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Synthesis and Characterization of Aminophenol Based Schiff Bases and Their Copper Complexes

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Abstract

Synthesis and characterization of two Schiff bases; salicylidine -2-aminophenol and salicylidine -2-amino-5-methylphenoland their copper(II) complexes were prepared by condensation between aldehyde and primary amine. Ligands are characterized by elemental analysis, infrared and UV-Vis spectral studies and complexes are characterized by elemental analysis, infrared, magnetic susceptibility and UV-Vis spectral studies. Based on the physicochemical and spectral data an octahedral geometry is proposed for both complexes

Keywords*: Schiff base, salicylidine -2-aminophenol, salicylidine -2-amino-5-methylphenol*

1. Introduction

Schiff base ligands are able to coordinate metals through imine nitrogen and another group, usually linked to the aldehyde. Modern chemists still prepare Schiff bases, and nowadays active and well-designed Schiff base ligands are considered ‘‘privileged ligands’’. In fact, Schiff bases are able to stabilize many different metals in various oxidation states, controlling the performance of metals in a large variety of useful catalytic transformations. In this article we show that Schiff bases are also able to transmit chiral information to produce nonracemic products through a catalytic process; chiral aldehydes or chiral amines can be used. These ligands are of interest because of their ability to form transition metal complexes which have varying configurations, structural lability and sensitivity to molecular environments (Cozzi, 2004). They have proven to be effective in constructing supramolecular architectures

such as coordination polymers and helical assemblies.

Many Schiff base complexes show excellent catalytic activity at reasonable temperatures *i.e.* below 100 ºC. Similarly, the activities of the complexes are varied by the change in the nature of ligand. Schiff base complexes are found to catalyse a large number of organic transformations such as polymerization reaction, epoxidation, ring opening of epoxides, reductions, oxidations, alkylation, Michael addition, heck reaction, annulation, carbonylation, benzoylation, cyclopropanation, diels alder reaction, aldol condensation etc. This review mainly concentrate on the use of transition metal complexes of tridentate Schiff bases as catalyst for some important organic transformations (Hathaway, 1987).

Study of the transition metal complexes with salicylaldehyde derivatives has gained much attention because of their potential diverse applications in catalysis, chemotherapy, insecticides, fungicides, organic light emitting diodes and so on (Zenat, 2006). Thus in the recent years the discovery of such new compounds is on progress (Manju, 2010). Thus, it is worthwhile to carry out the structural and spectral studies of salicylidine Schiff bases with different structural features as well as their metal complexes.

2. Materials and methods

2.1 Materials and physical measurements

2-Aminophenol, 5-methyl-2-aminophenol(Aldrich) and salicylaldehyde(Aldrich) and hydrated copper(II) chloride (Aldrich) are used as supplied. The preparation of Schiff base is carried out by condensation. Elemental analyses of the ligands were done on a Vario EL III CHNS analyzer. The IR spectra were recorded on a JASCO FTIR–4100 spectrometer using KBr pellets in the range 400–4000 cm^{-1}. The electronic spectra of the ligands were recorded on a Thermoelectron Nicolet evolution 300 UV-Vis spectrophotometer.

2.2 Synthesis of salicylidine -2-aminophenol (L1)

About 2g of 2-aminophenol is mixed with 2.5ml of salicylaldehyde and is dissolved in 25ml of ethanol. Then it is kept aside. After few minutes ligands are formed and it is filtered, washed with little amount of ethanol and dried in air. The solubility of the ligand was checked and it is found that the ligand is soluble in dilute HCl and Ethanol. (Figure 1).

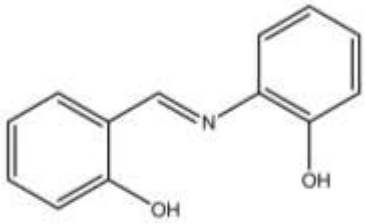

Fig 1: Structure of *salicylidine -2-aminophenol*

2.3 Synthesis of salicylidine-5-methyl-2-aminophenol(L2)

About 2.2g of 5-methyl-2-aminophenol is mixed with 2.5ml of salcylaldehyde and is dissolved in 25ml ehanol. Then it is kept aside. After few minutes ligands are formed and it is filtered, washed with little amount of ethanol and dried in air. The solubility of the ligand was checked and it is found that the ligand is soluble in dilute HCl and Ethanol.

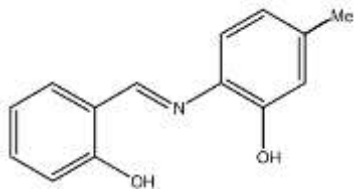

Fig 2: Structure of *salicylidine-5-methyl-2-aminophenol(L2)*

2.4 Synthesis of complexes

2.4.1 Synthesis of copper complex of salicylidine-2-aminophenol (Cu-L1)

About 0.535 g of salicyladehyde 2-aminophenol is dissolved in 20ml of ethanol.Then it is mixed with 0.425 g of copper chloride dissoved in 30 ml of ethanol. Further it is refluxed under flame for about 1 hour. The solubility of the complex is determined. It is found that the complex is soluble in dilute HCl, Water, Ethanol and insoluble in Chloroform and Ether.

2.4.2 Synthesis of copper complex of salicylidine-5-methyl-2-aminophenol (Cu-L2)

About 0.575g of salicylidine-5-methyl-2-aminophenol is dissolved in 20 ml of ethanol. Then it is mixed with 0.425g of copper chloride dissolved in 30ml of ethanol. Futher it is refluxed in the flame for about 1 hour. The solubility of the complex is determined. It is found that the complex is soluble in dilute HCl, Choloroform and Alcohol. But it is sparingly soluble in Ether and Water.

3. Results and discussion

3.1 Elemental analyses

The preparation of ligands and complexes were carried out in ethanol and the yield is found to be good. Table 1 gives the analytical data of the ligand and complexes. The analytical data indicate that the complexes have the formulae given in Table 1. From the data it is found that the found and calculated values are in good agreement.

Table 1: Analytical data of ligands and complexes

Compound	Colour	Elemental analysis founded		
		% of C	% of H	% of N
L1	Orange	72.99 (73.23)	5.02 (5.29)	6.056 (6.5)
L2	Brown	73.85 (73.99)	5.44 (5.77)	5.966 (6.16)
$[Cu(L1)(H_2O)_3]$	Black	47.04 (47.49)	4.55 (4.60)	4.01 (4.26)
$[Cu(L2)(H_2O)_3]$	Black	40.29 (40.05)	4.11 (5.00)	4.34 (4.09)

3.2 Magnetic susceptibility measurements

The magnetic moment values (at 27 ºC) of the complexes are shown in Table 2. The magnetic moment of the copper(II) complex is 1.90 and 1.87 B.M. respectively which shows the lack of Cu–Cu interactions and monomeric nature of the complex. The magnetic data clearly establish +2 oxidation state for copper in these complexes. The data indicates the absence of any metal-metal interaction and excludes the possibility of these complexes to have a tetrahedral structure.

Table 2. Magnetic moment data

Complex	Magnetic moment (BM)
$[Cu(L1)(H_2O)_3]$	1.90
$[Cu(L2)(H_2O)_3]$	1.87

3.3 Infrared spectra

The IR spectral data of the ligands and complexes were given in table 3. Most of the bands due to the free ligand are present in the spectra of complexes. when compared with that of the free ligands, spectra of complexes shows certain differences. Selected vibrational bands of the free ligands and the copper complexes, useful for determining the mode of coordination of the ligands, are given in Table. The band at 3000–3500 cm^{-1} due to the νOH of the free Schiff base was found to be broadened in the spectrum of the complex, which suggests that water molecules are coordinated with copper after complexation. The presence of coordinated water molecules in both complexes are evidenced by the formation of two new bands at 1162 and 1186 cm^{-1} corresponding to δ(O—H) of the coordinated water (Nakamoto, 1986). The azomethine stretching frequencies of the free ligands

were found to be increased in the complexes indicating the participation of the azomethine nitrogen in chelation. Furthermore, in these complexes, the bands in the regions 500−550 cm^{-1} and 420−480 cm^{-1} can be assigned to the stretching modes of the metal to ligand bonds, ν(Cu–O) and ν(Cu–N), respectively (Arun, 2009).

3.4 Electronic Spectra

The electronic spectral bands and their assignments are given in table 4.The electronic spectra of the compounds were recorded in ethanol. The UV–Vis spectrum of the Schiff base shows two strong bands at 300 and 450 cm^{-1} due to π–π* transitions. For all the complexes, the π–π* bands are not altered to a greater extent on coordination. The copper(II) complex contains intense charge transfer bands at 560 and 570, which masks all the possible d-d transitions [Lever, 1984].

Table 3: FTIR spectral data of ligands and complexes

Compound	**ν (OH)/H_2O**	ν (C=N)	**ν (C–O)**	**ν (M–O)**	**ν (M–N)**
L1	3386/3450	1609	1254	-	-
L2	3200	1612	1260	-	-
[Cu(L1)$(H_2O)_3$]	3500 br	1689	1259	530	417
[Cu(L2)$(H_2O)_3$]	3500 br	1634	1245	528	424

Table 4: UV Vis spectral data of ligands and complexes

Complexes	λ max	Band assignments
L1	300 460	π→π* π→π*
L2	270 420	π→π* π→π*
[Cu(L1)$(H_2O)_3$]	300 420 560	π→π* π→π* CT
[Cu(L2)$(H_2O)_3$]	320 420 570	π→π* π→π* CT

4. Conclusions

In current work synthesis and characterization of two Schiff bases; salicylidine -2-aminophenol and salicylidine -2-amino-5-methylphenol; were carried out. The copper(II) complexes of the Schiff bases are also prepared. Schiff bases are prepared by condensation between aldehyde and primary amine. Aldehyde chosen was salicylaldehyde and primary amines used were 2-aminophenol and 2-amino-5-methylphenol. Ligands are characterized by elemental analysis, infrared and UV-Vis spectral studies and complexes are characterized by elemental analysis, infrared, magnetic susceptibility and UV-Vis spectral studies. An infrared spectrum of Schiff bases gives a medium band in the range 1600-1650 cm^{-1} characterized azomethine stretching of Schiff base. On complexation azomethine stretching is red shifted in both cases. On the basis of all the studies carried out an octahedral geometry is proposed for both complexes (Figure 3).

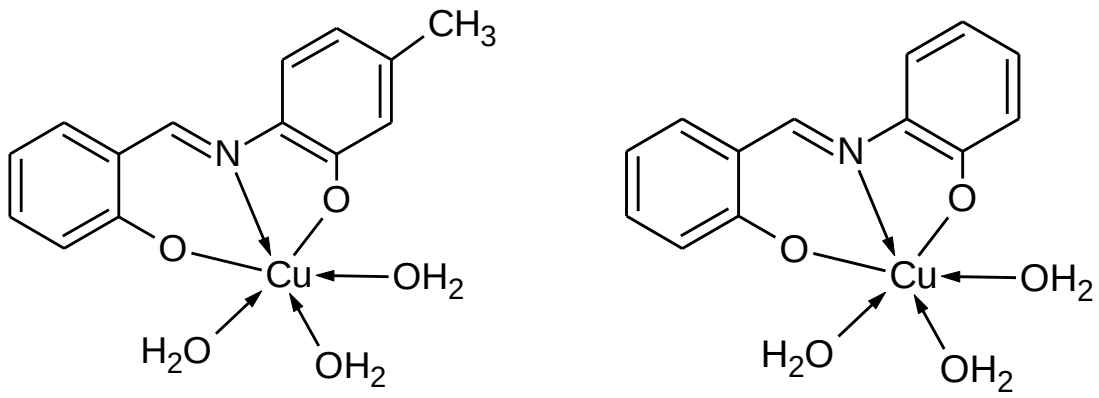

Figure 3: Geometry of the complexes

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Phytochemical Analysis and Synthesis of Zinc Oxide nanoparticle from *Carica Papaya*

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Abstract

An ecofriendly method is developed for the synthesis of Zinc oxide nanoparticles from the plant Carcia Papaya fruit peel extract ad we also conducted phytochemical analysis of the Carcia Papaya plants fruit peel extract. Here we have used biological or green method for the synthesis of ZnO nanoparticles which can be regarded as an effective substitute for physical and chemical methods. Therefore our study on "Biosynthesis and characterization of ZnO nanoparticles using Carica Papaya" was successful in synthesizing zinc oxide nanoparticles in a very effortless, cost effective, eco-friendly and sustainable manner. Phytochemical studies indicates the presence of Carbohydrates, Saponin, Phenol, Flavonoids in Carica Papaya.

Key words : *Zinc oxide, nanoparticle, phytochemicals, green method*

1. Introduction

Nanoparticles are particles between 1 and 100 nanometers in size. The term "nano" comes from Greek word "nanos" meaning dwarf. They are particulate dispersion of solid particles with at least one dimension at a size range of 10-100 nm. The most important feature of nanoparticles is their surface to volume ratio, allowing them to interact with other particles easier. Nanoparticles have unique chemical, physical, thermal, optical, magnetic and electric properties compared to their bulk material counterparts. Properties of materials change as their size approach nanoscale.

Nanoparticles are classified as zero, one, two, three dimensional depending on their over shape. One dimensional nanomaterial has thin films and are used in circuitry of computer chips and for hard coating on eye

glasses. Two dimensional nano-materials have long nano-structures with thick membrane. Three dimensional nano-materials are small nano-structures where thin films are deposited under conditions that generates atomic scale porosity, colloids and free nanoparticles with various morphologies.

Zinc oxide nanoparticles have been in recent studies due to its potential applications like antibacterial, anti-fungal, anti-diabetic, anti-inflammatory, wound healing, antioxidant, and optic properties. Green synthesized zinc oxide nanoparticles exhibit anti-bacterial and anti-fungal activities even at low concentrations. Zinc oxide nanoparticles are also found to have superior UV blocking properties compared to its bulk state. Therefore, they are used in preparations of sunscreen lotions.

Carica Papaya (Papaya) is a tropical fruit having commercial importance because of its high nutritive and medicinal value. *Carica Papaya* Linn. (Family, Caricaceae), is a widely grown perennial tropical tree, grows up to about 10 m in height with an erect trunk. Its leaves are large, measuring about 50-70 cm in diameter, deeply palmately lobed with seven lobes. Its fruit (papaya) is known by different parts of the world such as fruta bomba in cuba and lechoza in Venezuela. In Nigeria, it is also known by different local names depending on the tribe. The ripe fruit is edible and is usually eaten raw, without the peel and seed. The unripe green fruit (which is a rich source of vitamin A) can be eaten cooked, usually in curries, salads and stews. Papaya is a major fruit crop in many tropical countries, and its ranked first amongst 38 common fruits based on its accordance to the united states recommended daily allowance for many vitamins, and consumption of papaya has been recommended for preventing vitamin A deficiency which causes childhood blindness in many tropical and subtropical countries. The fruits, leaves, seeds and latex are used in folklore for several ailments. Unripe papaya is one of the common plant materials used in treatment of sickle cell disease in Nigeria; it is also found out that its water extract has no harmful effect on kidney functions.

Among nanoparticles used for all purposes, the metallic nanoparticle considered as the most promising as they contain remarkable antibacterial properties due to their large surface area to volume ratio. Synthesis, characterization and applications of zinc oxide nanoparticle (Khalil, et.al.,2013). Use of unripe papaya peel could be of beneficial

in the management/treatment of some oxidative stress induced human ailments (Felix abayomi Dada et.al. 2016).

The methanolic extracts of the papaya (*Carica Papaya* L. var solo 8) peels exhibited the high DPPH radical scavenging activities (81.89 ± 0.14 %). These data indicated that papaya (*Carica Papaya* L. var solo 8) peels could constitute a potential good source of natural antioxidant for local population (Tano et.al., 2017).The peel extracts showed highest zone of inhibition against Gram negative bacteria as compared to Gram positive bacteria and fungi (sumitra et.al., 2014).The ability of peel extracts to reduce metal ions has been known since the early 1900s, although the nature of the reducing agent involved was not well understood. In view of its simplicity, the use of peel extract and peel tissue for reducing metal salts to nanoparticles has attracted considerable attention within the last 30 years. Therefore; the use of unripe peel extract for this purpose is potentially advantageous (Kalishwqaralal et.al.,2010).It is the best platform for the syntheses of nanoparticle being free from toxic chemicals as well as providing natural capping agents for the stabilization of ZnO nanoparticles.

2. Materials and Methods

2.1 .Materials Required:

1. Zinc Nitrate [$Zn(NO_3)_2.6H_2O$],
2. Sodium Hydroxide [NaOH]
3. *Carica Papaya* Extract

2.2. Methods:

(A) Collection and Preparation of Fruit Peel Extract

The unripened fruit peel of *Carica Papaya* was collected locally. The samples collected was identified by Dr. Rekha, Assistant professor, Dept of Botany, St. Mary's college, Thrissur. The samples were cleaned of all the impurities present by washing with normal water followed by distilled water. The sample was dried in shade by keeping overnight in at room temperature and made in to fine powder.10g of powdered sample were weighed and transferred to a 500ml beaker, 250ml of double distilled water was added to it and a glass rod was placed inside the beaker. Then the beaker was placed in water bath maintained at 80°c for thirty minutes and cooled. The extract was then filtered using whatmann No: 1 filter paper into a conical flask. This extract was stored in refrigerator for future use.

(B) Phytochemical Analysis of Friut Peel Extract of Carica Papaya

Test For Carbohydrates - Molisch's Test In a test tube, 2ml of the fruit peel extract of *Carica Papaya* was shaken with 4 drops of Molisch's reagent. Carefully incline the tube and pour drop wise conc.H_2SO_4, using a dropper along the sides of the tube. Observe the violet colour at the junction of the two liquids. The violet colour indicates the presence of carbohydrates.

Test For Glycosides – Salkowiski's test 2ml of chloroform was mixed with 2ml of fruit peel extract of *Carica Papaya* . Then 2ml of conc. H_2SO_4 was added and shaken gently. Reddish brown colour indicates the presence of glycosides.

Test for Tannins About 0.5g of dried powered sample was boiled in 20ml of distilled water in a water bath and filtered. 10 ml of this extract was added to 2 drops of diluted neutral ferric chloride (0.1%) solution. A dark green or blue green coloration indicates the presence of tannins.

Test For Saponins – Foam Test About 2g of dried powdered sample was boiled in 20ml of distilled water in a water bath and filtered. 10 ml of this extract was mixed with 5ml of distilled water and shaken vigorously. Formation of stable foam indicate the presence of saponins.

Test for Phenol 2ml of fruit peel extract of *Carica Papaya* was shaken with a few drops of 2% neutral ferric chloride solution. Blue green or Purple coloration indicated the presence of phenols.

Test For Flavonoids – Lead Acetate Solution Test 2ml of fruit peel extract of *Carica Papaya* was mixed with a few drops of lead acetate (10%) solution. Formation of intense yellow colour indicates the presence of flavonoids.

Test for Anthocyanoside 1ml of fruit peel extract of *Carica Papaya* was mixed with 5ml dil.HCl solution. Formation of pale pink colour indicates the presence of anthocyanoside.

Test for Anthraquinonine 1 ml of fruit peel extract of *Carica Papaya* was mixed with 2 drop of 10% ammonia solution, a pale pink colour precipitate indicates the presence of anthraquinonine.

(C) Biosynthesis of Zinc Oxide Nanoparticles

Preparation of Zinc Nitrate Solution: 0.1 Molar solution Zinc Nitrate was prepared by dissolving required quantity of Zn $(NO_3)_2.6H_2O$ in 500 ml standard flask.

Preparation of ZnO nanoparticle:

To synthesis ZnO nanoparticle, one of the most common methods employed by researchers include mixing the solution of Zn $(NO_3)_2$ with fruit peel extract in varying ratios. In our work we tried to synthesize ZnO nanoparticle in three different concentrations viz. 90:10, 95:5 and 98:2.

To synthesis 90:10 concentration, 90ml of Zn $(NO_3)_2$ solution was taken in a conical flask, to this 10 ml of fruit peel extract of *Carica Papaya* was added in dropwise with constant shaking. This was placed on magnetic stirrer for 30 minutes. The solution was observed for colour change and then stored in the refrigerator. To synthesis 95:5 concentration, 95ml of Zn $(NO_3)_2$ solution was taken in a conical flask, to this 5 ml of fruit peel extract of *Carica Papaya* was added in dropwise with constant shaking. This was placed on magnetic stirrer for 30 minutes. The solution was observed for colour change and then stored in the refrigerator. To synthesis 98:2 concentration, 98ml of Zn $(NO_3)_2$ solution was taken in a conical flask, to this 2 ml of fruit peel extract of *Carica Papaya* was added in dropwise with constant shaking. This was placed on magnetic stirrer for 30 minutes. The solution was observed for colour change and then stored in the refrigerator.

Characterization Technique: UV-Vis spectroscopy: The absorbance spectra were recorded using UV-Vis spectrophotometer (Department Of chemistry, St. Mary's College Thrissur).

3. Results and Discussion

3.1. Phytochemical Analysis:

Aqueous extracts of the unripened fruit peel of ***Carica Papaya*** showed the presence of Carbohydrates, Saponin, Phenol, Flavonoids. Antroquinonine, Anthocyanoside, Tannins, Glycosides were not found in the extract of ***Carica Papaya***. The results of phytochemical analysis of ***Carica Papaya*** is showed in the table given below:

3.2. Visual Observations and UV visible Spectral Analysis:

We successfully synthesised ZnO nanoparticles by reduction using unripened fruit peel extract of ***Carica Papaya***. Reduction of zinc was identified by colour change of reaction mixture from pale yellow into white. White colour of powder indicates formation of ZnO nanoparticles.

*Table 1: Phytochemical Analysis of **Carica Papaya** extract.*

Sl.No:	Name of the phytochemical Constituent	Aqueous extract
1	Carbohydrate	+
2	Glycosides	-
3	Tannins	-
4	Saponin	+
5	Phenol	+
6	Flavonoids	+
7	Anthocyanoside	-
8	Anthroquinonine	-

(+) and (-) indicates the presence and absence of phytoconstituents

(A) Effect of Concentration

We prepared various samples that differ in the concentrations of Zinc Nitrate and peel extract. Higher yield of nanoparticles was obtained from sample containing 5 ml ***Carica Papaya*** extract and 95 ml Zinc Nitrate solution.

Nanoparticles synthesised were characterised by UV-Vis spectroscopy.

(B) Uv-Vis Spectroscopy

UV-Vis absoption curves of synthesised ZnO nanoparticles are given below. Absorption peak was found near 350 nm. Since this result correlates with already reported results, formation of ZnO nanoparticle was confirmed. Absorption peak of sample containing 95:5 concentration is found to be more characteristics towards reported Zinc Oxide absorption.

4. Conclusion

The work was about the Biosynthesis of ZnO nanoparticles and the phytochemical study of peel extract of unripened Carica Papaya. We successfully synthesized ZnO nanoparticles using *Carica Papaya* extract. Prepared ZnO nanoparticles were White in colour and their formation was confirmed by visual observation and characterization techniques like UV-Vis absorption spectroscopy. A peak near 350 nm in UV-Vis spectra comparable to already reported values of ZnO nanoparticles accounts for the formation of Zinc oxide nanoparticles. Here we have used biological or green method for the synthesis of ZnO nanoparticles which can be regarded as an effective substitute for physical and chemical methods. Therefore our study on "Biosynthesis and characterisation of ZnO

nanoparticles using Carica Papaya" was successful in synthesising zinc oxide nanoparticles in a very effortless, cost effective, eco-friendly and sustainable manner. Phytochemical studies indicates the presence of Carbohydrates, Saponin, Phenol, Flavonoids in *Carica Papaya*.

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Conservation status of *Clarias dussumieri* (Valenciennes) in central Kerala

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Abstract

Clarids are air breathing cat fishes with elongate compressed body, four pairs of barbels, air breathing organ and horizontally inserted paired fins. Clarias dussumieri is an endemic fresh water fish of peninsular India. The population of the fish is experiencing a marked decline during the past few years. A survey was conducted during 2008-2012 all along the stretch of Keecheri waster shed of Kerala and it was collected only from the upper reaches of the river.

Key words: *Clarias dussumieri, population decline, threatened.*

1. Introduction

Kingdom	- Animalia
Phylum	- Chordata
Class	- Actinopterygii
Order	- siluriformes
Family	- Clariidae
Species	-Clarias dussumieri
Species Authority	-Valenciennes, 1840
Common name	-Valenciennesclarid
Vernacular name	- Mushi
Conservation status	- Near threatened (Iucn 2012), Vulnerable (Radhakrishnan 2010)

Clarids is an endemic fresh water fish of peninsular India with distribution in Goa, Karnataka, Kerala and Pondichery (Talwar and Jhingran 1991). It is a benthopelagic fish feeding on small fishes and detritous matter. Though it is not of much commercial importance it is a highly favored fish of the local people. The fish was very common a few years ago occurring in the ponds, lower reaches of streams, rivers, Paddy fields, canals and swamps. However it recorded a sharp decline since the past twenty years. Very rare (Gopi, 2000), Vulnerable

(Radhakrishnan, 2010), endangered (Lakra, 2010), Near threatened (Iucn 2012) and it was also considered as vanishing from Kerala according to Binoy (2010). It was reported declining (Thomas, 2004) and as absent from the wetlands and rivers of central Kerala (Subhashbabu, 2002; Raghavan 2008) where it abundantly existed once.

2. Materials and methods

Ten sites were selected in the Keecheri watershed. Extensive surveys were conducted during 2009 to 2011 all along the stretches of Keecheri watershed. Fishes were collected using cast net gill net and traps. They were immersed in formalin and brought to the laboratory for identification.

3. Result and discussion

All throughout the survey the fish, *Clarias dussumieri* was reported as absent from all sites. However by the end of survey period in 2011 it was collected from the upstreams of Keecheri river, from the Vazhani reservoir.

Various proposition for the decline include that many areas of *Clarias dussumieri*'s natural habitat has been converted or degraded due to land reclamation and pollution due to the excessive use of pesticides and fertilizers (Padmakumar *et al.* 2010). A similar decline in Clarias batrachus from central Thailand was indicated to be due to habitat destruction and fishing pressure (Muangboon, 1981.)

Stocks of the indigenous catfish species of Lake Victoria have declined coinciding with the Nile perch boom and concomitant ecological changes in the lake (Goudswaard, 2006.). The introduction of Clarias garipinus as a coinhabitor poses severe threat not only as a competitor but also it promotes a disguised threat of genetic introgression, as Clarias garipinus genes were identified by Senanan (2004) in C. macrocephalus.

The fish being a bottom dwelling species it is seriously affected by fertilizer and pesticide pollution. The pesticides alter thyroid, testosterone and cortisol levels in Clarias batrachus (Saravanan, 2009). This could be the reason for low reproduction, mortality and decline in the population. The recent changes in the pattern of farming by reduction in the use of pesticide and resort to organic farming are proposed as the reasons for replenishment of this fish stock.

Present record of the fish from the upstreams of Keecheri river focuses insight into the fact that the decline in fish is owing to the ecological transformations of their habitat in

the middle and lower stretches of the river with agricultural setup.

The reoccurrence of the fish could also be by the natural replenishment of stock after two decades post EUS epidemic of 1991whose severe victims were the bottom living fish channids, heteropneustids and clarids. The disease had washed away ample freshwater fishery resources of Kerala. Usually the fish prefers lowland areas and is rarely seen in highland and highrange areas (Thomas, 2004). However the present record of the fish from highlands insists further investigation on its biology and conservation strategies.

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Green Synthesis of Silver Nanoparticles: Microbiological Approach Using Bacteria and Fungi

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Abstract

Nanotechnology is one of the most active areas of research in modern material sciences. This field is developing day by day, making an impact in all spheres of human life make vast implications in life sciences especially biomedical devices and biotechnology since it is expected to open new avenues to fight and prevent disease using atomic scale tailoring of materials. Silver nanoparticles are traditionally being synthesized by chemical and physical methods which needs severe reaction conditions, such as aggressive chemical agents, harmful solvent systems, higher temperature and higher pressure etc. In the green synthesis approach, prokaryotic or eukaryotic organisms including bacteria, fungi and yeasts could synthesize nanoparticles by means of bioreduction. This biological route involving microorganisms has the potential to be cost-effective, simple and environmentally friendly and allow size and shape controlled nanoparticle synthesis.

Key words: Nanoparticles, Nanotechnology, green synthesis, Biosynthesis

1. Introduction

Nanotechnology is an important field of modern research dealing with design, synthesis, and manipulation of particle structures ranging from approximately 1-100 nm. It is the most promising technology that can be applied in almost all spheres of life, ranging from electronics, pharmaceutical, defense, transportations and heat transfer to sports and aesthetics. The applications of

nanotechnology are totally dependent on the types of the nanoparticles. Metallic nanoparticles are of great interest due to their excellent physical and chemical properties, such as high surface-to-volume ratio and high heat transfer.

Nanoparticles are particles between 1 and 100 nanometers in size. Over the past few decades interest in metallic nanoparticles and their synthesis has greatly increased. This has resulted in the development of numerous ways of producing metallic nanoparticles using chemical and physical methods. However, drawbacks such as the involvement of toxic chemicals and the high energy requirements of production make it difficult for them to be widely implemented. An alternative way of synthesizing metallic nanoparticles is by using bacteria, fungi and plants.The green method or biological nanoparticle production is a promising approach that allows synthesis in aqueous conditions, with low energy requirements and low-costs. But all have their own advantages and disadvantages (Suresh K *et al.*, 2004). Intracellular or extracellular synthesis, growth temperature, synthesis time, ease of extraction and percentage synthesized versus percentage removed from sample ratio, all play an important role in biological nanoparticle production (Edmundson MC *et al.*, 2014).

2. Silver nanoparticles

Silver nanoparticles are one of the promising products in the nanotechnology industry. The development of consistent processes for the synthesis of silver nanoparticles is an important aspect of current nanotechnology research. Silver nanoparticles can be synthesized by several physical, chemical and biological methods. One of such promising process is green synthesis.

Silver nanoparticles have unique optical, electrical, and thermal properties and are incorporated into products that range from photovoltaic to biological and chemical sensors, including pastes, conductive inks and fillers which utilize silver nanoparticles for their high electrical conductivity, stabilization and low sintering temperatures. Due to optical properties of nanoparticles, these are mainly used in molecular diagnostics and photonic devices. An increasing application is the use of silver nanoparticles for antimicrobial coatings and many textiles, wound dressing, and biomedical devices that continuously release a low level of silver ions to provide protection against bacteria.

3. Synthesis of silver nanoparticles using bacteria

Research have heavily focused on prokaryotes as a means of synthesizing metallic nanoparticles. Due to their abundance in the environment and their ability to adapt to extreme conditions, bacteria are a good choice to study. They are also fast growing, inexpensive to cultivate and easy to manipulate. Growth conditions such as temperature, oxygenation and incubation time can easily controlled. In a study by He *et al.*, it was discovered that changing the pH of the medium during the incubation results in the production of nanoparticles of differing size and shape (He S *et al.*, 2007). Controlling such properties is important, as varying sizes of nanoparticles are required for different applications such as optics, catalysts or anti-microbials.

It was reported that highly stable silver nanoparticles could be synthesized by bioreduction of aqueous silver ions with a culture supernatant of nonpathogenic bacterium, *Bacillus licheniformis*. Well-dispersed silver nanocrystals were synthesized using the bacterium *Bacillus licheniformis* (Saifuddin *et al* 2009) have described a novel combinational synthesis approach for the formation of silver nanoparticles by using a combination of culture supernatant of *B. subtilis* and microwave irradiation in water. They reported the extracellular biosynthesis of monodispersed Ag nanoparticles (5-50 nm) using supernatants of *B. subtilis*, but in order to increase the rate of reaction and reduce the aggregation of the produced nanoparticles, they used microwave radiation which might provide uniform heating around the nanoparticles and could assist the digestive ripening of particles with no aggregation. Silver nanocrystals of different compositions were successfully synthesized by *Pseudomonas stutzeri* AG25. The silver-resistant bacterial strain, *Pseudomonas stutzeri* AG259, isolated from a silver mine, accumulated silver nanoparticles intracellularly, along with some silver sulfide, ranging in size from 35 to 46 nm (Slawson *et al.*, 1992). Larger particles were formed when *P. stutzeri*AG259 challenged with high concentrations of silver ions during culturing, resulted intracellular formation of silver nanoparticles, ranging in size from a few nm to 200 nm. *P. stutzeri* AG259 detoxificated silver through its precipitation in the periplasmic space and its reduction to elemental silver with a variety of crystal typologies, such as hexagons and equilateral

triangles, as well as three different types of particles: elemental crystalline silver, monoclinic silver sulfide acanthite (Ag2S), and a further undetermined structure (Klaus *et al.* 1999). The periplasmic space limited the thickness of the crystals, but not their width, which could be rather large (100-200 nm). In another study, rapid biosynthesis of metallic nanoparticles of silver using the reduction of aqueous Ag+ ions by culture supernatants of *Klebsiella pneumonia*, *E. coli*, and *Enterobacter cloacae* (Enterobacteriacae) was reported (Shahverdi *et al.*, 2007). The synthetic process was quite fast and silver nanoparticles were formed within 5 min of silver ions coming in contact with the cell filtrate. It seems that nitroreductase enzymes might be responsible for bioreduction of silver ions. It was also reported that visible-light emission could significantly increase synthesis of silver nanoparticles (1-6 nm) by culture supernatants of *K. pneumonia*. Monodispersed and stable silver nanoparticles were also successfully synthesized with bioreduction of [Ag $(NH_3)_2$] + using *Aeromonas* sp. SH10 and *Corynebacterium*s p. SH09. It was speculated that [Ag $(NH_3)_2$] + first reacted with OH− to form Ag_2O, which was then metabolized independently and reduced to silver nanoparticles by the biomass.

Lactobacillus strains, when exposed to silver ions, resulted in biosynthesis of nanoparticles within the bacterial cells (Nair and Pradeep 2002). It has been reported that exposure of lactic acid bacteria present in the whey of buttermilk to mixtures of silver ions could be used to grow nanoparticles of silver. The nucleation of silver nanoparticles occurred on the cell surface through sugars and enzymes in the cell wall, and then the metal nuclei weretransported into the cell where they aggregated and grew to larger-sized particles. Biological methods of silver nanoparticle synthesis require a special ability: "Resistance of the organism to silver ions". It is to be noted that those organisms which synthesize silver nanoparticles are also vulnerable to higher concentrations of silver ions. For example, *Bacillus licheniformis* is one such organism used to synthesize silver nanoparticle at 1 mM concentration, i.e., when the concentration of the silver ion in the environment is 1 mM, the organism can synthesize silver nanoparticles without undergoing cell death. But, when the concentration of the silver ions is raised, say 10 mM, the organism undergoes cell death within minutes, i.e., when the concentration

crosses the threshold level (Kalimuthu *et al.*, 2008). Even though the organism has the resistance to silver ions, it becomes useless at the higher concentration. That is why silver can be rightly called "moiety with two functions" – one is inducing the organism to synthesize nanoparticles at lower concentration, another is the induction of cell death at higher concentration.

4. Synthesis of silver nanoparticles using fungi

The use of fungi in producing metallic nanoparticles has received significant interest as they offer certain advantage over the use of bacteria for the synthesis of nanoparticles. The ease of scaling up and down stream processing, the economic feasibility and the presence of mycelia offering an increased surface area, are important advantages to consider (Mukherjee *et al.*, 2001). Mukherjee *et al* suggested that because fungi secrete significantly higher amounts of proteins than bacteria, this would amplify the nanoparticle synthesis productivity.

Silver nanoparticles (5-50 nm) could be synthesized extracellularly using *Fusarium oxysporum*, with no evidence of flocculation of the particles even a month after the reaction. The long-term stability of the nanoparticle solution might be due to the stabilization of the silver particles by proteins. The morphology of nanoparticles was highly variable, with generally spherical and occasionally triangular shapes observed in the micrographs. Silver nanoparticles have been reported to interact strongly with proteins including cytochrome *c* (Cc). This protein could be self-assembled on citrate-reduced silver colloid surface. Interestingly, adsorption of (Cc)-coated colloidal Au nanoparticles onto aggregated colloidal Ag resulted Ag: Cc: Au nanoparticle conjugate (Keating *et al.*, 1998). In UV-vis spectra from the reaction mixture after 72 h, the presence of an absorption band at ca. 270 nm might be due to electronic excitations in tryptophan and tyrosine residues in the proteins. In *F. oxysporum*, the bioreduction of silver ions was attributed to an enzymatic process involving NADH-dependent reductase (Ahmad *et al.*, 2003). The exposure of silver ions to *F. oxysporum*, resulted in release of nitrate reductase and subsequent formation of highly stable silver nanoparticles in solutio. The secreted enzyme was found to be dependent on NADH cofactor. Theymentioned high stability of nanoparticles in solution was due to capping of particles by release of capping proteins by *F. oxysporum*. Stability of the capping

protein was found to be pH dependent. At higher pH values (>12), the nanoparticles in solution remained stable, while they aggregated at lower pH values (<2) as the protein was denatured.

Kumar *et al.* (Kumar *et al.*, 2007) have demonstrated enzymatic synthesis of silver nanoparticles with different chemical compositions, sizes and morphologies, using NADPH-dependentnitrate reductase purified from *F. oxysporum* and phytochelat in, *in vitro*. Silver ions werereduced in the presence of nitrate reductase, leading to formation of a stable silver hydrosol10-25 nm in diameter and stabilized by the capping peptide. Use of a specific enzyme in *invitro* synthesis of nanoparticles showed interesting advantages. This would eliminate the downstream processing required for the use of these nanoparticles in homogeneous catalysis and other applications such as non-linear optics. *Fusarium acuminatum* Ell. And Ev. (USM-3793) cell extracts can cause biosynthesis of silver nanoparticles. The nanoparticles were produced within 15-20 minutes and were spherical with abroad size distribution in the range of 5-40 nm with the average diameter of 13 nm. Anitrate-dependent reductase enzyme might act as the reducing agent. The white rot fungus, *Phanerochaete chrysosporium*, also reduced silver ions to form nano-silver particles. Possible involvement of proteins in synthesizing silver nanoparticles was observed in *Plectonema boryanum*UTEX 485(a filamentous cyanobacterium).

Stable silver nanoparticles could be achieved by using *Aspergillus flavus* (Vigneshwaran *et al.*, 2007). These nanoparticles were found to be stable in water for more than 3 months with nosignificant aggregation because of surface binding of stabilizing materials secreted by the fungus (Vigneshwaran *et al.*, 2007). Extracellular biosynthesis of well-dispersed silver nanoparticles (5-25 nm) with variable shapes using *Aspergillus fumigates* (a ubiquitous saprophytic mold) has also been investigated (Bhainsa and D'Souza 2006).

Compared to intracellular biosynthesis of nanoparticle, extracellular synthesis could be developed as a simple and cheap method because of uncomplicated downstream processing and handling of biomasses. The extracellular filtrate of *Cladosporium cladosporioides* biomass can be used to synthesize silver nanoparticles. It was suggested that proteins, organic acids and polysaccharides released by *C. cladosporioides* were responsible for

formation of sphericalcrystalline silver nanoparticles.

Kathiresan *et al.*(Kathiresan *et al.*, 2009) have shown that when the culture filtrate of *Penicillium fellutanum* was incubated with silver ions and maintained under dark conditions, spherical silver nanoparticles could be produced. They also changed crucial factors such as pH, incubation time, temperature, silver nitrate concentration and sodium chloride to achieve the maximum nanoparticle production. The highest optical density at 430 nm was found at 24 h after the start of incubation time, 1 mM concentration of silver nitrate, pH 6.0, 5˚C temperature and 0.3% sodium chloride. Fungi of *Penicillium* genus can be used for green synthesis of silver nanoparticle. *Penicillium* sp. J3 isolated from soil was able to produce silver nanoparticles. The bioreduction of silver ions occurred on the surface of the cells and proteins might have critical role in formation and stabilization of the synthesized nanoparticles. *Coriolus versicolor* form of monodisperse spherical silver nanoparticles.

Biological synthesis gives particles with good control on the size distribution and the nanoparticles could be stabilized directly in the synthesis process by proteins (Duran et al., 2005). This has led to the consideration of biological systems, mainly microorganisms for the biosynthesis of nanoparticles. The novel microbial approach of making nanoparticles is potentially attractive because fermentation and microbial respiration is a well understood, highly scalable, and environmental friendly process.

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Structure and Synthesis of Bimetallic Nanoparticles

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Abstract

Nanoparticles (NP) are structures with diameter in nanoscale that finds application in a variety of fields. In recent years bimetallic nanoparticles have piqued the interest of researchers due to its novelty, versatility and improved properties like selectivity and stability. This article discusses the structure and synthesis of bimetallic nanoparticles. They may be synthesized either by top-down or by bottom-up approach. By optimizing various parameters like temperature, time and reagent concentrations, the size and shape of nanoparticles can be modified.

Key Words : Bimetallic nanoparticle, structure, alloys, core shell, Synthesis, Top-down, Bottom-up

1. Introduction

Nanoparticles (NP) are structures with diameter in nanoscale that finds application in a variety of fields. Nanoparticles are now being used in different fields like medicine (Zhang, Lisha, et al. 2008), agriculture (Sabir, Sidra, Muhammad Arshad, and Sunbal Khalil Chaudhari.2014), environmental remediation (Zhang, Wei-xian 2003), solar cells (Suliman, Ali Elkhidir, Yiwen Tang, and Liang Xu. 2007), catalysis (Astruc, Didier, 2008) electronics, optoelectronics, information storage, biosensors (Li, Jun, and Nianqiang Wu, 2013) etc. Such widespread use of nanoparticles are due to the fact that the properties of nanoparticles are different from that of bulk material. They may be

completely new or enhanced and are often tunable. Some of the most recent advances of nanoparticles are in the area of catalysis. Nanoparticles of gold, silver, iron, copper, titanium and nickle have all found application as catalyst.

In recent years bimetallic nanoparticles have piqued the interest of researchers due to its novelty and versatility. Bimetallic nanoparticles are made up of two different metal elements and generally show improved properties as an addition of the second metal usually allows for better control of its activity, selectivity and stability. These nanoparticles may show not only those properties that are the combination of the properties of two individual metals but also manifest new properties due to a synergy between two metals. This article discusses the structure and synthesis of bimetallic nanoparticles.

2. Structure of bimetallic nanoparticles

The bimetallic nanoparticles may be classified based on structure and atomic ordering. Based on structure they are divided into into mixed structure or composite structure. Depending upon their atomic ordering they may also be classified into an alloy, inter-metallic compounds, sub-clusters & core-shell. These may further be subdivided into multi-shell core-shell, multiple core materials coated by a single shell material, crown jewel, hollow structure etc (Zaleska-Medynska, et al. 2016).

Alloyed nanoparticles are composed of nanocrystals in which the two metals are randomly mixed; while in *intermetallic structures*, the two metals are mixed in an ordered way. In *sub clusters* two separate components share an interface while in *coreshell structure* one metal forms the core while another metal forms the outershell. In a core shell structure an inexpensive metal is made the core and the catalytically active metal is taken as the shell. This is achieved by first reducing the core metal followed by nucleation of the shell metal around it. The core metal also electronically modifies the shell and thereby improves catalytic activity. (Jiang, H L., et al. 2011).

In a *Crown-jewel structure*, one metal atom (single atom) is controllably assembled or decorated on the surface of the other metal atoms. Generally, the the more expensive metal with catalytic activity is assembeled on the surface of the cheaper metal. This structure allows for the effective use of precious metal as every single atom of precious metal can engage in a chemical

reaction The adjacent second element can often modify the electronic state of the primary catalytic component and improve their catalytic properties. (Liu, X, Wang D, and Li.Y 2012). The *Hollow structure* bimetallic nanoparticle have a very high surface to volume ratios and porosity. The void can be used to encapsulate various multifunctional nanomaterial or even as a nanoreactor. As this structure is hollow, it is often cheaper than other possible structures. The final architecture of nanocrystal is influensed by the atomic distribution of the two metals. Their properties also vary depending upon their atomic arrangement. For example Au—Pd alloy nanocrystals show higher electrocatalytic activity and stability than Au@Pd core—shell nanocrystals.

3. Synthesis of bimetallic nanoparticles

Bimetallic nanoparticles may be synthesized either by top-down or by bottom-up approach. One of the most commonly employed top- down method is laser ablation where in a solid bimetallic alloy is treated with a laser beam to obtain bimetallic nanoparticles. They may also be synthesized by a two- step synthesis, that is, a laser irradiation of a mixture of two metals.

The bottom- up method involves building up of nanoparticles from their corresponding atoms. Gas condensation was the first technique used to synthesize nanocrystalline metals and alloys. In this technique, a metal is vaporized using thermal evaporation sources in an atmosphere of 1-50 m bar. At high residual gas pressure, ultra fine particles (100nm) are formed by gas phase collision. The nanoparticles so formed are removed by scrapper in the form of a metallic plate. The method is extremely slow and evaporation rates may be dissimilar. Sputtering or laser evaporation may be used instead of thermal evaporation.

Chemical Vapor Deposition (CVD) is a well known process in which a solid is deposited on a heated surface via a chemical reaction from the vapor or gas phase. Chemical Vapor Condensation (CVC) on the other hand involves pyrolysis of vapors of metal organic precursors in a reduced pressure atmosphere (Rajput, Namita. 2015) .

The sol-gel process is a wet-chemical technique that uses either a chemical solution (sol short for solution) or colloidal particles (sol for nanoscale particle) to produce an integrated network (gel). Metal alkoxides and metal chlorides are typical precursors. They undergo hydrolysis and polycondensation

reactions to form a colloid, a system composed of nanoparticles dispersed in a solvent. The sol evolves then towards the formation of an inorganic continuous network containing a liquid phase (gel). Formation of a metal oxide involves connecting the metal centers with oxo (M-O-M) or hydroxo (M-OH-M) bridges, therefore generating metal-oxo or metal-hydroxo polymers in solution. After a drying process, the liquid phase is removed from the gel. Then, a thermal treatment (calcination) may be performed in order to favor further polycondensation and enhance mechanical properties. It is one of the simple, fastest and economically less expensive method, and has advantages like low processing temperature, homogeneity of produced material and formation of complex structures or composite materials.

Another simple and versatile technique for synthesizing bimetallic nanoparticle is by co-reduction. A suitable soluble source of metals like soluble salts are treated with a reducing agent like glucose, citrate or sodium borohydride. The cations are reduced to metal and these metals trend to aggregate together to form nanoparticles. These nanoparticles are stabilized by the use of capping reagents like surfactants, polymers, and polyelectrolytes. By optimizing various parameters like temperature, time and reagent concentrations, the size and shape of nanoparticles can be modified (Loza, Kateryna, Marc Heggen, and Matthias Epple. 2020).

The use of chemical and physical methods in the synthesis of nanoparticles was very expensive and energy intensive. The process often use harsh hazardous chemicals detrimental to the environment. This lead to development of green methods of synthesis using biological extracts. The extract of plants and microbial cells are most often employed for synthesis. Compounds such as alkaloids, terpenoids, phenolics, flavonones, pigments, amines, amides, proteins etc present in these extracts may act as both reducing agent and stabilizing agents for synthesis of nanomaterials (Roopan, Selvaraj Mohana, et al. 2014).

4. Conclusion

The bimetallic nanoparticles may be classified either based on structure or based on atomic ordering. They may be either alloy, intermetallic structures, sub clusters or coreshell structures and can be synthesized either by laser ablation, Gas condensation,

Chemical Vapor Deposition, sol-gel, Co-reduction or biosynthetic methods.

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Effect of Microwaves on Smartphone

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Abstract

Microwaves are a form of non-ionizing electromagnetic radiation in the electromagnetic spectrum. Microwave oven is an electronic oven that cooks food using microwaves. The microwaves are absorbed by dielectric molecules in the food such as water, fat, sugar etc. whose consequent vibrations produce heat. Sensors are devices which measure the physical energy and converts it into a signal. All smartphones have either infra-red or microwave based in-built sensors. Although Microwave ovens are shielded, still emit low levels of microwave radiation. So microwave radiations emitted from ovens affect the working of sensor of smartphones if they are kept long time near the oven.

Key words **-**Microwaves, Microwave oven, Smart phone, Sensor

1. Introduction

Electromagnetic radiation is the flow of energy at the universal speed of light through free space or through a material medium in the form of the electric and magnetic fields. An electromagnetic wave is characterized by its intensity and the frequency of the time variation of the electric and magnetic fields. The electromagnetic spectrum consists of radio waves, microwaves, infrared, visible light, ultraviolet rays, X-rays and gamma rays (Encyclopedia Britannica, 2018).

Microwaves are a form of non-ionizing electromagnetic radiation with a frequency range of 300 MHz - 300 GHz. Microwaves are used by astronomers to learn about the structure of nearby galaxies. Microwaves are the principal carriers of high-speed data transmissions between stations on Earth and also between ground-based stations

and satellites and space probes. It is used for international broadband of all kinds of communications such as television and telephone. Radar beams consist of short pulses of microwaves. Microwaves can penetrate clouds of smoke but are scattered by water droplets, so they are used for mapping meteorological disturbances and in weather forecasting. Microwaves play an increasingly wide role in heating and cooking food. They are absorbed by water and fat in foodstuffs and produce heat from the inside. In most cases, this reduces the cooking time a hundredfold (Michael Vollmer, 2004).

Sensors are devices which measure the physical energy and converts it into a signal. There are more than hundreds of types of sensors available in market today. All smartphones have built-in sensors that measure the orientation, motion, light and other environmental conditions. These sensors measure and provide a high precision and accurate data. Smartphones have both hardware-based sensors and software-based sensors. The hardware-based sensors are physically present in the device and directly measure the environmental properties whereas the software-based sensors are virtual sensors which take their inputs from one or more hardware sensors for calculation. The sensors used in smartphones include accelerometer, ambient light sensor, compass, gyroscope, proximity sensor etc. which have either microwave or infrared based detector (Koenig, A. Q. Memon and K. David, 2013).

2. Principle

Microwave oven is an electronic oven that cooks food by means of high-frequency electromagnetic waves called microwaves. The microwaves are absorbed by dielectric molecules in the food such as water, fat, sugar etc. whose consequent vibrations produce heat. Dielectric molecules have a partial positive charge at one end and a partial negative charge at the other. When they absorb microwaves, they rotate to align themselves with the alternating electric field of the microwaves. Rotating molecules hit other molecules and put them into motion, thus dispersing energy. This energy, dispersed as molecular rotations, vibrations and/or translations in solids and liquids raises the temperature of the food. This is termed as dielectric heating The heating thus occurs inside the food greatly reduces cooking time without warming the surrounding air (Kerry Parker et.al, 2004).

Although Microwave ovens are shielded, still emit low levels of microwave radiation. So microwave radiations emitted from ovens affect the working of sensor of smartphones if they are kept long time near the oven.

3. Components of microwave oven

A microwave oven consists of a high-voltage power source, a cavity magnetron with control circuit, a metal cooking chamber with a turntable and a control panel. The high-voltage power source is a simple transformer or an electronic power converter, which passes energy to the magnetron (Kerry Parker et.al, 2004). The cavity magnetron converts high-voltage electric energy to microwave radiation and short waveguide couple microwave power from the magnetron into the cooking chamber. Modern microwave ovens use either an analog dial-type timer or a digital control panel for operation.

4. Hazards of microwave oven

Microwaves generated in microwave ovens cease to exist once the electrical power to the magnetron is turned off. They neither can make the food or the oven radioactive. Therefore, food cooked in a microwave oven is not a radiation hazard.

Water and other homogeneous liquids can superheat when heated in a microwave oven in a container with a smooth surface. That is, the liquid reaches a temperature slightly above its normal boiling point without bubbles of vapour forming inside the liquid. The boiling process can start explosively when the liquid is disturbed, such as when the user takes hold of the container to remove it from the oven or while adding solid ingredients such as powdered creamer or sugar. This can result in spontaneous boiling (nucleation) which may be violent enough to eject the boiling liquid from the container and cause severe scalding (David R. Baghurst et.al, 1992).

Although Microwave ovens are shielded, still emit low levels of microwave radiation (Bangay, M.; Zombolas, C, 2003). This is not harmful to humans. It can sometimes cause interference to Wi-Fi and Bluetooth and other devices that communicate on the 2.45 GHz wavebands or at close ranges. The microwaves interfere with cellular radiations and interference occur. It may either slow down or freeze the network. So it is advisable to keep the WiFi unit and mobile phones well clear of your microwave oven (T. M. Taher, M. J. Misurac, J. L. LoCicero and D. R. Ucci, 2008).

Microwaves can affect the sensor system of smart phones. Sensors are either based on infrared radiations or micro waves. So microwave radiations emitted from ovens affect the working of sensor of smartphones if they are kept long time near the oven. So Keep the smartphones away from oven.

5. Conclusion

Microwaves are a form of non-ionizing electromagnetic radiation with a frequency range of 300 MHz - 300 GHz. Microwave oven is an electronic oven that cooks food using microwaves. The microwaves are absorbed by dielectric molecules in the food and oven cooks food by the process of dielectric heating. Sensors are devices which measure the physical energy and converts it into a signal. All smartphones have either infra-red or microwave based in-built sensors. Although Microwave ovens are shielded, still emit low levels of microwave radiation. So microwave radiations emitted from ovens affect the working of sensor of smartphones if they are kept long time near the oven. So Keep the smartphones away from oven.

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Phytochemical screening, antioxidant and cytotoxicity analysis of flower extracts of *Calotropis gigantea* (L.)W.T.Aiton

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Abstract

The study was designed to determine phytoconstituents, antioxidant activity and cytotoxicity of flower extracts of C.gigantea. Though the advances in modern medicines are significant, there remains an ever increasing demand for herbal medicines. Effective and potent herbal medicines require evaluation by standard scientific methods so as to be validated for the treatment of diseases. Qualitative phytochemical analysis performed to identify various valuable primary and secondary metabolites. The antioxidant potential and cytotoxic efficacy of flower of C.gigantea in various extracts were evaluated according to standard protocols. The qualitative analysis showed the presence of carbohydrates, quinine, tannin, coumarin, alkaloid, phenol, steroid and flavonoid. Distilled water and Ethanol extracts showed maximum activity of 66.11% and 61.55% respectively at 320 µg/ml.. The maximum 69.8% of cell death occurred in ethanol extract. The findings established he antioxidant and cytotoxic property, which may be due to secondary metabolites.

Key words: *C. gigantea*, phytochemicals, antioxidant, DPPH free radical scavenging, cytotoxicity

1. Introduction

India has a rich culture of medicinal herbs and spices, which includes about more than 2000 species and has a vast geographical area with high potential abilities for ayurvedic, unani, siddha traditional medicines but only very few have been studied chemically and pharmacologically

for their potential medicinal value (Gupta et al., 2005). Plants are used medicinally in different countries and are a source of many potent and powerful drugs (Srivastava et al., 1996). The most common reasons for using traditional medicine are that it is more affordable, more closely corresponds to the patient's ideology, allays concerns about the adverse effects of synthetic medicines, satisfies a desire for more personalized health care, and allows greater public access to health information. Herbal medicines are also very common in Europe, with Germany and France leading in over-the-counter sales among European countries, and in most developed countries, one can find essential oils, herbal extracts, or herbal teas being sold in pharmacies with conventional drugs.

Herbs and plants can be processed and can be taken in different ways and forms, and they include the whole herb, teas, syrup, essential oils, ointments, salves, rubs, capsules, and tablets that contain a ground or powdered form of a raw herb or its dried extract. Plants are rich in a variety of compounds. Many are secondary metabolites and include aromatic substances, most of which are phenols or their oxygen-substituted derivatives such as tannins (Hartmann 2007). Many of these compounds have antioxidant properties . Ethnobotanicals are important for pharmacological research and drug development, not only when plant constituents are used directly as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds (Li and Vederas 2009). Herbal materials for commercial products are collected from wild plant populations and cultivated medicinal plants. The expanding herbal product market could drive overharvesting of plants and threaten biodiversity. Poorly managed collection and cultivation practices could lead to the extinction of endangered plant species and the destruction of natural resources. It has been suggested that 15,000 of 50,000–70,000 medicinal plant species are threatened with extinction . The efforts of the Botanical Gardens Conservation International are central to the preservation of both plant populations and knowledge on how to prepare and use herbs for medicinal

purposes (Li and Vederas 2009). Attention paid to medicinal plants is still minimum. Therefore, an urgent need to conserve the genetic diversity of medicinal plant resources. Parallel with recent increasing interest in alternative medicine for the prevention and treatment of various illnesses, there is increasing concern about the safety of medicinal plants. The alarming situation has resulted in short supply, high prices, and forced substitution and adulteration of crude drugs entering in to formulations of many classical, patent, or proprietary medicines. To develop a successful drug to the WHO standard, need for screening of its biological activities to test safety (Natesh, 2001).

Oxidative stress is an important risk factor in the pathogenesis of numerous chronic diseases. Free radicals and other reactive oxygen species are recognized as agents involved in the pathogenesis of sicknesses such as asthma, inflammatory arthropathies, diabetes, Parkinson's and Alzheimer's diseases, cancers as well as atherosclerosis. Reactive oxygen species are also said to be responsible for the human aging.

An antioxidant can be broadly defined as any substance that delays or inhibits oxidative damage to a target molecule. The main characteristic of an antioxidant is its ability to trap free radicals. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative disease. Herbal plants considered as good antioxidant since ancient periods.

Herbal drugs considered free from side effects than synthetic one. They are less toxic, relatively cheap and popular (Momin, 1987). In India medicinal plants have been used as natural medicine since the days of Vedic glory .Many of these medicinal plants and herbs are part of our diet as spices, vegetables and fruits.

Calotropis gigantea belonging to Asclepiadaceae family, is an important Indian medicinal plant and widely used in ayurveda for management of various diseases. The plant is reported as effective in treating skin, digestive, respiratory, circulatory and neurological disorders. The

leaves of *C.gigantea* are used for the treatment of poisonous snake bites, periodic fever, intestinal worms and ulcers. Roots of this plant are crushed well and applied well by rubbing firmly over the bitten area. Latex of this plant is used to cure dental problems, rat bite, swellings, gonococcal arthritis and other rheumatic complaints. Flowers are used to cure bronchial asthma (Kumar, 2011).

2. Materials and methods

2.1 Collection of plant materials

The fresh flowers of *C.gigantea* were collected in month of November 2018, from Mukkattukara and were identified in the Department of Botany, St. Mary's College Thrissur , Kerala.. The plant name was checked with International Plant Name Index (IPNI) and submitted a voucher specimen in our Dept herbarium.

2.2 Preparation of extracts

Flowers were shade dried for several days. The dried material was ground to a course powder and 50 gm of the powdered material was soaked in solvents of increasing polarity starting petroleum ether, chloroform, ethyl acetate , ethanol and distilled water(1:5) for 72 hours. The solvent was then removed by rotary evaporation. Each residue was weighed and the yield percentage was determined. Percent of yield calculated as follows:

Extract yield % = (w1\ w2) × 100

Where, w1 is net weight of powder in grams after extraction and w2 is total weight of powder in grams taken for extraction.

The dried extract was stored in refrigerator for further studies.

2.3 Qualitative phytochemical screening of plant extracts

Qualitative analysis of the petroleum ether, chloroform, ethyl acetate, ethanol and aqueous extracts of flowers of *C. gigantea* were further carried out to test the presence of phytochemicals such as alkaloids, flavonoids, terpenoids, sterols, tannins, glycosides, phenols etc using standard protocol given by Harborne,1998.

2.4 Anti – Oxidant Property Screening

The dried plant extracts were re- dissolved in dimethyl sulfoxide to get the solution of 10 mg / 10 ml for each extract which was

subjected to analysis of in vitro anti – oxidant activities .

2.4.1 DPPH Radical Scavenging Assay

Free radical scavenging activity of the *C. gigantea* flower extracts assessed based on the radical scavenging effect of the stable 1,1- diphenyl-2-picrylhydrazyl (DPPH) . The diluted working solutions of the test extracts (10 g/ml -320 g /ml concentration) and 6.34 m solution of DPPH were prepared in methanol and 100 µl test ,100 µl DPPH solution and 800 µl of methanol was taken in a test tube and mixed well . These solution mixtures were kept in dark for 20 min and optical density was measured at 517 nm using cecil- elect spectrophotometer . Methanol (900 µl) with DPPH solution (6.34 m,100 µl) taken as control and methanol as blank. The optical density recorded and % of inhibition calculated using the formula given below .

Percent (%) inhibition of DPPH activity

$$= A\text{-}B/A \times 100$$

Where A = optical density of control

B= optical density of sample

2.4.2 Calculation of IC 50 Concentration

The extract concentration corresponding to 50 percent inhibition (IC50) was calculated. Each sample was assayed in triplicate for each concentration.

2.4.5 In Vitro Cytotoxicity Study

The extract was studied for short term in vitro cytotoxicity using Dalton's lymphoma ascites cells (DLA). The tumour cells aspirated from the peritoneal cavity of tumour bearing mice were washed thrice with PBS or normal saline. Cell viability was determined by trypan blue exclusion method. Viable cell suspension ($1*10^6$ cells in 0.1 ml) was added to tubes containing various concentrations of test compounds and the volume was made up to 1 ml using phosphate buffered saline (PBS) . Control tube contained only cell suspension. These assay mixtures were incubated for 3 hours at 37 º c. Further cell suspension was mixed with 0.1 % trypan blue and kept for 2-3 minutes and loaded on a haemocytometer . Dead cells take up the blue colour of trypan blue ,while live cells do not take up the dye . The numbers of stained and unstained cells were counted separately .

% cytotoxicity

$$= \frac{No.of\ dead\ cell}{No.of\ live\ cells + No.of\ dead\ cell} \times 100$$

3 . Results

3.1 yield of extract

The extraction yields from flowers of *C.gigantea* using different solvent extracts are presented in (Table.1**)**. Comparatively, distilled water extract exhibited higher extraction yield (4.36%) than from other extracts. The extraction ability of different solvents for recovering extractable component from flower bud followed the order. Distilled water > Ethanol > Ethyl acetate > Chloroform > Petroleum ether. The variation in yield may be due to the polarity of the solvents used in the extraction process.

Table 1 : % yield of extract of flower bud of *C.gigantea* in various solvents

Selected Solvents	**Percentage yield (gm)**
Petroleum Ether	1.06
Chloroform	1.22
Ethyl acetate	1.43
Ethanol	1.75
Distilled water	4.36

3.2 Phytochemical screening

Petroleum ether, chloroform, ethyl acetate, ethanol, aqueous extract of *C. gigantea* flowers were subjected to preliminary phytochemical screening and the result of various phytochemical constituents are depicted in Table 2.

Petroleum ether extract showed the presence of carbohydrate, steroid, quinone, phenol, alkaloid,and tannin. Chloroform extract contained quinone, steroid, coumarin, flavanoid and phenol. Metabolites present in ethyl acetate extract were quinone, steroid, coumarin, flavanoid,coumarin, phenol and tannins. Quinone, coumarin, flavanoid, steroid, phenol, alkaloids and carbohydrate were present in ethanol extract. Metabolites like carbohydrate, quinone, coumarin, flavanoid, steroid, phenol, alkaloids, steroids and tannin were present in distilled water extract.

Moreover, information about different phyto constituents of plants is very important as it is much valuable in the production of complex chemical compounds and screening of their biological activities.

Table 2. Phytochemical screening of different flower extracts of *C. gigantean,* + indicates the presence of metabolite and - indicates the absence of metabolite

Primary/secondary metabolite	Petroleum ether	Chloroform	Ethyl acetate	Ethanol	Distilled water
Quinone	+	+	+	+	+
Cardiac glycoside	_	_	_	_	_
Steroid	+	+	+	+	+
Flavanoid	_	+	+	+	+
Alkaloid	+	_	_	+	+
Phenol	+	+	+	+	+
Saponin	_	_	_	_	_
Tannin	+	_	+	_	+
Coumarin	_	+	+	+	+
Terpenoid	_	_	_	_	_
Sugar	_	_	_	_	_
Protein	_	_	_	_	_
Ketose	_	_	_	_	_
Starch	_	_	_	_	_
Carbohydrate	+	_	_	+	+
Aminoacid	_	_	_	_	_
Picric acid	_	_	_	_	_

3.3 In Vitro Cytotoxicity Study

Ethanol and distilled water extract of *C.gigantea* flowers displayed cytotoxicity against DLA cells. The percentage of cell death in five different concentrations (µg/ml) of extracts are given below (Table 3).

The maximum 69.8 % of cell death occurred in 200 µg ethanol drug concentration where as in distilled water it was only 33.3 %. Drug discovery from medicinal plants has played an important role in the treatment of cancer and indeed most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been made towards combating cancer.

Table 3: In-vitro cytotoxicity study of Ethanol and Distilled Water extract of *C.gigantea*

Sl.No	Concentration (µg/ml)	Percent cell death (DLA)	
		Ethanol	Distilled water
1	200	69.8	33.3
2	100	42.8	19.4
3	50	34.2	14.2
4	20	20.5	8.6
5	10	7.6	4.7

3.4 In vitro Anti-oxidant property screening of flowers of C. gigantea - DPPH free radical scavenging assay

The free radical scavenging activity of different extracts of flowers of *C. gigantea* was studied by its ability to reduce the DPPH, a stable free radical and any molecule that can donate an electron or hydrogen to DPPH, can react with it and there by bleach the DPPH absorption. DPPH is a purple colour dye having absorption maximum of 517 nm and up on reaction with a hydrogen donor the purple colour fades or disappears due to conversion of it to 2,2- diphenyl-1-picryl hydrazine resulting in decrease in absorbance. Distilled water and Ethanol extracts showed maximum activity of 66.11and 61.55 respectively at 320 µg/ml. All the five extracts exhibited very good DPPH free radical scavenging activity (Table 4).

3.5 IC 50

IC 50 indicate the potency of scavenging activity. The antioxidant potential obtained through 2,2- Diphenyl -2-Picryl Hydrazyl method shows lowest IC 50 value for the distilled water extract (118.48 ± 13.96). Lower IC 50 value indicates the high antioxidant potential and property. The highest IC 50 value is for (356.87 ± 9.88) petroleum ether extract. The different solvents with their respective IC 50 values are depicted in Table.5.

Table 4. Percentage inhibition of DPPH free radical by different flower bud extracts of *C. gigantea* at 517 nm, (values are represented as mean ± standard deviation (n=3)

Sl.No	Concentration (µg/ml)	Percentage inhibition DPPH free radical (%)				
		Petroleum ether	chloroform	Ethyl acetate	Ethanol	Distilled water
1	10	5.77 ± 1.53	5.55 ± 2.54	7.08 ± 0.68	9.13 ± 0.71	20.39 ± 1.74
2	20	9.77 ± 2.03	12.85 ± 2.64	8.48 ± 0.88	11.26 ± 0.83	30.47 ± 2.01
3	40	16.44 ± 2.03	18.66 ± 2.84	21.06 ± 1.01	27.59 ± 1.99	36.62 ± 1.01
4	80	33.74 ± 0.71	26.66 ± 3.33	29.66 ± 3.41	36.72 ± 2.21	44.73 ± 2.28
5	160	34.70 ± 1.73	34.13 ± 2.27	49.89 ±1.55	52.15± 2.44	57.22 ± 1.72
6	320	46.16 ±0.85	47.51±0.98	54.24±0.83	61.55±1.34	66.11±0.58

Table 5: Comparison of IC 50 values of different flower extracts of C. *gigantea.* (values are represented as mean ±standard deviation).

Sl.No	Solvents	IC 50 (µg/ml)
1	Petroleum ether	356.87 ± 9.88
2	Chloroform	341.71 ± 12.95
3	Ethyl acetate	174.60 ± 21.28
4	Ethanol	153.30 ± 15.97
5	Distilled water	118.48 ± 13.96

4 . Discussion

Plants have been used for medicinal purposes long before recorded history. Many drugs listed as conventional medications were originally derived from plants. The secondary metabolites produced by the plants are usually responsible for the biological characteristics of plant species used throughout the world. The plants remain to offer mankind with new medicines. Some of the beneficial properties ascribed to plants have recognised to be flawed and medicinal plant treatment is

based on the experimental findings of hundreds to thousands of years (Balick, M.J. and Cox, P.A. 1997). Medicinal plants have proved their sole role in coping with a number of deadly diseases including cancer and the diseases associated with viral onslaught *viz*.Hepatitis, AIDS etc. Even today, plants are not only indispensable in health care, but form the best hope of source for safe future medicines . In spite of the fact that now we have at our command a number of modern drugs, it is still genuinely urgent to discover and develop new therapeutic agents (Bown D,1995).Traditional plant medicines still enjoy significant position in the modern-day drug industries due to the minor side effects as well as the synergistic action of the combination of compounds. Most of the important drugs of the past 50 years, which have revolutionized modern medicinal practice, have been isolated/derivatized from plants. These chemical ingredients exhibit therapeutic properties of plant and animal drugs (Angell M and Kassirer JP.,1998). The WHO endorses and promotes the addition of herbal drugs in national health care programs because they are easily accessible at a price within the reach of a common man and are time tested and thus considered to be much safer than the modern synthetic drugs. Thus, the research of pharmacologically/ biologically active agents obtained by screening natural sources such as plant extracts had led to the detection of many pharmaceutically valuable drugs that play a key role in the treatment of human diseases .The phytochemical-pharmacological research work has recently yielded effective solutions to certain diseases which synthetic drug industry has failed to afford (Bassam Abdul Rasool Hassan,2012).

All plants produce chemical compounds, These phytochemicals have potential for use as drugs, and the content and known pharmacological activity of these substances in medicinal plants is the scientific basis for their use in modern medicine, if scientifically confirmed (Koehn, F.E. and Carter, G.T. 2005). In consideration of the importance of phytochemical investigation on medicinal plants, the

Phytochemical analysis of *C.gigantea* was carried out in the present work. Alkaloids,

flavonoids steroids, coumarin, phenolic compounds, quinone, tannins and carbohydrate were found to be present in *C.gigantea* . The phytochemical analysis has established that the extracts from all the solvents contain active chemicals capable of curing ailments. Phenolic acids are ubiquitous in edible vegetable, fruits and nuts, and it is estimated that an average of 1-2g/day of these components may be consumed in a human diet, which have demonstrated potential antidiabetic effects (Violetta et al., 2012).

Pure isolated alkaloids and their synthetic derivatives are used as basic medicinal agents for their analgesic, bacterial effects etc (James, W. O.1950).

Flavonoid represent a beneficial group of naturally occurring compounds with hypoglycemic potentials (Bailey and Day.,1989). Quinones are characteristically highly reactive, colored compounds with two ketone substitutions in aromatic ring. These are another significant group of secondary metabolites with potential antimicrobial properties.

Coumarins (1-benzopyran-2-one) are chemical compounds in the benzopyrone class of organic compounds found in many plants. Coumarins possess a variety of biological properties, including antimicrobial, antiviral, antiinflammatory, antidiabetic, antioxidant, and enzyme inhibitory activity(Clark GS,1995). Steroidal glycosides, referred to collectively as saponins. These are bioactive compounds present naturally in many plants and known to possess potent hypoglycemic activity (Rao and Gurfinkel.,2000). Oxidative stress has been implicated in the pathology of many diseases and condition including diabetes, cardiovascular diseases, inflammatory conditions, cancer and aging. Antioxidant may offer resistance against the oxidative stress by scavenging free radicals, inhibiting lipid peroxidation and by many other mechanisms and thus prevent disease, and today widely used as free radicals inhibitors in food for maintaining the freshness, flavor and odor for a longer period. Antioxidants can be defined as compounds that can delay or prevent the oxidation of lipids or other molecules by

inhibiting the initiation or propagation of an oxidizing chain reaction and by many other mechanisms and thus prevent disease (Youdim and Joseph.,2001). For the past few decades, number of plants have been widely used for the treatment of various diseases due to their antioxidant properties.

In the present investigation, flower extracts of *C.gigantea* exhibited outstanding scavenging effects on DPPH. Since this investigation is a preliminary study, a detailed study of the antioxidant mechanisms of specific phenolic components is an absolute necessity. For further work on the profile and nature of chemical constituents of *C.gigantea* flowers will provide more information on the active principles responsible for their pharmacological properties. This may also lead to the development of a new generation of drugs that possess both chemotherapeutic and chemo preventive properties which can result in ways of combating the serious problems of diseases. Since many people in developing countries depend on traditional medicinal plants for their primary health care; it is very important to study the cytotoxic effects of the plant in use. *In vitro* cytotoxicity is necessary to define basal cytotoxicity such as the intrinsic ability of a compound to cause cell death as a result of damage to several cellular functions (Bouaziz *et al*., 2006). The crude extracts and pure compounds of medicinal plants are important in drug discovery; however their toxicity requires extensive attention since this can cause various side effects (biological implications) to human and animals. In general, cell type cytotoxic specificity of plant extracts is likely due to the presence of different classes of compounds (such as terpenes or terpenoids, and alkaloids) in the extracts. There are several types of cytotoxicity assays that can be used to determine the level of toxicity in the plant extracts. The DLA cell line cytotoxicity study of ethanol and distilled water extract of *C.gigantea* was conducted and the cell death was 69.8% at 200µg for ethanol extract. This study explores the goodness of the flowers of *C.gigantea* which has a commendable sense of purpose

and can be advised as a plant of phytopharmaceutical importance.

5. Conclusion

Phytochemical screening of petroleum ether, chloroform, ethyl acetate, ethanol, aqueous extracts of *C. gigantea* flowers revealed the presence of carbohydrate, quinone, coumarin, flavanoid, phenol, alkaloids, steroids and tannin by positive reaction with the respective test reagent. Phytochemical screening showed that maximum presence of phyto constituents in distilled water and ethanol extracts. The present study provides evidence that solvent extracts of *C. gigantea* contains medicinally important bioactive compounds and this justifies the use of plant species as traditional medicine for treatment of various diseases. However, further studies are required in this direction for its comprehensive analysis including qualitative analysis, characterize it's chemical structure and assess its biological activities. The phytochemical analysis of the medicinal plants are important and have commercial interest in both research insititutes and pharmaceutical companies for the manufacturing of the new drugs for treatment of various diseases.

The flowers of *C. gigantea* were found to scavenge the DPPH radicals in vitro in concentration dependent manner. When the concentration increases, percentage inhibition also increases . IC 50 value was found to low in distilled water extract than other extracts. The results from this study indicate the flowers of *C. gigantea* possess antioxidant properties and could serve as free radical inhibitors or scavenger, acting possibly as primary antioxidants. However further studies by in vivo models are still needed to confirm this property.

Among the different experiments studied ethanol and distilled water extract were found to be the best extract, which were used for DLA cell line study. Further studies are needed to clarify the *in vivo* potential of the flower extract in the management of various age-related human diseases resulting from oxidative stress.

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GPCRs : G protein linked Cell Signaling – A Review

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Abstract

G-protein-coupled receptors (GPCRs) are the largest and most diverse group of membrane receptors in eukaryotes. These cell surface receptors act like an inbox for messages in the form of light energy, peptides, lipids, sugars, and proteins. Such messages inform cells about the presence or absence of life-sustaining light or nutrients in their environment, or they convey information sent by other cells.GPCRs play a role in an incredible array of functions in the human body, and increased understanding of these receptors has greatly affected modern medicine.

Keywords - Cell signaling receptors, GPCRs (GPCRs - G-protein-coupled receptors)

1. Introduction

Cell to cell communication is fundamental for multi-cellular organisms as cells are required to communicate with their environment and among themselves in order to respond to particular stimuli. Such response is then integrated at the system level for the proper functioning of the organism. Dysregulation of cellular communication causes many diseases including cancer. Cell to cell communication involves many signalling pathways. These signalling pathways regulate multiple cellular processes by acting through sensors to stimulate molecules that are responsible for controlling different important cellular processes.

2. Types of Cell Signalling

Cell signaling can be classified as either

mechanical or biochemical based on the type of the signal. Mechanical signals are the forces exerted on the cell and the forces produced by the cell. These forces can both be sensed and responded to by the cells. Biochemical signals are the biochemical molecules such as proteins, lipids, ions and gases. These signals can be categorized based on the distance between signaling and responder cells. Signaling within, between, and amongst cells is subdivided into the following classifications:

- *Intracrine* signals are produced by the target cell that stay within the target cell.
- *Autocrine* signals are produced by the target cell, are secreted, and affect the target cell itself via receptors. Sometimes autocrine cells can target cells close by if they are the same type of cell as the emitting cell. An example of this are immune cells.
- *Juxtacrine* signals target adjacent (touching) cells. These signals are transmitted along cell membranes via protein or lipid components integral to the membrane and are capable of affecting either the emitting cell or cells immediately adjacent.
- *Paracrine* signals target cells in the vicinity of the emitting cell. Neurotransmitters represent an example.
- *Endocrine* signals target distant cells. Endocrine cells produce hormones that travel through the blood to reach all parts of the body.

3. Signalling Molecules

The important classes of signaling molecules are :

- Hormones are the major signaling molecules of the endocrine system, though they often regulate each other's secretion via local signaling (e.g. islet of Langerhans cells), and most are also expressed in tissues for local purposes (e.g. angiotensin) or failing that, structurally related molecules are (e.g. PTHrP).
- Neurotransmitters are signaling molecules of the nervous system, also including neuropeptides and neuromodulators. Neurotransmitters like the catecholamines are also secreted by the endocrine system into the systemic circulation.
- Cytokines are signaling molecules of the immune system, with a primary paracrine or juxtacrine role, though they can during significant immune responses have a strong presence in the circulation, with systemic effect (altering iron metabolism or body temperature). Growth factors can be considered as cytokines or a different class.

Signaling molecules can belong to several

chemical classes: lipids, phospholipids, amino acids, monoamines, proteins, glycoproteins, or gases. Signaling molecules binding surface receptors are generally large and hydrophilic (e.g. TRH, Vasopressin, Acetylcholine), while those entering the cell are generally small and hydrophobic (e.g. glucocorticoids, thyroid hormones, cholecalciferol, retinoic acid), but important exceptions to both are numerous, and a same molecule can act both via surface receptor or in an intracrine manner to different effects.In intracrine signaling, once inside the cell, a signaling molecule can bind to intracellular receptors, other elements, or stimulate enzyme activity (e.g. gasses). The intracrine action of peptide hormones remains a subject of debate.

Hydrogen sulfide is produced in small amounts by some cells of the human body and has a number of biological signaling functions. Only two other such gases are currently known to act as signaling molecules in the human body: nitric oxide and carbon monoxide.

4. Receptors

Cells have proteins called receptors that bind to signaling molecules and initiate a physiological response. Different receptors are specific for different molecules. Dopamine receptors bind dopamine, insulin receptors bind insulin, nerve growth factor receptors bind nerve growth factor, and so on. In fact, there are hundreds of receptor types found in cells, and varying cell types have different populations of receptors. Receptors can also respond directly to light or pressure, which makes cells sensitive to events in the atmosphere.

Receptors are generally transmembrane proteins, which bind to signaling molecules outside the cell and subsequently transmit the signal through a sequence of molecular switches to internal signaling pathways. Membrane receptors fall into three major classes: G-protein-coupled receptors, ion channel receptors, and enzyme-linked receptors. The names of these receptor classes refer to the mechanism by which the receptors transform external signals into internal ones — via protein action, ion channel opening, or enzyme activation, respectively. Because membrane receptors interact with both extracellular signals and molecules within the cell, they permit signaling molecules to affect cell function without actually entering the cell. This is important because most signaling molecules are either too big or too charged to cross a

cell's plasma membrane.

Not all receptors exist on the exterior of the cell. Some exist deep inside the cell, or even in the nucleus. These receptors typically bind to molecules that can pass through the plasma membrane, such as gases like nitrous oxide and steroid hormones like estrogen

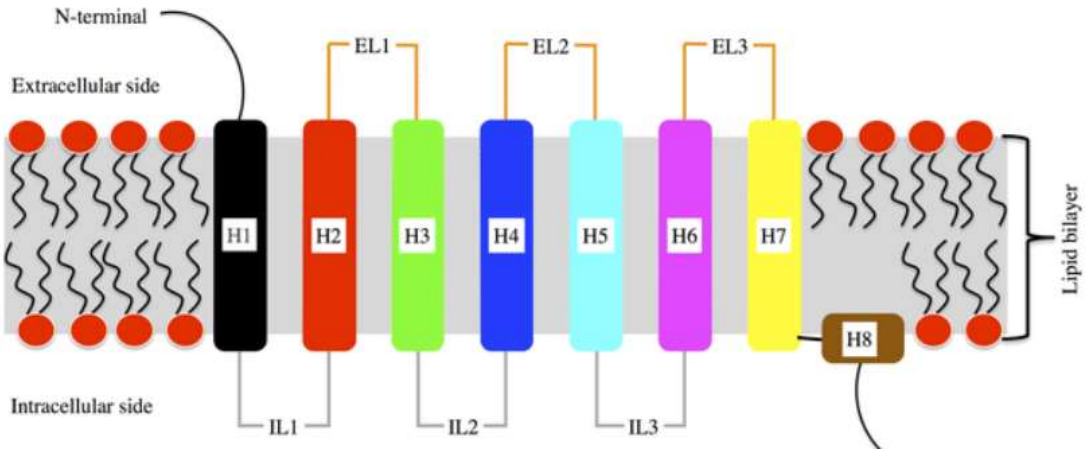
Once a receptor protein receives a signal, it undergoes a conformational change, which in turn launches a series of biochemical reactions within the cell. These intracellular signaling pathways, also called signal transduction cascades, typically amplify the message, producing multiple intracellular signals for every one receptor that is bound.

5. G-protein-coupled receptors (GPCRs)

G protein-coupled receptors (GPCRs), also known as seven-(pass)-transmembrane domain receptors, 7TM receptors, heptahelical receptors, serpentine receptor, and G protein–linked receptors (GPLR), constitute a large protein family of receptors that detect molecules outside the cell and activate internal signal transduction pathways and, ultimately, cellular responses. Coupling with G proteins, they are called seven-transmembrane receptors because they pass through the cell membrane seven times.

G protein-coupled receptors are found only in eukaryotes, including yeast, choanoflagellates,and animals. The ligands that bind and activate these receptors include light-sensitive compounds, odors, pheromones, hormones, and neurotransmitters, and vary in size from small molecules to peptides to large proteins. G protein-coupled receptors are involved in many diseases, and are also the target of approximately 34% of all modern medicinal drugs

It constitutes a large and diverse family of proteins whose primary function is to transduce extracellular stimuli into intracellular signals. They are among the largest and most diverse protein families in mammalian genomes. On the basis of homology with rhodopsin, they are predicted to contain seven membrane-spanning helices, an extracellular N-terminus and an intracellular C-terminus. This gives rise to their other names, the 7-TM receptors or the heptahelical receptors. GPCRs transduce extracellular stimuli to give intracellular signals through interaction of their intracellular domains with heterotrimeric G proteins, and the crystal structure of one member of this group, bovine rhodopsin, has recently been solved.



6.GPCR Classification

G protein-coupled receptors (GPCRs) are the largest class of membrane proteins in the human genome. The term "7TM receptor" is commonly used interchangeably with "GPCR", although there are some receptors with seven transmembrane domains that do not signal through G proteins. GPCRs share a common architecture, each consisting of a single polypeptide with an extracellular N-terminus, an intracellular C-terminus and seven hydrophobic transmembrane domains (TM1-TM7) linked by three extracellular loops (ECL1-ECL3) and three intracellular loops (ICL1-ICL3). About 800 GPCRs have been identified in man, of which about half have sensory functions, mediating olfaction (~400), taste (33), light perception (10) and pheromone signalling. The remaining ~350 non-sensory GPCRs mediate signalling by ligands that range in size from small molecules to peptides to large proteins; they are the targets for the majority of drugs in clinical usage , although only a minority of these receptors are exploited therapeutically. The first classification scheme to be proposed for GPCRs divided them, on the basic of sequence homology, into six classes. These classes and their prototype members were as follows: **Class A** (rhodopsin-like), **Class B** (secretin receptor family), **Class C** (metabotropic glutamate), **Class D** (fungal mating pheromone receptors), **Class E** (cyclic AMP receptors) and **Class**

F (frizzled/smoothened). Of these, classes D and E are not found in vertebrates.

An alternative classification scheme "GRAFS" divides vertebrate GPCRs into five classes, overlapping with the A-F nomenclature, *viz*:

Glutamate family (class C), which includes metabotropic glutamate receptors, a calcium-sensing receptor and $GABA_B$ receptors, as well as three taste type 1 receptors and a family of pheromone receptors (V2 receptors) that are abundant in rodents but absent in man.

Rhodopsin family (class A), which includes receptors for a wide variety of small molecules, neurotransmitters, peptides and hormones, together with olfactory receptors, visual pigments, taste type 2 receptors and five pheromone receptors (V1 receptors

Adhesion family GPCRs are phylogenetically related to class B receptors, from which they differ by possessing large extracellular N-termini that are autoproteolytically cleaved from their 7TM domains at a conserved "GPCR proteolysis site" (GPS) which lies within a much larger (~320 residue) "GPCR autoproteolysis-inducing" (GAIN) domain, an evolutionary ancient mofif also found in polycystic kidney disease 1 (PKD1)-like proteins, which has been suggested to be both required and sufficient for autoproteolysis

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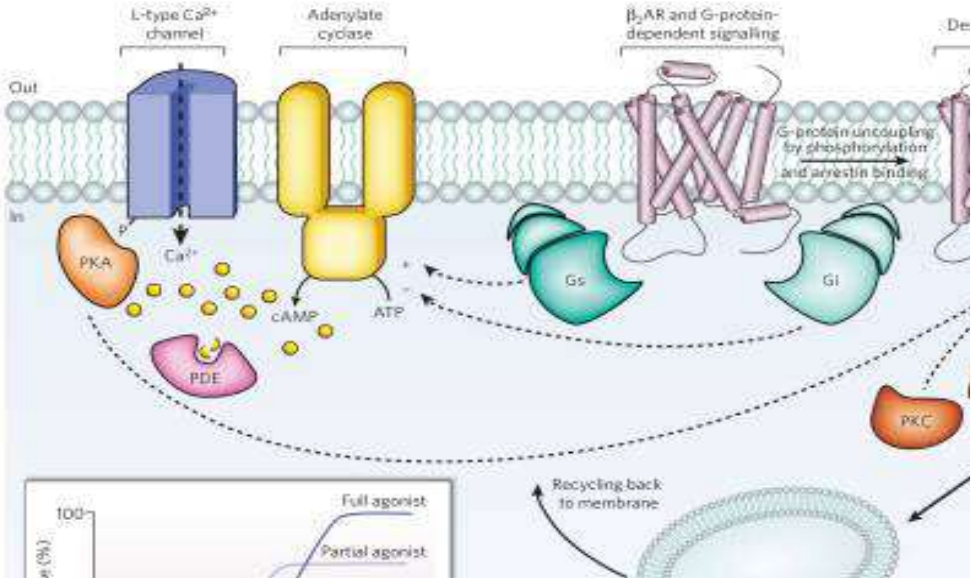

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Frizzled family consists of 10 Frizzled proteins (FZD(1-10)) and Smoothened (SMO). The FZDs are activated by secreted lipoglycoproteins of the WNT family, whereas SMO is indirectly activated by the Hedgehog (HH) family of proteins acting on the transmembrane protein Patched (PTCH).

Secretin family, encoded by 15 genes in humans. The ligands for receptors in this family are polypeptide hormones of 27-141 amino acid residues; nine of the mammalian receptors respond to ligands that are structurally related to one another (glucagon, glucagon-like peptides (GLP-1, GLP-2), glucose-dependent insulinotropic polypeptide (GIP), secretin, vasoactive intestinal peptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP) and growth-hormone-releasing hormone (GHRH)

7. G Protein Mediated Pathways

Secondary messenger Systems Involved In Signal Transduction: Adenylate cyclase cAMP mediated pathway and Phospholipase mediated pathway.

cAMP Mediated Pathway : The cAMP-dependent pathway, also known as the adenylyl cyclase pathway, is a G protein-coupled receptor triggered signaling cascade used in cell communication. When a GPCR is activated by its extracellular ligand, a conformational change is induced in the receptor that is transmitted to an attached intracellular heterotrimeric G protein complex.

Gs cAMP Dependent Pathway : The Gs alpha subunit of the stimulated G protein complex exchanges GDP for GTP and is released from the complex. In a cAMP-dependent pathway, the activated Gs alpha subunit binds to and activates an enzyme called adenylyl cyclase, which, in turn, catalyzes the conversion of ATP into (cAMP).Increases in concentration of the second messenger cAMP may lead to the activation of an enzyme called protein kinase A (PKA). ¬ The PKA enzyme is also known as cAMP-dependent enzyme because it gets activated only if cAMP is present. Many different cell responses are mediated by cAMP. These include increase in heart rate, cortisol secretion, and breakdown of glycogen and fat.This pathway can: Activate enzymes and Regulate gene expression If cAMP-dependent pathway is not controlled, it can ultimately lead to hyper-proliferation, which may contribute to the development and/or progression of cancer.Alterations in number, structure or function of receptors

will lead to disorder in cellular signal transduction. Which include, Up-regulation/hypersensitivity, Down regulation/desensitization , Receptor Gene Mutation

GicAMP Dependent Pathway: Gi mainly inhibits the cAMP dependent pathway by inhibiting adenylate cyclase activity, decreasing the production of cAMP from ATP, which, in turn, results in decreased activity of cAMP-dependent protein kinase. Therefore, the ultimate effect of Gi is the opposite of cAMP-dependent protein kinase.

8. Conclusion

GPCRs are a large family of cell surface receptors that respond to a variety of external signals. Binding of a signaling molecule to a GPCR results in G protein activation, which in turn triggers the production of any number of second messengers. Through this sequence of events, GPCRs help regulate an incredible range of bodily functions, from sensation to growth to hormone responses.

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Protease production by selected soil isolates in solid-state fermentation using agro industrial substrates

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Abstract

Protease is the collective name for various enzymes that break down or modify proteins or peptides. These enzymes are widely used in several industries, notably in detergent, food processing, brewing and pharmaceutical industries. They are also used for diagnostic, scientific and analytical purposes. In this study the protease producing bacteria were isolated from detergent contaminated soil. The proteolytic bacteria identified as Bacillus species, based on various morphological, staining and biochemical characteristics was used for Protease enzyme production under Solid state fermentation conditions. Enzyme was quantitated, cultural conditions like fermentation time and effect of inoculum optimised. The partial purification of the enzyme was followed by confirmation of its gelatinolytic activity by dot blot assay. Gelatin zymography, an in-situ method of detection of proteolysis and separation, showed a zone of gelatin clearing zone. Thus, the bioconversion of locally available agricultural wastes such as rice bran, wheat bran, musambi peel powder, orange peel powder, etc to high value protease enzyme preparation was achieved using a novel Bacillus isolate under solid-state fermentation conditions.

Keywords: Casein nutrient agar, solid state fermentation, protease, *Bacillus* species, biochemical identification

1. Introduction

Proteases represent the class of enzymes which occupy a pivotal position with respect to their physiological roles as well as their commercial applications. Proteases represent one of the three largest groups of industrial enzymes and microbial proteases have eco-friendly as well as commercial importance.

Microbial proteases are among the most important hydrolytic enzymes (Gupta *et al, 2002*) with degradative and synthetic functions. Microbes are an attractive source of proteases owing to the limited space required for their cultivation and their ready susceptibility to genetic manipulation. Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Godfrey *et al, 1996*). Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications.

Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus*. Bacterial neutral proteases are active in a narrow pH range (pH 5 to 8) and have relatively low thermo tolerance. Due to their intermediate rate of reaction, neutral proteases generate less bitterness in hydrolyzed food proteins than do the animal proteinases and hence are valuable for use in the food industry. Their low thermo tolerance is advantageous for controlling their reactivity during the production of food hydrolysates with a low degree of hydrolysis. Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10, and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry.

Fungi elaborate a wider variety of enzymes than do bacteria. For example, *Aspergillus oryzae* produces acid, neutral, and alkaline proteases. The fungal proteases are active over a wide pH range (pH 4 to 11) and exhibit broad substrate specificity. However, they have a lower reaction rate and heat tolerance than do the bacterial enzymes. Fungal enzymes can be conveniently produced in a solid-state fermentation process. Fungal acid proteases have an optimal pH between 4 and 4.5 and are stable between pH 2.5 and 6.0. They are particularly useful in the cheese making industry due to their narrow pH and temperature specificities. Fungal neutral proteases are metalloproteases that are active at pH 7.0 and are inhibited by chelating agents. Fungal neutral proteases supplement the action of plant, animal, and bacterial proteases in reducing the bitterness of food protein hydrolysates. Fungal alkaline proteases are also used in food protein modification (Mala *et al, 1998*).

Proteases have a large variety of applications, mainly in the detergent and food industries.

In view of developing environmentally friendly technologies, proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes. Proteases are used extensively in the pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds, etc. Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations; whereas those that are used in medicine are produced in small amounts but require extensive purification before they can be used (Mala *et al, 1998*). Besides their industrial and medicinal applications, proteases play an important role in basic research. Their selective peptide bond cleavage is used in the elucidation of structure-function relationship, in the synthesis of peptides, and in the sequencing of proteins.

A number of microorganisms have been reported to produce a variety of proteases. The current investigation is aimed at isolating protease producing microorganisms followed by solid state fermentative production of the enzyme.

2. Materials and Methods

2.1 Microorganisms

Protease producing microorganisms isolated from poultry and detergent contaminated soil, air, etc. were used in the present study.

2.2 Substrates

Powdered Orange peel, Wheat bran, Musambi peel, Rice bran.

2.3 Methods

2.3.1 Casein nutrient agar plate test for isolation of proteolytic bacteria

Soil samples were collected from poultry farm and from detergent contaminated area. 1g of soil was weighed and uniformly mixed in 100ml of sterile water followed by serial dilution. Dilutions were spread plated onto sterilized casein nutrient agar plates (nutrient agar with 1% casein) and then kept for incubation at room temperature for 48 hours and development of the zone of hydrolysis was noted. Casein agar plates were also exposed to air, in order to isolate protease producing organism. After exposure, plates were incubated at room temperature for 48 hours and checked for the development of zone of clearance. The colonies which showed zone of clearance indicated the presence of casein hydrolysing enzymes being produced by those microorganisms. These colonies were taken up for further study.

2.3.2 Gelatin plate test for Protease

The organisms showing zone of clearance were plated onto nutrient agar medium containing 0.4% gelatin. After incubation at room temperature for 48 hours, plates were flooded with 1% tannic acid. Protease producing colonies were identified on the basis of development of halos around the colonies on addition of 1 % tannic acid. Isolates having a higher ratio of clearing zone to colony size were selected for the production of enzyme under solid state fermentation conditions.

These isolates were grown in liquid broth and the amount of protease production was determined from the culture filtrate. The isolates which showed higher protease activity was selected and maintained on nutrient agar slant to be used for further studies. Selected isolate was identified based on morphological and biochemical characteristics as per Bergey's manual of determinative bacteriology.

2.3.3. Production of enzyme

2.3.3.1. Inoculum preparation

5ml of medium containing nutrient broth and 1% casein was sterilized and inoculated with one loop full of isolated culture samples followed by incubation at room temperature for 24 h. 2% of inoculum was then transferred to 100 ml production media.

2.3.3.2. Submerged fermentation for the production of enzyme

100ml of production medium containing casein as substrate was prepared and sterilized in autoclave. The selected cultures were inoculated into the medium and it was kept under shaking conditions till 48 h. The enzyme samples were taken at regular intervals, centrifuged at 10000rpm for 10 min and the supernatant containing the crude enzyme was assayed for its activity and protein content by Lowry's method (Lowry *et al*., 1951). The isolates which gave maximum enzyme production were subjected to solid state fermentation (SSF).

2.3.3.3 Solid state fermentation for the production of enzyme

SSF was carried out in 250ml conical flask containing 5g of agricultural residues (Musambi peel powder, orange peel powder, rice bran, wheat bran) moistened with 10 ml of salt solution (10mg $MnSO_4$, 50mg $CaCl_2$, 20mg $MgSO_4$ in water) and autoclaved for 20 minutes. This enables sterilization as well as softening the hard texture of the substrates so as the microbe digests them easily yielding optimal enzyme production. After cooling, the flasks were inoculated with 10%

inoculum (v/w) of 24 h grown culture broth. The contents were mixed thoroughly and incubated in a slanting position to provide maximum surface area, at room temperature for various time periods (12, 24, 36, 48, 60, 72,and 84 h). For enzyme extraction, at the end of incubation, distilled water was added to the culture flask (3 times v/w), flask subjected to uniform shaking at 200 rpm. The content of flask was filtered using a Whatmann No.1 filter paper, centrifuged and the supernatant analysed for enzymatic activity.

2.4 Tyrosine standard curve

Tyrosine standard curve was plotted using known concentrations of tyrosine and estimating the amino acid content using Lowry's method. Absorbance was measured at 670 nm using spectrophotometer.

2.5 Protease assay

The culture filtrate serves as the source of enzyme. The enzyme protease reacts with the casein and liberates tyrosine. The liberated tyrosine in alkaline conditions causes the reduction of phosphomolybdate and phosphotungstate in Folin- Ciocalteau's reagent to give blue color, the color developed is measured at 670nm in a spectrophotometer. The absorbance serves as the parameter of the estimation of tyrosine produced. The protein content of the enzyme sample was estimated by the Lowry's method.

The culture supernatant (1 ml) was incubated with 1 ml 2% casein in phosphate buffer (50mM, pH 7) at 50°C for 10 min and then reaction terminated by addition of 5 ml (5%) trichloro acetic acid. After 30 min, mixture was filtrated and 2 ml of filtrate added to 5 ml reagent C and incubated for 10 min followed by the addition of 0.5 ml reagent D. After 30 min incubation at room temperature in dark, absorbance was measured at 670 nm. The amount of amino acids released was calculated from a standard curve plotted using known concentrations of tyrosine and the enzyme activity expressed as one unit of enzyme activity being the amount of enzyme required to release the 1µg of tyrosine/min under standard conditions.

2.6 Identification of bacteria

2.6.1 Microscopic examination

Gram staining

A drop of sterilized distilled water was taken on the middle of the clear slide. Then a loopful of bacterial suspension (young culture) was transferred to the sterilized drop of water and a very thin film was prepared on the slide by spreading uniformly. The film was fixed by passing it over the gentle flame

for two or three times. The slide was flooded with crystal violet solution and allowed to stand for 1 minute. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. Gently flood the smear with Gram's iodine and let stand for 1 minute. Tilt the slide slightly and gently rinse with tap water or distilled water using wash bottle. The smear will appear as a purple circle on the slide. Decolorize using 95% ethyl alcohol or acetone. Tilt the slide slightly and apply the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear. Be careful not to over-decolorize. Immediately rinse with water. Gently flood with safranin to counter-stain and let stand for 45 seconds. Blot dries the slide and observes the smear using a light microscope under oil-immersion.

Motility test – Hanging drop method

A loopful of bacterial suspension is placed in the centre of a cover slip, containing paraffin wax on all the four corners. The glass slide is placed over the cover slip in such a way that cover slip sticks to the slide. Immediately, the glass slide is lifted and turned around. The drop of bacterial suspension now "hangs" on the lower surface of the cover slip. The drop is then observed under the low power (10x) dry objective of the compound microscope. The edge of the drop must be focused. Bacteria tend to accumulate at the edge of the drop. Once the edge is located, it is then observed under 40x high power objective.

Endospore staining

One drop of sterile saline water was taken on a clean glass slide for spore staining. A loopful of bacterial old slant culture was taken in the drop and smear was made on the slide. The film was dried over flame, gentle heating was done. The slide was then placed over a beaker and 5 % malachite green was added drop wise. Boiling of the malachite green was avoided by adding more malachite green. The slide was taken out of the stream and washed gently with tap water. The preparation was flooded with safranin solution for about 1 minute and washed with gentle stream of tap water and placed under the immersion lens with immersion oil.

2.6.2Biochemical tests

Catalase test

Catalase test that carried out of one drop of 30% hydrogen peroxide placed on a slide. One loopful of the fresh bacterial culture was taken by a sterile needle and placed on the drop of hydrogen peroxide. Bubble production indicated positive result.

Oxidase test

In oxidase test bacterial colonies were picked with a platinum wire and streaked on filter paper saturated with 0.5% tetramethyl-*p*-phenylenediamine hydrochloride or commercially available paper discs. Rapid appearance of a dark purple colour was considered a positive reaction.

Carbohydrate fermentation test

Nutrient broth is used as basal medium for fermentative test. Phenol red is used as an indicator. Using sterile techniques, inoculate each experimental organism into appropriately labelled fermentation tubes using inoculation loop. Do not shake the tubes as it may cause the air bubbles to get trapped in Durham tube. Incubate all tubes for 24 hours at 37˚c.

Triple sugar iron test

Using sterile technique, inoculate each experimental organism into triple sugar iron agar slant tubes by means of a stab and streak inoculation. One tube kept as control. Incubate for 18-24 hours at 37˚C. Examine the colour of both the butt and slant of all inoculated tubes and determine the type of reaction that has taken place (acid, alkaline or none) and the carbohydrate that has been fermented in each culture. Examine all cultures for the presence or absence of blackening with in the medium and determine the ability of the organism to produce H_2S.

IMViC

Indole Production test

For the Indole, one loopful fresh bacterial culture (24 hours old) was inoculated in peptone broth and incubated at 37˚C for 1-3 days, after incubation, Kovac's solution was added and shaken vigorously for one minute. A red colour in the reagent layer indicated positive reaction.

Methyl Red test

Using sterile technique, inoculate each experimental organism into its appropriately labelled tube containing MR-VP broth by means of a loop inoculation. The last tube will serve as a control. Incubate all cultures for 24 to 48 hours at 37˚C. After incubation add 5 drops of methyl red indicator and observe the colour change.

Voges Proskauer (VP) test

Using sterile technique, inoculate each experimental organism into its appropriately labelled tube containing MR-VP broth by means of a loop inoculation. The last tube will serve as a control. Incubate all cultures for 24 to 48 hours at 37˚C. After incubation add 12 drops of Barritt's reagent A and 2-3 drops of Barritt's reagent B. Shake the tubes gently for 30 seconds with the caps off to

expose the media to oxygen. Allow the reaction to complete for 15-30 minutes and observe the colour change.

Citrate utilization test

For the citrate utilization test, slope culture with a 1 inch butt of Simmon's citrate agar was inoculated by streaking over surface with an inoculation loop and incubated at 37°C for 3 days. The colour of the medium changed from green to bright blue due to the utilization of citrate and when citrate is not utilized, the colour of the medium remain unchanged.

3. Results and Discussion

Proteases, an important group of industrial enzymes, are produced by a wide range of microorganisms, including fungi and bacteria. Proteases have numerous applications in detergent, food, pharmaceutical and leather industries however, the cost of alkaline protease is a major issue in enzyme applications in different industries. Solid state fermentation employing agricultural residues is a highly cost effective and economically viable alternative for the production of enzymes and application of crude enzyme for various industrial processes. Solid state fermentation results in value addition of these agro-industrial residues besides providing a natural substrate for microbial growth and fermentation.

In the present study an attempt was made to isolate proteolytic bacteria from soil followed by solid state fermentative production of the enzyme. The morphological, staining and biochemical characteristics of the isolates were analysed to perform preliminary identification of the isolates.

3.1. Isolation and screening of proteolytic bacteria

3.1.1 Casein nutrient agar plate test

Zone of clearance around the colony after 24 – 48 h of incubation was showed in the detergent soil sample and in plates after air exposure. Organism producing enzyme shows a clear zone around the colony as the protein near the colony is utilized. Depending upon the ratio of zone of clearance to colony size, two high clearing organisms were selected for further experimental studies.

3.1.2 Gelatin plate test

Isolates showing clear zone on casein agar plates were further spotted on media containing 0.4% gelatin. After incubation for 48 h at room temperature, they showed

clearance following the addition of 1% tannic acid.

Table 3.1 Isolates and zone of clearance

Sl No.	Isolates	Zone of clearance (cm)
1	D_1	3.8
2	D_6	4

Figure 3.1 Casein agar plates with protease producing colonies

Fig.3.2 0.4% gelatin agar plates with colonies showing zone of clearance

3.2 Enzyme production

The isolate D_6 showing higher clear zone were subjected to submerged fermentation conditions for the enzyme production in casein nutrient broth media and the amount of protease production was determined from the culture filtrate. One unit of enzyme activity was defined as the amount of enzyme required to release the 1µg of tyrosine/min under standard conditions.

The amount of tyrosine liberated is calculated by plotting a standard curve using known concentrations of tyrosine and estimating the amino acid content using Lowry's method. The standard curve gave an equation y = 0.1539x, where x is the amount of tyrosine liberated and y is the optical density at 670nm.

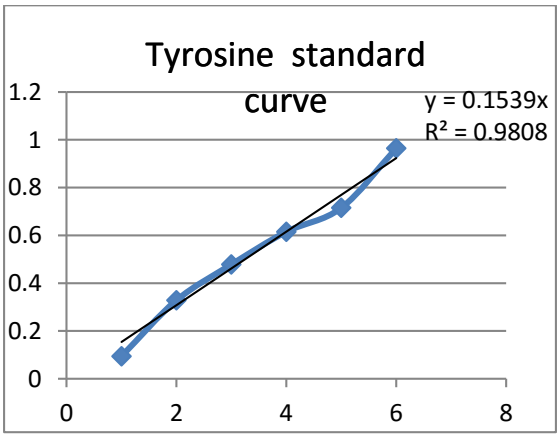


Fig 3.3 Standard graph of tyrosine

The maximum production of enzyme of 0.011 U/ml/mn was obtained at 96 h of incubation with the isolate D_6 (Table 3.2).

3.3 Solid state fermentation for the production of enzyme

The use of agro-industrial residues as the basis for cultivation media is a matter of great interest, aiming to decrease the costs of enzyme production and meeting the increase in awareness on energy conservation and recycling. Solid state fermentation (SSF) was carried out using $D_1$and D_6 in 250ml conical flask containing 5g of agricultural residues (Musambi peel powder, orange peel powder, rice bran, wheat bran) moistened with 10 ml of salt solution. The protease activity is reported per gram of dry solids used in initial extraction.

Table 3.2 Protease activity in solid state fermentation

Isolates	**Substrates**	**Protease activity U/g**
D_6	Musambi	0.3
	Orange	0.45
	Wheat	0. 18
	Rice bran	0.03

The isolate D_6 was successfully used in degradation of pectinaceous waste into useful products like enzyme and biomass by using cheap substances like wheat bran, rice bran, and fruit peel wastes. Orange and Musambi fruit peel wastes yielded good levels of enzyme -0.315 U/g and 0.3 U/g with D_6 and have the potential to be used as production medium for proteases. The choice of an appropriate inducing substrate is of great importance in the production of protease, since the enzyme production have been shown to be affected by the nature of the substrate used in fermentation, in earlier studies.

Maximum enzyme activity of 0.825 (U/g) and 0.915 (U/g) was obtained with the orange peel substrate by 9th day of incubation (Table 3.3).

Table 3.3 Time Course of Protease activity

Day	**Substrate**	**Protease Activity of D_6 (U/g)**
5th day	Musambi	0.135
	Orange	0.105
	Wheat	0.09
	Rice bran	0.06
9th day	Musambi	0.69
	Orange	0.915
	Wheat	0.315
	Rice bran	0.135
11th day	Musambi	0.675
	Orange	0.615
	Wheat	0.3
	Rice bran	0.135

The incubation period is directly related to production of enzymes and other metabolites. After that, the enzyme production and the growth of the microorganism decreases; this can be attributed to the reduced availability of nutrients and the production of toxic metabolites.

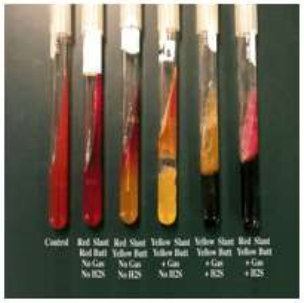
3.5 Identification of the bacterial isolate

The isolate D6 which showed good protease activity was identified based on morphological and biochemical characteristics as per Bergey's manual of determinative bacteriology. Gram staining showed D6 as Gram positive spore forming rod shaped bacteria. The strains were further subjected to biochemical observations as shown in Table 3.4.

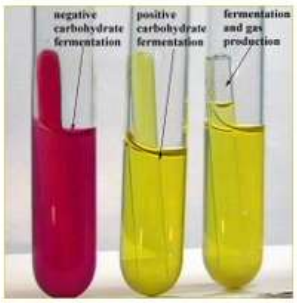
Table 3.4 Microscopic and biochemical identification of the isolates

Sl No	**Test**	**D_6**	
		Observation	**Inference**
1	Gram staining	Gram positive rod	Gram positive rod
2	Motility test	Motile	Motile
3	Endospore staining	Spore present	Spore forming
4	Catalase test	Bubbles formed	+ve
5	Oxidase test	Development of blue colour	+ve
6	Indole production test	No development of red layer on the top	-ve
7	Methyl red test	Development of red colour	+ve
8	Voges Proskauer test	No red colour formation	-ve
9	Citrate utilization test	Development of blue colour	+ve
10	Triple sugar iron test	Yellow slant, yellow butt, no H_2S	Acid slant, Acid butt No H_2S
11 a)	Carbohydrate fermentation Sucrose	Development of yellow colour in the medium	Acid production
b)	Mannitol	Broth retained red colour	No acid and gas production
c)	Dextrose	Development of yellow colour in the medium	Acid production
d)	Lactose	Broth retained red colour	No acid and gas production
e)	Maltose	Broth retained red colour	No acid and gas production

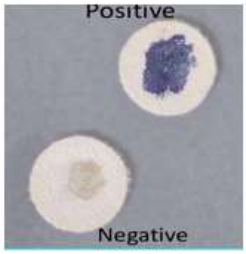
From the above observations, the isolate D_6 was tentatively identified as Bacillus species.



Triple Sugar Iron Test



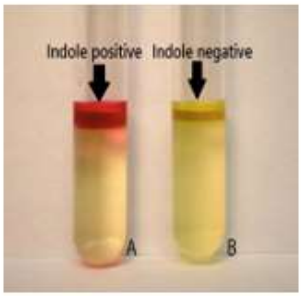
Carbohydrate Fermentation Test



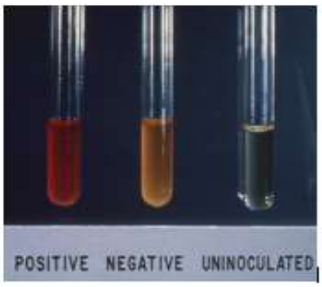
Oxidase Test

Catalase Test

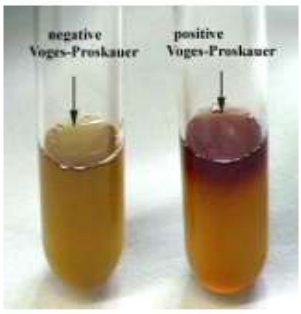
Fig 3.5 Biochemical identification of the isolates



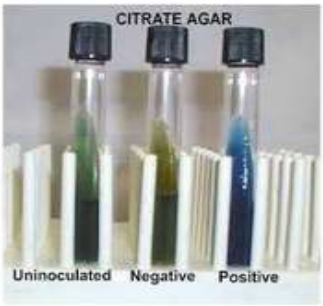
Indole ProductionTest



Methyl Red Test



Voges- Proskauer Test



Citrate Utilization Test

Fig 3.6 Biochemical identification of the isolates

4. Conclusions

The present study was to isolate protease producing microorganisms and to check their ability for enzyme production under solid state fermentation condition from cheap agricultural wastes. Bacillus species isolated yielded good levels of enzyme with orange and musambi fruit peel wastes. Further, optimization of enzyme production with respect to various parameters like fermentation time, incubation temperature, moisture content, inoculum level, substrate concentration etc will be pursued.

5. References

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Mala B. Rao, Aparna M. Tanksale, Mohini S. Ghatge, and Vasanti V. Deshpande Molecular and Biotechnological Aspects of Microbial Proteases. (1998) 62(3): 597–635.

Annals of Basic and Applied Sciences

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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.

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