate of Adsorption of Oxalic acid, Acetic acid and Salicylic acid on activated charcoal

Sl.No	C_o	V_2	V_1 for Oxalic acid	V_1 for Acetic acid	V_1 for Salicylic acid
1	0.5	10	11	14.6	18
2	0.4	10	8.6	11..2	14.3
3	0.3	10	6.3	8.2	10.5
4	0.2	10	4.1	5.2	6.8
5	0.1	10	2.0	1.6	3.2
6	0.05	10	0.5	0.8	1.4

C_o =Initial Concentration of acid = $(V_{oxalic\ acid} \times N_{oxalic\ acid})/50$ V_2=Volume of titrant = 10ml , V_1= Volume of NaoH

3.2 Adsorption Isotherm

We check the validity of different isotherms and it is obvious that Freundlich adsorption isotherm is obeys the adsorption process and gives straight line. The adsorption parameters are given in tables 2-4, and isotherms are given in Figures 1-3. Intercept of the isotherm gives the value of equilibrium constant (K_{ads})for the absorption process .Higher the value of K_{ads} ensures the spontaneity of the adsorption process and is the characteristics of strong interaction and stability of the adsorbed layer. Slope gives the value for n.The values obtained are less than one, confirming the validity of Freundlich's adsorption isotherm. From the tables it is observed that value of equilibrium constant for adsorption is higher for oxalic acid, which proves the result obtained from volumetric analysis. The order of rate of adsorption is oxalic acid > Acetic acid > Salicylic acid. It is explained as follows; the higher rate for oxalic acid may be due to the presence of two acid groups and lower value for salicylic acid due to intramolecular hydrogen bonding and due to aromaticity.

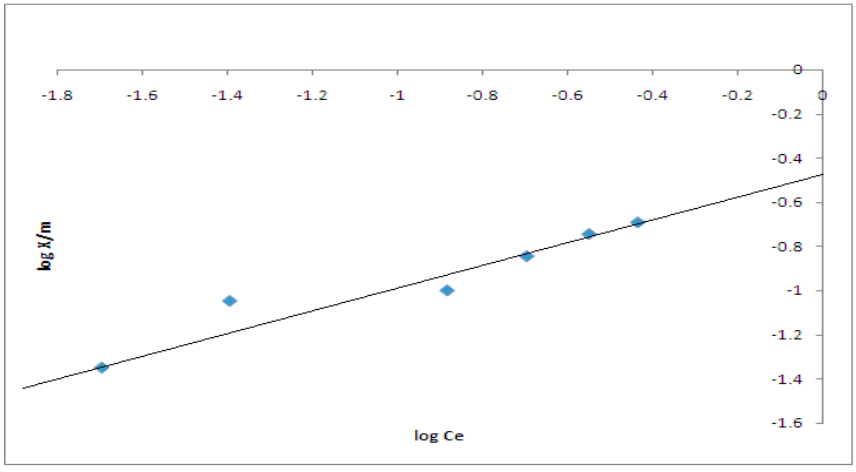


Freundlich Adsorption isotherm for Oxalic acid

Table 2. Adsorption parameters for Oxalic Acid

Sl.no.	C_o	V_2	C_e	log C_e	X	X/m	log X/m	K_{ads}	n
1	0.5	10	0.255	-0.5934	1.1025	0.5512	-0.2586	0.3981	0.5550
2	0.4	10	0.215	-0.6675	0.8325	0.4162	-0.3806		
3	0.3	10	0.158	-0.8027	0.6412	0.3206	-0.494		
4	0.2	10	0.103	-0.9892	0.4387	0.2193	-0.6589		
5	0.1	10	0.065	-1.187	0.1575	0.0787	-1.104		
6	0.05	10	0.028	-1.5606	0.1013	0.0506	-1.2958		

C_o =Initial Concentration of acid = $(V_{oxalic\ acid} \times N_{oxalic\ acid})/50$
V_2=Volume of titrant = 10ml , V_1= Volume of NaoH ,
C_e= Equilibrium concentration = $(V_{NaOH} \times N_{NaOH})/V_2$, X = $(C_0-C_e) \times 50 \times 90$

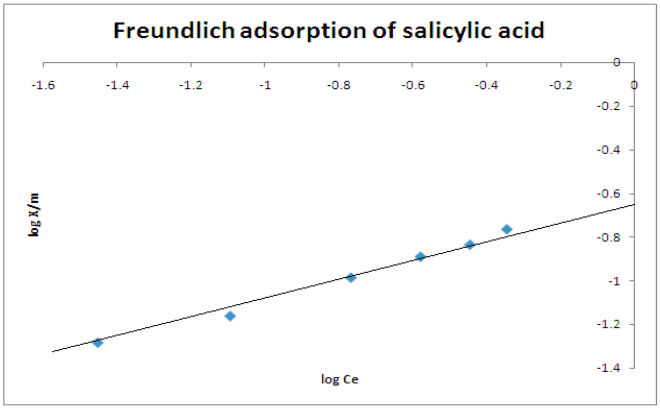


Freundlich Adsorption isotherm for Acetic acid

Table 3. Adsorption parameters for Acetic Acid

Sl.no.	Co	V_2	Ce	log Ce	X	X/m	log X/m	K_{ads}	n
1	0.5	10	0.365	-0.4377	0.405	0.203	-0.6925	0.2630	0.3640
2	0.4	10	0.28	-0.5528	0.36	0.180	-0.745		
3	0.3	10	0.2	-0.6989	0.285	0.143	-0.845		
4	0.2	10	0.13	-0.886	0.21	0.105	-0.979		
5	0.1	10	0.04	-1.3979	0.18	0.090	-1.046		
6	0.05	10	0.02	-1.699	0.09	0.045	-1.347		

C_o =Initial Concentration of acid = $(V_{oxalic\ acid} \times N_{oxalic\ acid})/50$
V_2=Volume of titrant = 10ml , V_1= Volume of NaoH ,
C_e= Equilibrium concentration = $(V_{NaOH} \times N_{NaOH})/V_2$, X = $(C_0-C_e) \times 50 \times 90$



Freundlich Adsorption isotherm for Salicylic acid

Table 4. Adsorption parameters for Salicylic Acid

Sl.no.	Co	V_2	Ce	log Ce	X	X/m	log X/m	K_{ads}	n
1	0.5	10	0.45	-0.3467	0.345	0.1725	-0.7632	0.2511	0.5520
2	0.4	10	0.3575	-0.4467	0.293	0.1465	-0.8341		
3	0.3	10	0.2625	-0.5808	0.2587	0.1293	-0.8890		
4	0.2	10	0.17	-0.7695	0.207	0.1035	-0.9850		
5	0.1	10	0.08	-1.0969	0.138	0.069	-1.1611		
6	0.05	10	0.035	-1.4559	0.1035	0.052	-1.2830		

C_o =Initial Concentration of acid = $(V_{oxalic\ acid} \times N_{oxalic\ acid})/50$
V_2=Volume of titrant = 10ml , V_1= Volume of NaoH,
C_e= Equilibrium concentration = $(V_{NaOH} \times N_{NaOH})/V_2$, $X = (C_0 - C_e) \times 50 \times 90$

4. Conclusion

In this experiment we investigate the rate of adsorption of three different acids, acetic acid, oxalic acid and salicylic acid on activated Charcoal. The important findings are:

- During titration, lesser volume of NaOH is used for oxalic acid shows higher rate of adsorption compared to other two acids.
- Freundlich isotherm obeys the adsorption process. n values obtained also proves the validity of the freundlich isotherm

- Higher the value of K_{ads} stronger the interaction between adsorbent and adsorbate, also proves the higher rate of adsorption of oxalic acid on activated charcoal.

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Qualitative and Quantitative Detection of Novel Strains of Engine Oil Degraders From Different Hydrocarbon Contaminated Soil

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Abstract

Increased mechanization, large scale of production and global competition has resulted in various socio-economic and environmental problems in Kerala. There are 250 significant polluting large and medium scale industries in the State. Accidental leakages of hydrocarbon fuels during transportation and other activities make these hydrocarbons the most common global environmental pollutants. Currently, biodegradation of aromatic compounds received a great attention from many people from industries and researchers due to their toxicity and refractory. Biodegradation is the chemical dissolution of materials by bacteria or other biological means. Some microorganisms have a naturally occurring catabolic diversity to degrade, transform or accumulate a huge range of compounds including hydrocarbons.. In present study, common oil contaminated sites such as autogarrage, diesel station and oil mill were focused to collect the soil sample to identify indigenous microbes. Three predominant bacterial isolates, namely *Pseudomonas(P1),Micrococcus(E1)Bacillus(C1)* were obtained from hydrocarbon contaminated soil in this study. An increase in oil degradation was corresponding to an increase in cell number during the degradation processes demonstrating the ability of utilizing engine oil as the energy source. The screening of potent engine oil degrading microbes were done qualitatively by Dioxygenase test. Outcome of this study offer a useful guideline in evaluating potential degraders from the environment.

Keywords: Biodegradation, Aromatic compounds, Hydrocarbon.

1.Introduction

1.1 Bio Degradation

Biodegradation is often used in relation to ecology, waste management, biomedicine, and is now commonly associated with environmentally friendly products that are capable of decomposing back into natural elements. Hydrocarbons are a primary energy source for current civilizations. The predominant use of hydrocarbons is as a combustible fuel source. Hydrocarbons are classified into various types which includes volatile organic compounds (VOCs), saturated hydrocarbons, aromatic hydrocarbons. Volatile organic compounds (VOCs) are organic chemicals that have a high vapor pressure at ordinary room temperature. They include both human-made and naturally occurring chemical compounds. Harmful VOCs typically are not acutely toxic, but have compounding long-term health effects. Saturated hydrocarbons (alkanes) are the simplest of the hydrocarbon species. They are composed entirely of single bonds and are saturated with hydrogen. The general formula for saturated hydrocarbons is CnH2n+2 (assuming non -cyclic structures). Aromatic hydrocarbon or arene (or sometimes aryl hydrocarbon) is a hydrocarbon with alternating double and single bonds between carbon atoms forming rings. Aromatic hydrocarbons can be monocyclic (MAH) or polycyclic (PAH). Some non-benzene-based compounds called heteroarenes, which follows Hückel's rule (for monocyclic rings: when the number of its π -electrons equals 4n+2), are also called as aromatic compounds.Engine oil(used in study) is a complex mixture of hydrocarbons and other organic compounds that is used to lubricate the parts of an automobiles engine. (Hagwell et al., 1992).The most important characteristic of the lubricating oil for automotive use is its viscosity. Used motor oil contains more metals and heavy polycyclic aromatic hydrocarbons (PAHs) that would contribute to chronic hazards including mutagenicity and carcinogenicity (Keithand Telliard, 1979; Hagwell et al., 1992; Boonchan et al.,

2000). Prolonged exposure and high oil concentrationmay result in the development of liver disease ,kidney disease, and can increase risk of cancer (Mishra et al., 2001; Propst et al., 1999Lloyd and Cackette, 2001).Microorganisms degrade these compounds by using enzymes in their metabolism (Alexander, 1999) and degradation rate depend upon various factors such as physical nature of the oil, chemical nature of the oil, availability of nutrients, availability of oxygen, water temperature, pressure, pH and salinity and presence of other microbes

2. Materials And Method

2.1. Sample collection

Soil samples were collected from three different oil contaminated sites of Kerala such as petrol bunk, auto garage and oil mill. Soil was collected randomly 5 to 10 cm beneath the surface using spatula and were packed in sterile poly bags and transferred to the laboratory.

2.2. Isolation of bacteria

100 ml of Bushnell Has broth (BHB) supplemented with 1% (v/v) engine oil was separately prepared in conical flasks. Broth was inoculated with soil samples (10g) and incubated for 7 days at room temperature along with agitation using shaker.1 ml of enriched media was transferred into freshly prepared enrichment media and incubated at the same conditions as described above. Dilutions from the third enrichment process were plated out onto BH agar plates, which were coveredwith 100 µl of used engine oil and incubated at 30°C. The single colonies were streaked onto nutrient agar plates, incubated at 30°C overnight, and stored at 4°C until further use.

2.3. Staining and biochemical activities of purified cultures

In order to identify the purified cultures tentatively on the basis of Bergey's manual various staining and biochemical tests were performed namely Gram staining, Endospore staining, Hanging drop method, Oxidase test, Catalase test, Mannitol motility, Lactose fermentation, Sucrose fermentation, Maltose fermentation, Indole test, Methyl red test, Voges prausker, Citrate utilization test, Triple sugar iron test, Gelatin liquefaction test, Urease test.

2.4. Dioxygenase test

The potential degraders were pre-grown on YEPG agar plates. After 3 hour of incubation at room temperature, the plates were swabbed using *E.coli* broth. After one day of incubation at 20°C, blue color (indigo) colonies considered as positive.

2.5. Engine oil degradation assay

A single colony of the isolate was inoculated into 10 ml nutrient broth (Merck) and incubated at 30°C overnight at 160 rpm. The overnight culture was centrifuged for 10 min at 10000 rpm. The cell pellet was washed twice in phosphate buffer saline (pH 7.6) and re-suspended in BH medium until OD_{600} nm was equivalent to 1. 1 ml of bacterial inoculum (1 OD_{600} nm equivalent) was added to 250 ml flasks containing 100 ml of liquid BH medium supplemented with 1% carbon source (engine oil). Uninoculated control flasks with 1% (v/v) engine oil were incubated in parallel to monitor abiotic losses of the substrate.

3. Result

In present study three bacterial isolates, namely *Pseudomonas*(P1), *Micrococcus*(E1) *Bacillus*(C1) were obtained from hydrocarbon contaminated soil . Comparative study of isolates and consortium with MTCC Strain *Pseudomonas putida* 2467 also revealed the degradation rate(Graph 1,2). Among three isolates, *Pseudomonas*(P1),

Micrococcus(E1) were showed the aromatic ring dioxygenase activity of the potential degraders(Fig 1) . The isolates were preserved from the soil samples on the basis of their colony morphology, texture and growth(Table 1,2). The growth profile were monitored by optical density and the graph reflect that predominant isolates grow maximally on engine oil substrate supplied as sole source of carbon and energy.

Table 1. Preliminary Tests

Preliminary Tests	***Pseudomonas species(P1)***	***Micrococcus species(E1)***	***Bacillus species(C1)***
Colony morphology	Green coloured, flat,slimy,transluscent colonies	Yellow spotted opaque irregular, flat colonies	Off white opaque mucoid colonies
Gram staining	Gram negative	Gram positive	Gram positive
Motility	Motile	Motile	Non motile
Catalase test	Positive	Positive	Positive
Oxidase test	Positive	Positive	Negative

4. Discussion And Conclusion

Common oil contaminated sites(in Thrissur)such as autogarrage, diesel station and oil mill were focused to collect the soil sample to identify indigenous microbes. Three predominant bacterial isolates, namely *Pseudomonas(P1),Micrococcus(E1)Bacillus(C1)* were obtained from hydrocarbon contaminated soil in this study. An increase in oil degradation was corresponding to an increase in cell number during the degradation processes demonstrating the

Table 2. Biochemical Analysis of Isolates

Sl. No.	Biochemical Tests	P1	E1	C1
1.	Indole	-	-	-
2.	Methyl-Red	+	+	_
3.	Vogues-Prausker	_	+	_
4.	Citrate utilization	+	_	_
5.	Urease	+	-	+
6.	Nitrate reduction	+	-	+
7.	Maltose	-	+	_
8.	Mannitol	+	+	+
9.	Sucrose	+	+	+
10.	Lactose	_	+	_
11.	Mannitol motility	M- M+	M+ M+	M+ M-
12.	Gelatin liquefaction	+	+	+

Figure 1. Qualitative Screening of Isolates

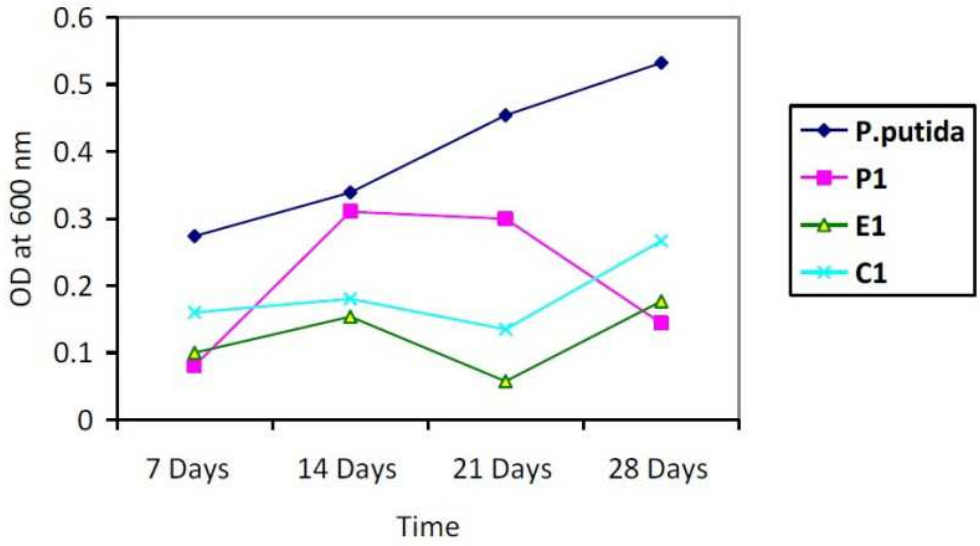

Figure 2. Photometry results of P1, E1, C1 with *P. putida*

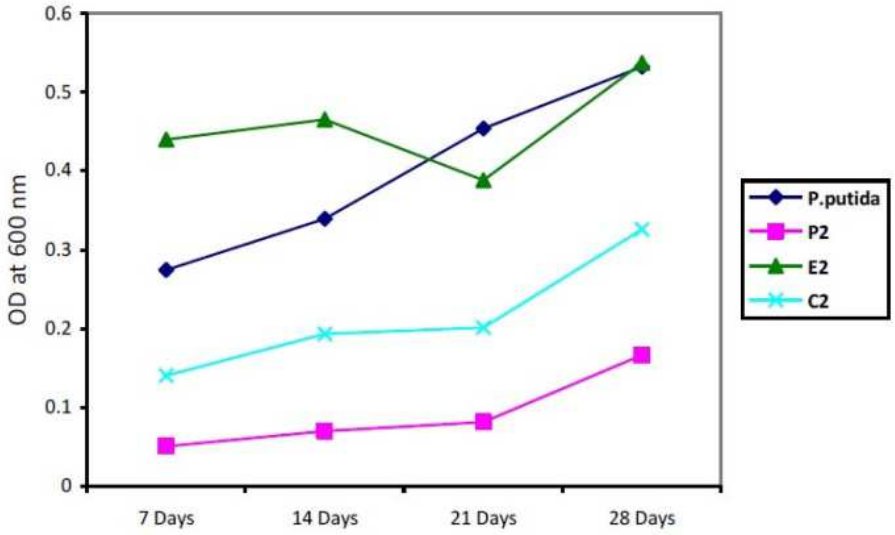

Figure 3. Photometry results of P2, E2, C2 with *P. putida*

ability of utilizing engine oil as the energy source. Comparative study of isolates with MTCC Strain *Pseudomonas putida* 2467 also revealed the degradation rate. Degradation potential of consortium from contaminated sites also concealed

higher degradation rate when compared with *Pseudomonas putida* 2467. Activity of the potential degraders was examined using indole because the formation of indigo from indole is presumptive for aromatic ring dioxygenases (Ensley et al.,1983) Among three isolates, *Pseudomonas*(P1), *Micrococcus*(E1) were showed the aromatic ring dioxygenase activity. Engine oil used by the bacterial consortium reflect higher degradation rates. Present study revealed that biological tools can reduce environmental pollution of soil and water that occurred due to increased transport, industrialization etc. Further studies are under way to scale up growth condition of isolates and to determine capability of isolates to produce surfactants and to identify genes involved in oil degradation. This work has also emphasized the need of research in concern area.

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Green Synthesis of Silver Nanoparticles Using Different Fruit Extracts

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Abstract

The chemical methods available for silver nanoparticles synthesis use chemicals that are toxic and potentially hazardous. Hence the search for a cost effective & ecofriendly method of synthesis. Thie study was successful in synthesizing silver nanoparticles using green methods from five locally available fruits of Kerala viz. *Averrhoa bilimb*i (Irumbanpuli), *Syzygium samarangense* (rose apple), *Syzygium cumini* (black plum), *Mangifera indica* (mango), *Citrus tenivesiculata* (vadukapuli), and *Anacardium occidentale* (Cashew fruit).

Key Words : *Green Synthesis, Silver nanoparticles, Averrhoa bilimbi, Syzygium samarangense, Syzygium cumini, Mangifera indica, Citrus tenivesiculata, and Anacardium occidentale*

1. Introduction

Among nanoparticles, metal nanoparticles especially silver nanoparticles has attracted considerable attention as a result of their significant applications in the field of fundamental sciences, nano-technology & opto-electronics (Hughes and Jain, 1979; Kreibig and Vollmer, 1995). Silver nanoparticles are nanoparticles of silver i.e. silver particles between 1nm and 100nm in size. While frequently described as being silver some are composed of a large percentage of silver oxide due to their large ratio of surface to bulk silver atoms.

The optical properties of silver nanoparticles have been extensively investigated by ultraviolet-visible (UV/VIS) spectroscopy technique. It is observed that the silver nanoparticles exhibit an intense absorption band in UV/VIS region. (Grijalva *et al*.,

2005; Liu *et al*., 2003; Wilcoxon *et al*., 2001; Cai*et al*., 2001). Their UV/VIS spectrum , known as the surface plasmon absorption band, is produced by the movement of the conduction electrons in the particles as a consequence of the incident electric field light, which results in a displacement of the negative and positive charges in the metal. The surface plasmon absorption band properties of silver nanoparticles depend on their size, shape, surrounding medium, coupling of the colloids and absorbed solutes (Bijanzadeh *et al.*, 2012). Silver exhibits one of the the highest efficiency of plasmon excitation in the visible spectrum. Moreover, optical excitation of plasmon resonances in nanosized silver particles is the most efficient mechanism by which light interacts with matter. A single silver nanoparticle interacts with light more efficiently than a particle of the same dimension composed of any known organic or inorganic chromophore. Hence silver is one of the material whose Plasmon resonance can be turned to any wavelength in the visible spectrum. The formation of silver nanoparticles was confirmed by Surface Plasmon Resonance as determined by UV–Visible spectra in the range of 400–500 nm.

Over the last decades, silver nanoparticles have found applications in catalysis, optics, electronics and other areas due to their unique size-dependent optical, electrical and magnetic properties. Currently, most of the applications of silver nanoparticles are as antibacterial or antifungal agents in biotechnology and bioengineering, textile engineering, water treatment and silver based consumer products. There is also an effort to incorporate silver nanoparticles in to wide range of medical devices like bone cement, surgical instruments, surgical masks, wound dressing etc. Silver nanoparticles can now be routinely synthesized by means of various chemical and physical methods. Through numerous chemical methods are available for metal nanoparticles synthesis, copious reactants and starting materials are used in these reactions that are toxic and potentially hazardous. Increasing

environmental concerns over chemical synthesis routes have resulted in attempts to develop biomimetic approaches.

Green synthesis of silver nanoparticles provides advancement over chemical and physical methods as it is cost effective, environment friendly and easily scaled up for large scale synthesis. In these methods, there is no need to use high pressure, energy, temperature and toxic chemicals. Silver has long been recognized as having inhibitory effects on microbes present in medical and industrial process. In these aspects synthetic methods based on naturally occurring biomaterials provide an alternative means for obtaining these nanoparticles. One of them is the synthesis using plant extract eliminating the elaborate process of maintaining the microbial culture and often found to be kinetically favourable than other bioprocesses. Biomolecules as reducing agents are found to have significant advantage over their counterpart as protecting agents

Elumalai *et al.* (2010) reported the synthesis of silver nanoparticle from Aqueous extract of shade dried leaves of *Euphorbia hirta*. In another study Prasanth *et al.* (2011) reported the synthesis of plant-mediated silver nanoparticles using 15 medicinal plant extracts. Mallikarjuna *et al.* (2011) reported the green synthesis of silver nanoparticles using *Ocimum* leaf extract. Green synthesis of silver nanoparticles using *argemone mexicana* leaf extract was reported by Singha *et al.*, in 2010. The silver nanoparticles synthesised were in the size range of 25-50nm and showed an absorbance peak at 450nm with an absorbance of 1.4. Others have also studied the use of fruit extracts in synthesis of silver nanoparticles. In a recent study on synthesis of plant mediated silver nanoparticles using papaya fruit extracts and evaluation of their antimicrobial activities, Jain. *et al.* (2009), observed that when the papaya fruit extract was mixed with the aqueous solution of the silver ion complex, it started to change the colour to yellowish brown due to reduction of

silver ion which indicate the formation of silver nanoparticles. The absorption spectra of silver nanoparticles formed in this process showed an absorbance peak at 450nm with an average size of 15nm.

In another study Amarnath Kanchana *et al.* (2011) reported that synthesis of silver nanoparticles from Spinacia Oleracea and Lactuca Sativa leaves by 'exploiting' the reduction capabilities of varied phytochemicals present in it. When the leaf extract was treated with aqueous silver nitrate solution, the entire reaction mixture turned to yellowish brown colour and exhibited an absorbance peak at 480nm in the UV-VIS spectra. Shalini Chauha *et al.* (2011) reported a cost effective and eco-friendly technique of green synthesis of silver nanoparticles from the extract of seed of pomegranate fruit. They observed an absorbance peak at λmax at 430 nm. The silver nanoparticles synthesised were 30 nm in size with spherical shape.

Here in, we attempted to find a cost effective & ecofriendly method for the synthesis of silver nanoparticles from 1mM $AgNO_3$ solution through the extract of five locally available fruits. The method involves reducing the silver ions present in the solution of silver nitrate by the extracts of different fruits. The fruits used here for the synthesis of silver nanoparticles are *Averrhoa bilimb*i (Irumbanpuli), *Syzygium samarangense* (rose apple), *Syzygium cumini* (black plum), *Mangifera indica* (mango), *Citrus tenivesiculata* (vadukapuli), *Anacardium occidentale* (Cashew fruit). Nanoparticles were characterized using UV-visible absorption spectroscopy using UV-visible spectrophotometer.

2. Materials & Methods

Silver nitrate is used for the synthesis of silver nanoparticles. All glassware have been washed with distilled water and dried in oven before use. Fresh fruits have been collected locally.

2.1. Preparation of fruit extract

Ripened fruits were used to prepare the extracts .Fruits were thoroughly washed in distilled water, dried, cut into fine pieces and were crushed to get a solution and filtered through Whatman no.40 filter paper. All the fruits were collected fresh & care was taken to select only ripe fruits free from blemishes. The fruits used for the preparation of extracts are *Averrhoa bilimbi* (Irumbanpuli), *Syzygium samarangense* (rose apple), *Syzygium cumini* (black plum), *Mangifera indica* (mango), Citrus *tenivesiculata* (vadukapuli), *Anacardium occidentale* (Cashew fruit)

2.2. Synthesis of silver nanoparticles

1mM aqueous solution of silver nitrate ($AgNO_3$) was prepared and used for the synthesis of silver nanoparticles. 1.7g of Silver nitrate was weighted accurately in an electrical balance, transferred to the standard flask and made up to 100ml. From this solution, pipette out 50ml $AgNO_3$ solution, and transfer to another 500ml standard flask. Make up to the mark to obtain 1mM $AgNO_3$ solution. 10ml of the fruit extracts was added in to 90 ml of aqueous solution of 1mM silver nitrate in a conical flask which was covered with aluminium foil, stirred well with a magnetic stirrer and kept in dark at room temperature. Here the fruit extracts act as reducing agents when exposed to silver ions, it gets reduced and silver nanoparticles are formed in the reaction medium which can be characterized using UV-VIS Spectrophotometer.

2.3. UV-Visible spectra analysis

The formation of silver nanoparticles was monitored by change in colour of the solution to golden brown. All the samples were left overnight in the dark and any change in colour noted on the following day. This was confirmed by measuring the UV-Visible spectrum of the reaction medium after diluting a small quantity of sample with distilled water. UV-Visible spectral scan was done by using UV mini1240 Spectrophotometer.

3. Results & Discussion

When the fruit extracts of *Averrhoa bilimbi* (Irumbanpuli), *Syzygium samarangense* (rose apple), *Syzygium cumini* (black plum), were mixed with aqueous solution of silver nitrate, it started to change the colour to yellowish brown due to reduction of silver ion which indicated the formation of silver nanoparticles. Absorption spectra of silver nanoparticles using *Averrhoa bilimbi* (Irumbanpuli) extract showed an absorbance peak at 481nm. The absorbance observed as 1.62. The absorption spectra of silver nanoparticles using *Syzygium cumini* (black plum) fruit extracts showed an absorption peak at 511nm with an absorbance 0.484. In the case of synthesis of silver nanoparticles using Syzygium *samarangense* (rose apple) fruit extract, the reaction mixture turned to yellowish brown colour and exhibited an absorbance peak at 469nm with an absorbance 0.714.

When we compare our result with the result of papaya fruit extracts, the absorption peak obtained is found to be 481nm, 469nm for *Averrhoa bilimbi* (Irumbanpuli), *Syzygium samarangense* (rose apple) respectively. These values are almost similar to the value of papaya fruit extract i.e, 450nm. But the absorption peak of *Syzygium cumini* (black plum) is slightly extended to a large value of 511nm.

The exact biomolecule that is responsible for the reduction of silver to silver nanoparticles has not been identified in this project. However, literature review has suggested some possibilities. It has been sugested that, biosynthetic products, reduced cofactors, molecules like glucose and ascorbate reduce respective salts to silver nanoparticles.

Ascorbic acid is known to act as a reducing agent for reduction of Ag^+ ions to Ag during synthesis of silver nanoparticles (Yaqiong Qin, 2010). Water-soluble antioxidative substances like ascorbate is present at high levels in all parts of plants.

Averrhoa bilimbi,a tropical fruit rich in Ascorbic acid and oxalic acid (Vera Lucia Arroxelas Galvo De Lima, 2001) It is quite possible that ascorbic acid may be involved as a reducing agent in the extract of *Averrhoa bilimbi*.

Another pathway for reduction of $AgNO_3$ to silver may be through the mechanism of glycolysis. Glycolysis is a definite sequence of ten reactions involving ten intermediate compounds. In this mechanism NAD^+ keeps on getting reoxidised and gets constantly regenerated due to redox reactions. This might have led to transformations of Ag^+ ions to Ag^0. Hence it is possible that the synthesis of silver nanoparticles using fruit extracts like *Syzygium samarangense* and *Syzygium cumini* may be through any of these routes.

When aqueous silver nitrate solution was treated with the fruit extracts of *Mangifera indica* (mango), *Citrus tenivesiculata* (vadukapuli) and *Anacardium occidentale* (Cashew fruit), no silver nanoparticles developed in the reaction medium. This was indicated by the absence of golden or yellowish brown colour in solution. On spectral scan no peaks were obtained in the UV-VIS region. The amount of silver nanoparticles synthesized and its various parameters like size and shape depends on reaction conditions like pH, concentration of silver nitrate, temperature etc. Due to various constrains, it was not possible to standardize the various parameters for synthesis of silver nanoparticles. It is possible that further tinkering with reaction conditions may have resulted in synthesis of silver of silver particles in those fruit extract which did not yeild silver nano particles in the current study. Further work is needed on this aspect.

In conclusion, it need to be emphasized that this green chemistry approach towards the synthesis of silver nanoparticles has many advantages like its ecofriendly nature

and high economic viability especially as the fruits used are all easily & locally available.

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Antimicrobial and Phytochemical analysis of Aqueous extract of *Eupatorium odoratum*

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Abstract

The aqueous extract of the leaves of *Eupatorium odoratum*was screened for phytochemical constituents and found to contain tannin, saponin, flavonoid, coumarins, terpenoid, steroids, phytosteroids, quinone and cardiac glycosides. Aqueous extract of the plant shows high quinone content. Antibacterial activity was carried out using the aqueous extract of *Eupatorium odoratum* was found to be effective against *E. coli, Klebsiella, Pseudomonas, Streptococcus* and *Bacillus* by well diffusion method.The maximum inhibitory effect of inhibition zone was recorded against *E. coli.* Antifungal studies were also conducted against three fungal species.

Key words:Eupatorium odoratum, Phytochemical screening,Antibacterial study, Antifungal activity.

1. Introduction

*Eupatorium odoratum*is a species of flowering shrub in the sunflower family, Asteraceae. Currently the plant is known as *Chromolaenaodorata.*It is also called as Christmas bush. *Chromolaenaodorata* is a fast-growing perennial shrub, native to South America and Central America. It has been introduced into the tropical regions of Asia, Africa and the Pacific, where it is an invasive weed. The shrub is reported to be highly allelopathic to nearby vegetation.This plant also reduces the diameter growth of teak in infested plantations.

In herbal medicine, leaf extracts with salt are used as a gargle for sore throats and colds. It is also used to scent aromatic baths. Extracts of Christmas bush have been

shown to inhibit or kill *Neisseria gonorrhea* (the organism that causes gonorrhoea) *in vitro* (Caceres et al., 1995) and to accelerate blood clotting (Triratanaet al., 1991). During fallows between cultivation, Christmas bush adds copious amounts of organic matter to the soil and may reduce the populations of nematodes (M'Boob, 1991). It is also useful as mulch for row crops . 4', 5, 6, 7-tetramethoxyflavone, is an active ingredient isolated from *Eupatorium odoratum* that has long been used to stop bleeding (Triratanaet al., 1991). This compound was studied in vitro for the effect on blood clotting factor activities.

A study of the effect of *Eupatorium odoratum* Linn. on blood viscosity during coagulation process were reported(Pradit and Roongkiat,1997).Platelet-activating factor (PAF) receptor binding antagonist activity of the methanol extracts and isolated flavonoids from *Chromolaenaodorata* (L.) King and Robinson has been reported (Sui Kiong Ling et al., 2007). Enhancement of homeostasis and blood coagulation using *C. odorata*extract has also been reported (Akah P.A., 1990). In Vietnam and other tropical countries, fresh leaves or decoction of the leaves are used for treatment of leech bite, soft tissue wounds, burn wounds, skin infection and dento-alveolitis (Phanet al., 2001).The juice of the aerial parts of this plant is used for cuts and wounds to arrest bleeding and promote healing. The macerated leaves are usually applied to swollen portion of the body to relieve inflammation amongst the rural populace in southern part of Nigeria. The anti-inflammatory activity of the aqueous extract of *Chromolaenaodorata* was investigated in rats using the carrageenan-induced oedema, cotton pellet granuloma and formalin-induced oedema methods (Owoyeleet al., 2005). This study has established the anti-inflammatory activity of *C. odorata* and, thus, justifies the traditional uses of the plant in the treatment of wounds and inflammation. Enhanced proliferation of fibroblasts and endothelial cells treated with an extract of the leaves of *Chromolaenaodorata* (Eupolin) were found as an herbal remedy for treating wounds (Phanet al.,1998).

Eupolin ointment is a topical agent used in the treatment of soft-tissue wounds and burns in Vietnam and is made from an aqueous extract of the leaves of *Chromolaenaodorata*.

Anti-oxidant effects of the extracts from the leaves of *Chromolaenaodorata* on human dermal fibroblasts and epidermal keratinocytes against hydrogen peroxide and hypoxanthine–xanthine oxidase induced damage were studied (Thanget al., 2001). In cutaneous tissue repair, oxidants and antioxidants play very important roles. In local acute and chronic wounds, oxidants are known to have the ability to cause cell damage and may function as inhibitory factors to wound healing. The administration of anti-oxidants or free radical scavengers is reportedly helpful, notably in order to limit the delayed sequelae of thermal trauma and to enhance the healing process. Protection of cells against destruction by inflammatory mediators may be one of the ways in which the extracts from the plant, *C.odorata*, contribute to wound healing.The, anti-wound healing inflammatory and haemostatic effect of *Eupatorium odoratum* have been investigated (R. Suresh et al., 2012).

2. Materials and methods

2.1. Materials

The fresh and healthy leaves from the plant *Eupatorium odoratum*were collected from the open fields of Chengaloor, Thrissur district, Kerala. The plants were identified at the Department of Botany, St.Mary's college, Thrissur district, Kerala, India. The fresh plant parts were dried in hot air oven for 3 days at 40 -50ºC. The dried samples were then crushed into powder.

Bacterial strains*:* Bacterial cultures used in this study were obtained from the culture collections of Department of Microbiology, St Mary`s College Thrissur, Kerala, India. Bacterial cultures namely *Klebsiellapneumoniae, Escherichia coli, Pseudomonas aeruginosa*, *Bacillus cereus* and *Streptococcus* species were used.

Fungal cultures used include *Aspergillus*, *Alternaria*and *Fusarium.* Fungal cultures were also from the same department. The bacterial strains were maintained on Nutrient Agar plates or slants and were stored at 4 °C before use.

2.2. Preparation of Eupatorium odoratum plant extracts

The leaves were dried and powdered using a clean grinder. The powder was stored in air sealed containers at room temperature before extraction. A fixed weight of 10g powdered material was weighed out in aseptic condition and was extracted with distilled water using the Soxhlet Apparatus at a temperature of 100 °C. The Soxhlet extraction was carried out continuously for 8 hrs. After extraction the solvent was removed to yield a viscous dark green residue of the aqeuous extract. Extract was collected and filtered through Whatmann No.1 filter paper. The extract was concentrated and dried in dessicator. The dried powder sample was then stored in air tight bottles at 4 ºC.

2.2.1. Phytochemical screening

The crude aqueous extract was analyzed for the presence of various phytochemicals by the standard procedure of Harborne (1973).

1. *Detection of tannins*

Ferric chloride test (mace, 1963)

The extract (5 mg) was dissolved in 5 ml of distilled water and few drops of neutral 5% ferric chloride solution were added. The formation of blue green color indicated the presence of tannins.

2. *Detection of phenols*

Lead acetate test

The extract (5 mg) was dissolved in distilled water and 3ml of 10 % lead acetate solution was added. A bulky white precipitates indicated the presence of phenols.

3. *Detection of flavonoids*

An aqueous solution of the extract was treated with ammonium hydroxide solution. The yellow fluorescence indicated the presence of flavonoids.

4. *Detection of coumarins*

10% NaOH (1 ml) was added to 1 ml of the plant extract formation of yellow color indicated presence of coumarines.

5. *Detection of saponin*

Distilled water 2 ml was added of each plant extract and shaken in a graduated cylinder for 15 minutes lengthwise. Formation of 1 cm foam indicates the presence of saponin.

6. *Detection of Quinone*

Concentrated sulphuric acid (1 ml) was added to 1 ml of each of the plant extract. Formation of red color indicated the presence of Quinones.

7. *Detection of Cardiac glycosides*

Glacial acetic acid (and few drops of 5 % ferric chloride were added to 0.5% of the extract . This was under layered with 1 ml of concentrated sulphuric acid. Formation of brown ring at the interface indicated presence of cardiac glycosides.

8. *Detection of terpenoids*

Chloroform (2 ml) and concentrated sulphuric acid was added carefully to 0.5 ml of extract. Formation of red brown color at the interface indicated the presence of terpenoids .

2 ml)

9.*Detectionof steroids and phytosteroids*

To 0.5 ml of the plant extract equal volume of chloroform was added and subjected with few drops of concentrated sulphuric acid . Appearance of brown ring indicates the presence of steroids and appearance of bluish brown ring indicated the presence of phytosteroids.

2.2.2. Antibacterial activity

One percent of the cultures were inoculated into Nutrient broth. DMSO was used as the solvent to dissolve the plant extract. Two percent DMSO was used which was found to have no adverse effect on the bacteria and fungus. The antimicrobial activities were analyzed using well diffusion method.

Well diffusion method

Antibacterial test were carried out by the well diffusion method with some modification. The bacterial cultures were inoculated into 250ml conical flask containing 100ml of Nutrient broth. From this 0.1ml cultures was uniformly distributed on to Nutrient agar plates. The wells made on the surface of Nutrient agar plates at equal distance. Extracts of different concentration (45, 22.5, 11.25 and 6 mg/ml) and a control (2% DMSO) were poured in each well with a micropipette. The experiments were performed in duplicates. The plates were incubated at 37 °C for 24 hrs and observed for zone of inhibition of growth around the discs. The antibacterial activity of the extracts against each bacterial species was assayed by measuring the diameter of zone of inhibition to the nearest cm.

2.2.3. Antifungal activity

Well diffusion method

Antifungal test were carried out by the well diffusion method with some modification (Collins and Lyne, 1987).The fungal cultures were inoculated into 250ml conical flask containing 100ml of Sabouraud's dextrose broth. From this 0.1 ml culture was uniformly distributed on to Sabouraud's dextrose agar (SDA) plates. The wells were made on the surface of SDA plates at equal distance. Extracts of different concentrations (45, 22.5, 11.25 and 6 mg/ml) and a control (2% DMSO) were added in each well with a micropipette. Then the plates were incubated at room temperature for 3-5 days. After incubation, the zone diameters were measured.

3. Results and Discussion

Eupatorium odoratum is a plant gaining great medicinal interest and has been extensively used in traditional medicine. It has antimicrobial activity; potentially against various bacterial, viral and fungal pathogens. In this study the phytochemicals in *Eupatorium odoratum*was investigated. Phytochemical analysis of the aqueous extract of *Eupatorium odoratum* was carried out using the standard procedure. The tests are based upon the qualitative approach. Phytochemical screening revealed the presence of tannin, flavonoids, quinone, terpenoid, steroid &phytosteroids, phenolics, saponins, cardiac glycosides and coumarins in the aqueous extract (Table 1). The aqueous extract was rich with secondary plant metabolites like quinones and coumarins. Another comparative analysis by the authors with methanolic and chloroform extracts revealed that quinone content is very high in all the extracts of *E. odoratum*. The saponins are present only in the aqueous extract and not in methanolic or chloroform extracts.

Table-1. Phytochemical analysis of *Eupatorium odoratum*aqueous extract

Phytochemicals	**Aqueous extract**
Tannin	+
Saponin	+
Flavonoid	+
Terpenoid	+
Steroid & phytosteroids	+
Phenol	+
Coumarins	++
Quinone	++
Cardiac glycosides	+

3.1 Antibacterial study

The in vitro antibacterial and antifungal activity of extracts of leaves of Eupatorium was assayed. Pathogenic bacterial strains belonging to five different species of bacteria namely *Bacillus, Escherichia coli*, *Streptococcus,Pseudomonas* and *Klebsiella* were used as the test organisms. The plant has potent antibacterial activity against *E. coli*, *Pseudomonas*, *Bacillus*, *Streptococcus* and *Klebsiella* respectively (Fig 1).Maximal antibacterial activity was obtained for *E.coli* with zone of growth inhibition of diameter 1cm.

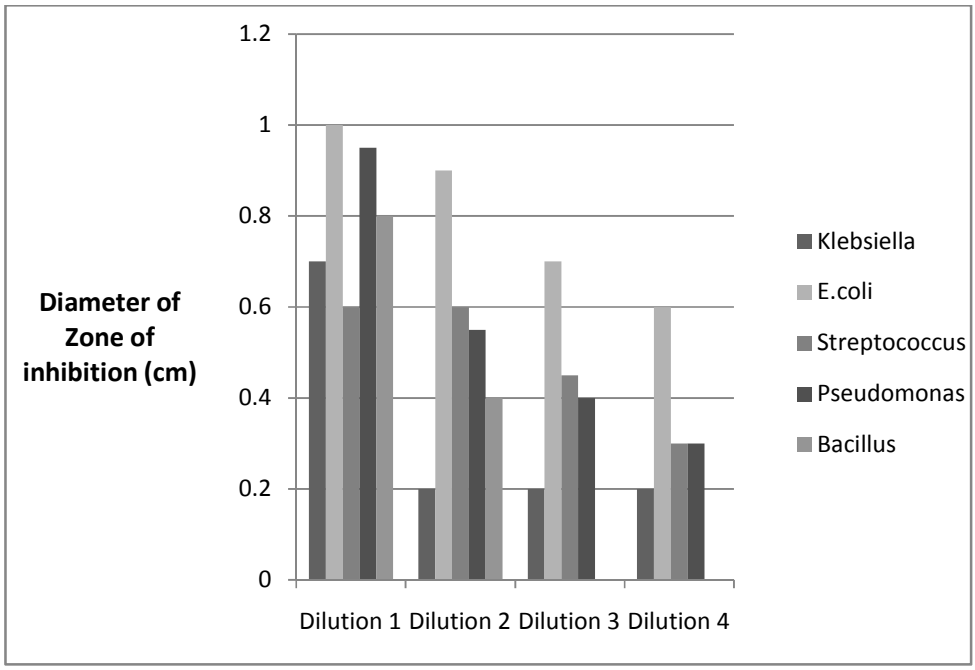

Figure-1. A plot determining the antibacterial activity of *E.odoratum*extracts. Zone of inhibition were plotted against different concentrations of the drug/ extract. Maximun inhibition was given for *E.coli.*

3.2 Antifungal activity

Antifungal testing of aqueous extract of *Eupatorium odoratum* against fungal species *Aspergillus*, *Alternaria* and *Fusarium* were performed by well diffusion method. Leaf extracts were ineffective against all the fungal species investigated

producing no zone of growth inhibition around the extract. This indicates that *Eupatorium* extracts are not showing antifungal activity but it contains antibacterial compounds.

Sasidharan et al., (1988) demonstrated that alcoholic extracts of plants were consistently found to be more inhibitory than aqueous extracts of the same concentration. The alcoholic extract of *E. odoratum* has the greatest activity against *S.aureus*and *E.coli.* These reports are in accordance to our data presented in table 1. A parallel study by the authors also demonstrates that methanolic extracts are rich in phytochemicals than aqueous extracts and also shows better inhibitory activity against the above reported bacteria strains.

Sukanya et al., (2011) reported that the leaves of *C. odorata* were extracted using different solvents such as methanol, ethanol, ethyl acetate and chloroform. Among treatments, maximum in vitro inhibition was scored in methanol extracts of *C. odorata* which offered inhibition zone of 10, 9, 12 and 12 mm against *E. coli, S. aureus, X. vesicatoria*and *R. solanaccearum*respectively. They reported that the minimum inhibitory concentration (MIC) value for the clinicalbacteria ranged between 0.35 to 4.0 mg/ml and 0.25 to 4.0 mg/ml for phytopathogenic bacteria when tested with all four solvents extracts of*C. odorata.* The antibacterial activity reported by us commensurate the reported results and shows good inhibition towards*E.coli* and has negligible reactivity against fungi tested.

4. Conclusion

Eupatorium has been extensively used in traditional medicinal preparations and also used as herbal tea where water decoctions, extracts or processed concentrates were employed. The present study has undoubtedly proved the potential of the water soluble bioactive compounds in *E. odoratum* leaf in their bactericidal action against pathogenic bacteria. The bactericidal activity of *E. odoratum*could be attributed to the bioactive compounds present in *E. odoratum* namelytannins, flavonoids,

quinone, terpenoid, steroid &phytosteroids, phenolics, saponins, cardiac glycosides and coumarinswhich could be effectively employed as effective chemotherapeutic agents in antibacterial treatment and therapy.

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Phytochemical and anitimicrobial investigation of *Symplocos cochinchinensis* and standardization of an ayurvedic formulation Nishakathakadi kashaya

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Abstract

The aim of the present study is to evaluate an Ayurvedic formulation and one of its ingredients by carrying out phytochemical and antimicrobial studies as per the standard procedures. The formulation selected for the study was Nishakathakadi kashaya and plant under study was bark of *Symplocos cochinchinensis* . The study includes phytochemical along with the anti-microbial activity. Methanol and water extract of both kashaya and plant part was subjected to phytochemical and antimicrobial screening. Phytochemical evaluation gave the evidence regarding the presence of some phytochemicals like alkaloids, flavonoids, glycosides, steroids, tannins and terpenoids in the kashaya and plant part. Antimicrobial activity screening was performed using *Staphylococcus aureus* and *Escherichia coli* by agar well diffusion method and the final results showed activities against the selected microorganisms. Maximum activity was shown by methanol extract of kashaya against *E. coli* followed by methanol extract of *Symplocos cochinchinensis* and water extract of *Symplocos cochinchinensis.*

***Key words**: Nishakathakadi kashaya, Symplocos cochinchinensis, Phytochemical screening, Antimicrobial sceening, Staphylococcus aureus, Escherichia coli*

1.Introduction

The relationship between man and plants has been very close throughout the development of human culture (Modi, 1984). Ancient man was totally dependent on green plants for his day to day needs of medicaments (Dandiya

and Vohra, 1989). Plants were used for healing and health from time in memorial (Parikh, 1991). Plants have been excellent sources of an amazing diversity of phytochemicals (secondary metabolites) which are of medicinal value. Phytochemical technique mainly applies to the quality control of herbal medicine of various chemical components, such as saponins, alkaloids, volatile oils, flavonoids and anthraquinone. The ancient Ayurvedic system of medicine utilizes many drugs of natural origin, preparations and therapeutic uses of which are described in various standard books on Ayurveda. Pure drug compounds from natural sources was an important step in growth of pharmacology and therapeutics (George, 2000). The ancient Ayurvedic system of medicine utilizes many drugs of natural origin, preparations and therapeutic uses of which are described in various standard books on Ayurveda. The objective of Ayurveda is to accomplish the physical, mental and spiritual well-being by the way of adopting preventive and promotive approach as well as treat the diseases with holistic approach. Ayurveda is effective not only in common ailment but also in many incurable, chronic and degenerative diseases. The effect of Ayurveda can only be achieved through proper medicinal plants and quality of medicinal plants. It is very close to nature and human physiology is integrated with the cosmic physiological changes thus it is suggested to use Ayurvedic drugs in its natural form.

As the drugs used in Ayurvedic system are being accepted more and more all over the world as effective medicines, they and their preparations need proper standardization. There is a variety of Ayurvedic formulations: Arishta/ Asava (Fermented formulation), Bhasma (Calcinated drug), Churna (Powdered herb), Ghrita (Ghee based), Kashaya (Decoction), Leha (Electuary), Rasakriya (Collerium), Taila (Oil based) (The Ayurvedic Pharmacopoeia of India, 2007)

Kvatha or kashaya is the commonest type of ayurvedic medicines. Kashaya are the herbal combination decoction derived after several hours of boiling at specific heat. Medicinal herbs are added at right intervals to retain optimum quality of the medicine (Mukerjee *et al*, 1995). Most kashaya are internally administered while a few of them are used for enigma (Trease and Evans, 2002).

The concept of standardization and quality control of drugs can be found even in the ancient Ayurvedic texts. In those days, the physician himself would check the raw drugs by their typical taste, color, smell, shape and texture and prepare the medicines. But in the modern time these tests are not sufficient to give scientific explanation and quality controls. In recent years the plant materials and herbal remedies derived from them represents a sustained proportion of global market and has gained place in the health management. This is because of its holistic approach, cost effectiveness and lesser side effects. The WHO also has been encouraging and promoting the traditional herbal medicines in health care programs. Hence the standardization of the raw drugs, processing, finished products, verification of the claims, mechanism of action and purity from metallic and microbial contamination are some of the major issue which have to be taken into consideration for increasing the worldwide acceptability of herbal products and also to achieve clinical success and maximum therapeutic effect.

The present study is aimed to standardize an ayurvedic formulation Nishakathakadi kashaya and to investigate the phytochemical and antibacterial activity of one of its component *Symplocos cochinchinensis*

2. Materials and methods

2.1. Collection

One of the Ayurvedic Formulation namely "Nishakadakadi kashaya" and one of its major ingredient *Symplocos cochinchinensis* collected from Amala Ayurvedic Hospital & Research centre, Thrissur District, Kerala were taken for the study. In the case of Symplocos *cochinchinensis* bark was collected and the collected bark was shade dried. The dried bark were then coarsely powdered and stored in airtight bottles for further use.

2.2. Physicochemical evaluation

2.2.1. Organoleptic characters of kashaya like color, odour, taste, pH

2.2.2. Determination of ash content

Total ash: Total ash content of each sample was determined using an apparatus called Bunsen, electric burner. Incinerated 3g accurately weighed powdered plant material in a silica crucible at a temperature not exceeding 450ºC until free from carbon, cooled and weighed. Calculated the percentage of total ash with reference to the air dried plant material.

Acid insoluble ash: Boil the ash obtained in above method for 25minutes with 25mL dilute hydrochloric acid, collect the insoluble matter in a silica crucible, or an ashless filter paper, wash with hot water and ignite to constant weight of acid insoluble ash with reference to the air dried plant material.

Water soluble ash: Boil the ash for 5minutes with 25mL of water, collect the insoluble matter in a silica crucible or an ashless filter paper, wash with hot water and ignite for 15minutes at a temperature not exceeding 450ºC until free from carbon, cooled and weighed. Calculate the percentage of water soluble ash with reference to the air dried plant material.

2.3. Extraction

Both Nishakadakadi kashaya and coarsely powdered bark of *Symplocos cochinchinensis* were subjected to solvent extraction. Coarsely powdered bark of *Symplocos cochinchinensis* were subjected to solvent extraction using soxhlet apparatus. Each time about 3g of dried powder is subjected to solvent extraction with water and methanol. These extracts were subjected to phytochemical and antimicrobial screening for further evaluation.

2.4. Preliminary phytochemical screening of the extracts

The extracts of the Nishakathakadi kashaya and plant *Symplocos cochinchinensis* were subjected to preliminary phytochemical analysis for the detection of major chemical groups.

2.4.1. Test for carbohydrates

Molisch Test: Dissolved a small amount of alcoholic extracts separately in 4mL distilled water and filtered. The filtrate was subjected to Molisch test. Formation of reddish brown ring indicates the presence of carbohydrates.

Fehling's Test: Dissolved a small quantity of extract in water and treated with Fehling's solution. Brown color indicate the presence of carbohydrates.

2.4.2. Test for alkaloids

Dragendorff's Test: To 2mL of extract added a few drops of dragendorff's reagent and mixed well. Reddish brown precipitate indicates the presence of alkaloids.

2.4.3. Test for flavonoids

Shinoda Test: To 2-3mL of extract, a piece of magnesium ribbon and 1mL of concentrated hydrochloric acid were added. Pink or red coloration of the solution indicate the presence of flavonoids.

2.4.4. Test for steroids

Salkowsky Test: To 2mL of the extract was added 2mL of chloroform.

Concetrated sulphuric acid (3 mL) was carefully added to form a layer. A reddish brown coloration of the interface indicates the presence of steroids.

2.4.5. Test for phenols

To 5mL of the extract added 5 drops of 10 % ferric chloride solution. Development of dark brown color indicates the presence of phenols.

2.4.6. Test for tannins

Braemer's Test: 2mL of the extract is mixed with 3mL of 1% ferric chloride solution, blue color develops if hydrolysable tannins are present, green colour develops if condensed tannins are present.

Lead acetate Test: To 5mL of the extract, added 1mL of lead acetate solution. Flocculent white precipitate indicated the presence of tannins.

2.4.7. Test for glycosides

Benedict's Test: To about 1mL of the extract 5mL of Benedict's reagent was added. The mixture was boiled for 2minutes and cooled. Development of brick red color indicates the presence of glycosides.

2.4.8. Test for terpenoids

Lieberman–Burchard Test: A few drops of acetic acid and 1mL concentrated sulphuric acid was added to 3mL of the extract prepared in chloroform. If a deep red color at the junction of the two layers indicate the presence of terpenoids.

2.4.9. Test for saponins

To 2mL of extract was added 5mL of distilled water in the test tube. The solution was shaken vigorously and observed for a stable persistent froth. If there is formation of froth indicates the presence of saponins.

2.4.10. Test for anthraquinones

1mL of the extract was boiled with 10mL of sulphuric acid and filtered while hot. The filtrate was shaken with 5mL of chloroform. The chloroform layer was pipetted into another test tube and 1mL of dilute ammonia was added.

Pink or deep red coloration of aqueous layer indicated the presence of anthraquinones.

2.4.11. Test for cardiac glycosides

To 1mL of extract 1mL of acetic acid and a few drops of ferric chloride was added and mix it well. 1mL of concentrated sulphuric acid was added slowly. Blue color develops when cardiac glycoside is present.

2.5. Bacterial cultures

For the antimicrobial assay, two bacterial strains namely Escherichia coli and Staphylococcus aureus were used. Microorganisms were obtained from Microbiology Department, Amala Ayurvedic Hospital & Research Centre, Thrissur. The bacterial cultures undergone sub culturing on nutrient agar slants and were maintained at 4°C.

2.6. Determination of antimicrobial activity

Antisensitivity tests were performed by agar-well diffusion method. Two wells respectively, were bored in each plate 5mm diameter with an aseptic cork borer after the plates had solidified. The test bacterial strains obtained from overnight broth culture of each bacterium were seeded on the sterile solidified agar medium by swab plate technique using sterile cotton swabs. Different extracts were prepared and reconstituted in specific solvent systems used and 200µL extracts were dispensed into each of the wells with the aid of a Pasteur pipette. After holding the plates at room temperature for about 2hours to allow diffusion of the extracts into the agar, they are incubated for 24 hours at 37°C. The tests were performed in duplicate for each microorganism. Both positive and negative controls were used in the assay.

3. Results and Discussion

3.1. Physico-chemical evaluation

Physico-chemical evaluation of Nishakathakadi kashaya was conducted by finding colour, odour, taste and pH.

Table-1. Organoleptic characters of Kashaya

Sl. No	**Organoleptic characters**	**Result**
1.	Colour	Brown
2.	Taste	Bitter
3.	Odour	Aromatic
4.	pH	5.0

Table-2. Ash content of plant material

Sl. No	**Ash test**	**Percentage**
1.	Total ash	6.4%
2.	Water soluble ash	0.466%
3.	Acid insoluble ash	2.7%

Total ash indicates the amount of impurity in the sample. The total ash content of the plant material is 6.4%. Water soluble ash shows the presence of inorganic matter. It was found to be 0.466%. Acid insoluble ash shows the presence of silicates. The percentage of acid insoluble ash was 2.7%.

Table-3. Phytochemical analysis

Sl. No	**Test**	**Nishakathakadi kashaya**		***Symplocos cochinchinensis***	
		Methanol extract	**Water extract**	**Methanol extract**	**Water extract**
1.	Test for Carbohydrate Molisch's Test Fehling's Test	 +++ + + +	 +++ + + +	 +++ + + +	 +++ + + +
2.	Test for Alkaloids Dragendorff's Test	 + +	 -	 -	 -
3.	Test for Flavonoids Shinoda Test Lead acetate Test	 - + + +	 - ++	 + -	 - +++
4.	Test for Steroids Salkowsky Test	 + +	 + + +	 + + +	 -
5.	Test for phenol	-	-	+ +	-
6.	Test for Tannins Braemer's Test	 + +	 +	 +	 +
7.	Test for Glycosides Benedict's test	 -	 + +	 + + +	 + +
8.	Test for Terpenoids Lieberman-Burchard test	 + +	 + + +	 + + +	 + + +
9.	Test for Saponins	-	+ +	-	+ +
10.	Test for Anthraquinones	-	-	-	-

Both kashaya and plant extract indicated the presence of carbohydrates. Only methanol extract of kashaya indicated the presence of alkaloids. Flavonoids and steroids were present in both plant and kashaya extract. Test for terpenoid was conducted using Lieberman-Burchard test. It indicated the presence of terpenoids in all extracts. Glycosides were present in both plant and kashaya. Test for saponins, diterpenes and anthraquinones were carried out and only water extracts of plant and kashaya answered the test for saponins. Only methanol extract of kashaya indicated the presence of hydrolysable tannins and all other extracts indicate the presence of pseudo-tannins. Thus in the qualitative phytochemical analysis, alkaloids, flavonoids, glycosides, steroids, tannins and terpenoids were seen to be present in kashaya extract and flavonoids, tannins, glycosides, steroids, saponins, terpenoids, and phenols were seen to be present in plant extract.

Table-4. Antibacterial screening

Sl. No.	**Test organisms**	**Zone of inhibition**			
		Kashaya Water extract (mm)	**Kashaya methanol extract (mm)**	***Symplocos cochinchinensis* water extract (mm)**	***Symplocos cochinchinensis* methanol extract (mm)**
1.	***Staphylococcus aureus***	8	13	20	22.5
2.	***Escherichia coli***	7.25	32.5	16	15

Water and methanol extract of plant *(Symplocos cochinchinensis)* and Nishakathakadi kashaya were tested for antimicrobial activity against *Staphylococcus aureus* and *Escherichia coli.* Maximum activity was shown by methanol extract of kashaya against *E. coli* (32.5mm-zone diameter) followed by methanol extract of *Symplocos cochinchinensis* (22.5mm-zone diameter) and water extract of *Symplocos cochinchinensis* (20mm-zone diameter).

4. Conclusions

The consumption of botanical drugs in India is increasing year after year. Therefore the phytochemical studies play a crucial role. In the present study an attempt has been made to conduct the scientific evaluation of an Ayurvedic formulation and one of its ingredients by carrying out phytochemical and antimicrobial studies as per the standard procedures. The formulation selected for the study was Nishakathakadi kashaya and plant under study was *Symplocos cochinchinensis* . The study includes phytochemical along with the anti-microbial activity. Phytochemical evaluation gave the evidence regarding the presence of some phytochemicals like alkaloids, flavonoids, glycosides, steroids, tannins and terpenoids in the kashaya. To find out the efficacy of the plant extract as well as the formulation antimicrobial activity screening was performed using *Staphylococcus aureus* and *Escherichia coli* by agar well diffusion method and the final results showed activities against the selected microorganisms. The results of present investigation clearly indicate the various components and activities shown by the *Symplocos cochinchinensis* and Nishakadadi Kashayam. Thus, the study ascertains the value of plants used in Ayurveda, which could be of considerable interest to the development of new drugs. Further studies are needed for the isolation of chemical constituents and subject them for the evaluation including bioassay & Microbiology assays.

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Exogenous GA_3 Induced Seed Germination and A-Amylase Activity In Okra Under Salinity Stress

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Abstract

Effect of GA_3 in inducing seed germination and associated α-amylase activity under salinity stress has been studied in Okra (*Abelmoschus esculentus*). Seeds were exposed to different salinity levels like 25mM, 50mM, 75mM and 100mM NaCl and each level of salinity with different combinations of GA_3 such as 0.01mM, 0.1mM and 0.3mM. Control seeds were raised in distilled water. A reduction in the percentage of seed germination was observed in the salinity levels 50mM and above, while an increase in the germination percentage was observed at low salinity levels of 25mM. All combinations of GA_3 imparted an inducing effect on seed germination up to the salinity level 75mM. However GA_3 at any concentration could not alleviate the inhibitory effect induced by NaCl at 100mM, but decreased the germination percentage further. Study also revealed the controlling influence of α-amylase enzyme on seed germination under salinity stress.

Key words: α-amylase, GA_3, germination percentage, okra, salinity

1. Introduction

Salinity is the major common abiotic stress for plant in arid or semi arid regions (Greenway *et al*., 1980). The United Nations Environment Programme estimates that approximately 20% of the agricultural land and 50% of the crop land in the world is salt stressed and that about 6% of the total global land area is affected by salinity (Flowers and Yeo, 1995). Based on their ability to grow on salt medium plants including crop species are traditionally classified as glycophytes, showing the effects of salt at concentrations less than 50mM or halophytes which can complete their life cycles at 50mM (Flowers, 1985; Mass, 1986). Studies conducted in

different plants have shown that salinity progressively decreased the percentage of germination in glycophytes (Akinci et al., 2004; Jamil et al., 2006; Basalah, 2010). α-amylase (E.C.3.2.1) is the enzyme which is active during the seed germination, helping mobilization of reserve food in the endosperm. Under salinity stress, decreased α-amylase activity has been reported in *Amaranthus candatus* (Bozena and Jan, 2009). Gibberellins (GA_3) constitute a group of tetracyclic diterpenes best known for their influence on seed germination.

Okra (*Abelmoschus esculentus* L. Moench) is a glycophytic crop plant belonging to the family Malvaceae. Water salinity has a retarding effect on the growth, yield and physiological growth parameters of Okra (Abid et al., 2002). In the present study, an attempt has been made to investigate the effect of GA_3 on the seed germination of *Abelmoschus esculentus* and α-amylase activity under salinity stress in laboratory conditions.

2. Materials and Methods

Good quality seeds of Okra (*Abelmoschus esculentus* var.Anamika) were procured from Department of Olericulture, Kerala Agricultural University, Vellanikkara, Thrissur, Kerala. 20 seeds each were soaked in different concentrations of 50ml NaCl and different combinations of NaCl and GA_3 as follows:

Control- Distilled water

25mM NaCl

25mM NaCl (25ml)+ 0.01mM GA_3(25ml)

25mM NaCl (25ml)+ 0.1mM GA_3 (25ml)

25mM NaCl (25ml)+ 0.3mM GA_3 (25ml)

50mM NACl

50mM NaCl (25ml)+ 0.01mM GA_3(25ml)

50mM NaCl (25ml)+0.1mM GA_3 (25ml)

50mM NaCl (25ml)+ 0.3mM GA_3 (25ml)

75mM NaCl

75M NaCl (25ml) + 0.01mM GA_3 (25ml)

75mM NaCl (25ml)+ 0.1mMGA_3 (25ml)

75mM NaCl (25ml)+ 0.3mM GA_3 (25ml)

100mM NaCl

100mM NaCl (25ml)+ 0.01mM GA_3 (25ml)

100mM NaCl (25ml)+ 0.1mM GA_3 (25ml)

100mM NaCl (25ml)+ 0.3mM GA_3 (25ml)

After 24 hrs. seeds were incubated in 5cm petridishes lined with filter paper moistened with 10 ml distilled water or the same volume of different test solutions and kept in darkness at room temperature. Experiment was conducted in triplicate.

Parameters studied were:

Germination percentage

α-amylase activity

2.1. Germination Percentage

Germination percentage was calculated on 2^{nd} day of germination. A seed was regarded as germinated when its radical reaches approximately 1cm in length. The following formula was used to calculate the germination percentage:

Germination Percentage= No. of seeds germinated/ Total no. of seeds x 100

2.2. Extraction of α-amylase enzyme

From each treatment germinated seeds were collected and seed coats were removed. The seedlings were rinsed with cold distilled water and were kept in the cold. 1gm of germinated seeds from each treatment was extracted with ice cold citrate buffer. The extract was filtered and the filtrate was subjected to centrifugation in the cold at

4°C in a cooling centrifuge (Remi) for ten minutes and at 10,000g and the supernatant was collected. This supernatant was served as the source of the amylase enzyme.

2.3. Assay of α-amylase activity

Alpha amylase activity was assayed by the modified method of Kang et al. (1997). On incubating a mixture of buffered enzyme solution with starch, the substrate is enzymatically reduced to maltose. This reducing sugar liberated was estimated using dinitrosalicylic acid reagent. The absorbance of the coloured solution was read at 560 nm using a spectrophotometer (UV-VIS digital spectrophotometer Labtronics model LT-29).

2.4. Statistical analysis

Results were computed statistically (MSTATC) using one-way analysis of variance (ANOVA).

3. Results and Discussion

In the present investigation, a reduction in the percentage of seed germination was observed in the salinity levels 50mM and above, while an increase in the germination percentage was observed at low salinity levels of 25mM (95%) than in the control (90%). The decreased seed germination in higher levels of salinity was reported by many workers in different crop plants (Basalah, 2010; Bozena and Jan, 2009; Jamil *et al.*, 2006; Basalah and Mohammed, 1999). The decrease in the germination percentage in higher salinity levels may be due to the combined effect of osmotic pressure and toxicity of salts or due to the effect of added Cl- ions (Gill et al., 2002). Increased germination rate at lower levels of salinity is in accordance with the findings of Ahmed *et al*. (2012**)** who observed increased germination

percentage and early seedling growth in three different cultivars of Sorghum at low levels of salinity.

Table 1. Percentage of germination and α-amylase activity of Okra in different concentrations of NaCl and GA_3

Treatment	**Germination**	****α-amylase activity(g-**
Control	90 ± 0.06	1.09
25mM NaCl	95± 0.04	1.34
25mM NaCl	90± 0.06	1.41
25mM NaCl +0.1mMGA3	100± 0.08	1.37
25mM NaCl +0.3mMGA3	90± 0.08	1.05
50mM NaCl	80± 0.02	1.11
50mM NaCl	85± 0.04	0.97
50mM NaCl +0.1mMGA3	90± 0.08	0.99
50mM NaCl +0.3mMGA3	95± 0.06	1.23
75mM NaCl	70± 0.02	0.74
75mM NaCl	85± 0.6	0.87
75mM NaCl +0.1mMGA3	85± 0.06	0.89
75mM NaCl +0.3mMGA3	90± 0.2	0.91
100mM NaCl	45± 0.08	0.58
100mM	35± 0.2	0.50
100mM	40± 0.4	0.48
100mM	40± 0.06	0.57

**values significant at 1% level

Application of GA_3 treatment overcame the effect of salt stress and improved percentage of germination in Okra. Maximum percentage of germination (100%) was noticed in the combined treatment of 25mM NaCl and 0.1mM GA_3. GA_3 in all combinations imparted an inducing effect on seed germination at higher levels of salinity. However GA_3 at any concentration could not alleviate the inhibitory effect induced by NaCl at 100mM, but decreased the germination percentage further. Results indicate that GA_3 can induce seed germination in Okra under salinity stress while a reverse impact may be resulted if the concentration of salinity is much higher. Stimulatory effect of GA_3 on seed germination of various plants under

salinity stress was reported by many workers (Rajan *et al*., 2000; Zhenguo and Jundi, 2001; Mohammed, 2007).

Significant decrease in the α-amylase activity was noticed in Okra seeds with increased levels of salinity. This result agrees with that of Abdel and Mohammed, (2007) and Siddiqui *et al*., (2006) who reported a decrease in the starch degradation rate during germination under saline stress. The stimulatory effect of exogenous GA_3 on α-amylase activity up to 24 hours incubation under salinity stress suggests that its mode of action in regulating Okra seeds germination involves the control of the activity of key enzyme responsible for seed germination. The increase of α-amylase activity due to GA_3 may help the seeds to overcome the inhibition exerted by salinity stress. These results agree with those of Bozena and Jan (2009) who reported that GA_3 increased α-amylase activity under salt stress during the first 14 hours of incubation. According to Lin and Kao (1995), the mechanism by which NaCl induced inhibition of α-amylase activities is related to deficiency of GA_3 in NaCl stressed endosperm. However, the reverse effect of GA_3 on seed germination and α-amylase activity of Okra at much higer levels of salinity as revealed in the current study is contradictory to the above explanation and needs further investigation.

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Biosynthesis of silver nanoparticles and its antibacterial activity

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Abstract

Nanotechnology is emerging as a rapidly growing field with its application in Science and technology for the purpose of manufacturing new materials at the nanoscale level. It is the most promising field for generating new applications in medicine. In recent years, plant mediated biological synthesis of nanoparticles is gaining importance due to its simplicity and ecofriendliness. The development of bio processes for the synthesis of silver nanoparticles is evolving in to an important branch of Nanotechnology. It is well known that inorganic nanomaterials are good antimicrobial agents . These biologically synthesized Silver nanoparticles were found to be highly toxic against different multidrug resistant human pathogens

Key words: Nanotechnology, Silver nanoparticles, Nanomaterial, Antimicrobial agent

1. Introduction

Nanotechnology is one of the most active areas of research in modern material sciences. It is a field that is growing very fast , making an impact in all spheres of human life (Singh *et al*., 2010). "Nanoparticles, generally considered as particles with a size of upto 100nm. Nanoparticles play an indispensable role in drug delivery, diagnostics, imaging, sensing, gene delivery, artificial implants and tissue engineering (Sinha *et al*., 2009).

Silver nanoparticles are one of the most commonly used nanomaterials in consumer products due to their desirable optical, conductive, catalytic and antimicrobial properties. Silver nitrate is mainly used for the silver nanoparticle synthesis due to the reason that it is cost effective and dissolves

in many solvents compared to other silver salts. In form of nanoparticle, silver is having increased catalytic properties and more reactive. It is well known that inorganic nanomaterials are good antimicrobial agents (Parashar *et al*., 2009).

Chemical and physical methods are quite expensive and potentially dangerous to the environment. Biosynthetic methods employing either biological microorganism, enzymes, plant extracts have emerged as a simple and viable alternative to chemical and physical methods (Prabhu *et al*., 2010). Thus there is a need for 'green chemistry' that includes a clean, non toxic and eco-friendly method of nanoparticles synthesis for other methods those generate toxic by products along with the nanoparticles synthesis.

Several plants have been successfully used for efficient and rapid extracellular synthesis of silver nanoparticles. Those nanoparticles have been used in various applications (Dubey *et al*., 2009). Medicinal plants exhibit antimicrobial activity since they contain innumerable biological, active chemical constituents. Contrary to the synthetic drugs, antimicrobials of plant origin are not associated with many side effects and have an enormous therapeutic potential to heal many infectious diseases.

2. Antimicrobial activity of number of plant nanoparticles

The herbal leaves like *Ocimum sanctum* and *Vitex negundo* were analyzed for the productivity of nanoparticles. The synthesis of silver phyto nanoparticles were prepared by adding silver nitrate solution (10^{-3} M) and the silver phyto nanoparticles were isolated from these herbal leaves. The silver phyto nanoparticles were collected from each herbal plant and tested their antibacterial l activity. The test cultures in this study were *Staphylococcus*

aureus, Vibrio cholerae, Proteus vulgaris and *Pseudomonas aeruginosa.* The antibacterial activities of nanoparticles obtained from *Ocimum sanctum* showed maximum inhibitory rate compared with *Vitex negundo* (Prabhu *et al.,* 2010*).*

Plant extracts are very cost effective and eco-friendly and thus can be an economic and efficient alternative for the large- scale synthesis of nanoparticles had found. The methanolic extract of *Eucalyptus hybrid* leaf was used in the extracellular biosynthesis of silver nanoparticles. Bioactive silver nanoparticle synthesized by reacting with the methanolic biomass of *Eucalyptus hybrid* leaf with aqueous solutions of silver nitrate ($AgNO_3$) at ambient temperature. Formations of silver nanoparticles are confirmed by UV - visible spectroscopy, X-ray diffraction patterns and Scanning Electron Microscopy (SEM) with Energy Dispersive X-ray (EDX) patterns.

Biological methods are a good competent for the chemical procedures, which are environment friendly and convenient . With the development of new chemical or physical methods, the concerns for environmental contaminations are also heightened. Green chemical procedures involved a chemistry of a clean, non toxic and environment friendly method of nanoparticle synthesis. Many biological organisms, both unicellular and multicellular are known to produce inorganic materials either intra or extracellularly often of nanoscale dimensions and of morphology (Dubey *et al.,* 2009*).*

A cost effective and environment friendly technique for green synthesis of silver nanoparticles from 1mM $AgNO_3$ solution through the extract of papaya fruit as reducing as well as capping agent had reported. Nanoparticles were characterized using UV-visible absorption spectroscopy, FTIR, XRD and SEM. XRD and SEM analysis showed the average particle size of 15nm as well as

revealed their cubic structure. These biologically synthesized nanoparticles were found to be highly toxic against different multi drug resistant human pathogens (Jain *et al.,* 2009*).*

As it is a cost effective and environment friendly and also in this method there is no need to use high pressure, temperature, energy and toxic chemicals. It is well known that inorganic materials are good antimicrobial agents. Silver nanoparticles take advantages of the oligodynamic effect that silver has on microbes, where the silver ions bind to reactive groups in the bacterial cells, resulting in precipitation and inactivation. Microbes generally have a harder time developing resistance to silver than they do to antibiotics (Parashar *et al.,* 2009).

The silver nanoparticles can be synthesized from *Gliricidia sepium.* When the leaf broth of *Gliricidia sepium* and aqueous silver nitrate solution of 1mM were mixed, the colour changed from yellowish green to brown, the final colour appeared gradually with time. The entire reaction mixture turned to brown colour after 12 hours of reaction. Transmission Electron Microscopy (TEM) analysis showed silver nanoparticles are polydispersed and ranged in size from 10-50 nm with an average size of 27nm, the particles were predominantly spherical. Phyto synthesized silver nanoparticles showed the antibacterial activity against the *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumonia (*Rajesh *et al., 2009).*

Ag nanoparticles synthesis by irradiation of Ag^{+} ions does not involve a reducing agent and is an appealing procedure. Eco-friendly bio-organisms in plant extracts contain proteins, which act as both reducing and capping agents forming stable shape controlled Ag nanoparticles. Both modified by surfactants

or polymers showed high antimicrobial activity against Gram positive and Gram negative bacteria. The mechanism of the Ag nanoparticles bactericidal activity is discussed in terms of Ag nanoparticle interaction with the cell membrane of bacteria. Silver containing filters are shown to have antibacterial properties in water and air purification (Sharma *et al*., 2008).

3. Conclusion

Applications of such eco-friendly silver nanoparticles in bactericidal , other medical and electronic applications, makes this method potentially exciting for the large scale synthesis of other inorganic nanomaterials. The reduction of silver ions and stabilization of silver nanoparticles was thought to occur through the participation of leaf proteins and secondary metabolites.

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Diaz E, Prieto MA (2000). Bacterial promoters triggering biodegradation of aromatic pollutants. *Curr. Opin. Biotech.* 11: 467-475.

Reference to a book:

Pitter P, Chudoba J (1990). Biodegradability of Organic Substances in the Aquatic Environment. CRC press, Boca Raton, Florida, USA.

Reference to a chapter in a book:

Mandell GL, Petri WA, 1996. Antimicrobial Agents: Penicillins, Cephalosporins, and other ß-Lactam Antibiotics, In: Goodman and Gilman`s. The Pharmacological Basis of Therapeutics. 9th, Ed. J.G. Hardman and L.E. Limbird, McGraw-Hill: NY. Vol. 23; PP. 1073–1101.

Mini reviews

The format requirements for original research papers apply to reviews too.

Submission

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