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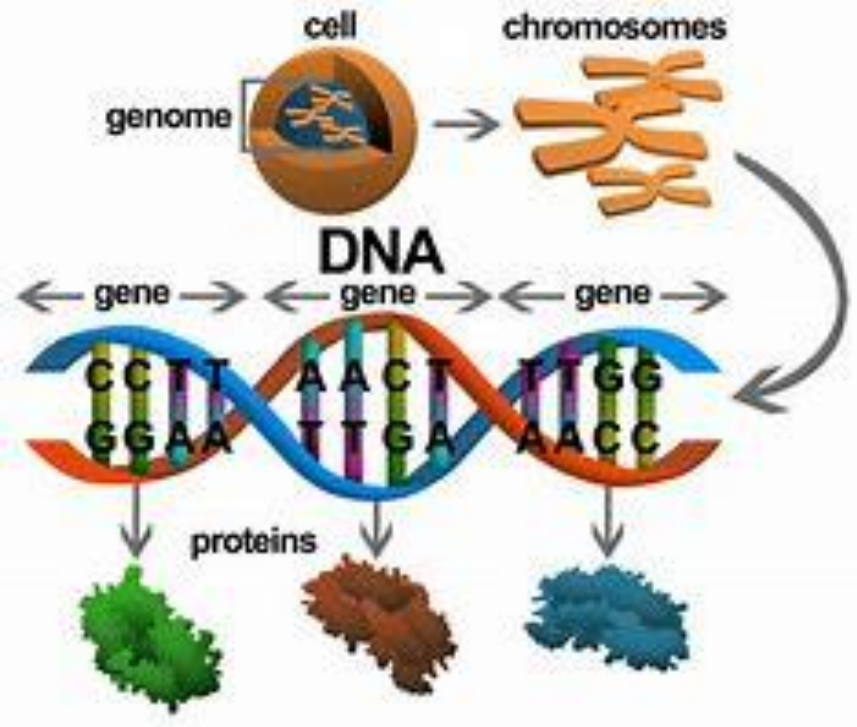

MOLECULES INTERACTION

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Cell and Biotechnology -

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Research Article

In-Vitro Interaction of Soluble and Amyloid form of Serum Amyloid A Protein with Serum Amyloid P Component to BC3H1cells

Asokan. C* , Thirumagal. J, Karthigai Devi. S, Venkatesan. C, Sangeetha. R, Raghuvarman G , Nirmal Kumar. C, Megaladevi. P and Kowsalya. G

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ABSTRACT

The BC3H1 smooth muscle cells of mice brain, the study was carried out membrane binding. This is important in relation to the activity of membrane proteins, because losing the activity of such systems will ultimately lead to malfunction or death of the cell. The interactions of Serum Amyloid A (SAA) and Serum Amyloid A protofibrils with Serum Amyloid P component (SAP ($CaCl_2$)) to BC3H1 cells of the mouse are dealt with in detail to study the binding of SAA protofibrils in various conditions. The FACScan and MTT assay results have shown the SAA and SAA fibrils binding with SAP ($CaCl_2$) 0.12-1.2 nM and cell toxicity with the BC3H1 cells. Specifically, cells were incubated with 1.25-6.25 µM SAA-FITC and SAA protofibrils-FITC 2.5 µM with SAP ($CaCl_2$) 0.12-1.2 nM assayed. The 50% viable BC3H1 cells at 4–6 µM with an LD_{50} of 3.5 µM. The interaction of serum amyloid A fibrils with a cell surface binding site/receptor might alter the local environment to cause cellular dysfunction and to be more favorable for amyloid formation. The RAGE (receptor for advanced glycation endproducts) a polyvalent receptor in the immunoglobulin super family has been implicated in binding with the isoform of SAA (SAA1.1) which has the highest fibirillogenic property. In the present study, concluding the SAA fibrils more binding and cell cytotoxicity than SAA protein and protecting role with SAP ($CaCl_2$).

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INTRODUCTION

The interaction with toxic amyloid fibrils is another type of cellular insult for which the mechanism is not yet known. Explanations like oxidative and ionic imbalance are being given with a lot of experimental evidence. Recently, membrane-stabilized protofibrillar structures were implicated in amyloid-related toxicity to mammalian cellular system with membrane-amyloid interaction as the central point of focus in the understanding of amyloid chemistry. The cell membrane is a dynamic entity, containing highly organized proteins, which are under constant thermal perturbation. The integrity of the membrane is maintained by the dynamic lipid matrix by acting

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as a thermal sink. Oxidative and ionic perturbations are other cellular insults. Various transporters nullify the ion fluctuation effects. The thermal fluctuations are transduced to the system through the cell membrane, and hyper thermal effects get attenuated through the expression of the heat shock proteins. The cells are protected from oxidative insults by the various anti oxidant mechanisms. The membrane is damaged when these intricate balances are lost. Yet another hypothesis predicts the specific denaturing ability of amyloid fibrils on interacting with native proteins. This is further supported by the research findings that amyloid have a very high structure breaking effect on vital proteins. Several groups have reported the loss of membrane-bound enzymatic activity due to amyloid interaction. Amyloid is one protein known to have anti chaperonic activity with very high membrane binding property. The toxic mechanism of any amyloid depends upon the specific and non-specific binding effect of amyloid assemblages in various forms.

SAP (PTX2) is a member of the pentraxin family of proteins that includes C-reactive protein (CRP; PTX1) and pentraxin-3 (PTX3). SAP is made by hepatocytes and secreted into the blood (Pepys et al, 1997). A search of proteomics and RNA-seq databases suggests that the liver is the major source of SAP. In humans and most mammals, the levels of SAP in the plasma are maintained at relatively constant levels, between 20 and 50μg/ml (Nelson et al, 1991). There is little evidence for sequence variation of SAP at the genomic or amino acid level. In mice, SAP acts as an acute phase protein, with levels rising up to 20-fold following an inflammatory insult (Le et al, 1982). SAP is a pentameric protein with sequence and structural similarity to CRP (Du Clos, 1996). The structure of SAP (and CRP) pentamers is a flat disk with a hole in the middle (Emsley et al, 1994). The crystal structure of PTX3 has yet to be determined, but models based on site-directed mutagenesis, electron microscopy, and small-angle X-ray scattering data suggests that PTX3 is an octamer of two tetramers (Garlanda et al, 2018). Each SAP molecule has two Ca++ atoms bound to it, and the pentamer thus has 10 Ca++ atoms on one side of the disk. With the help of the bound Ca++, this side of the disk binds to a variety of molecules including apoptotic debris, bacterial polysaccharides, amyloid deposits, and bacterial toxins (Hamazaki, 1995).

It has been shown that the synthesis of A-apoSAA is induced by proinflammatory cytokines (Betts et al 1991). In particular, the acute-phase genes coding for SAAs are regulated by IL-1 or TNF and reach the maximum stimulation in the presence of interleukin-6. While the primary source of circulating A-apoSAAs is the liver for the systemic acute-phase response, extrahepatic expression of these proteins has been demonstrated in different species and in various cell types, such as cultured smooth muscle cells, endothelial cells, monocytes, and macrophage cell lines. Therefore, it appears that these cells could provide a local source of SAA proteins. The local production of A-apoSAA raises the possibility that A-apoSAA may play a role related to the site of expression rather than to acute-phase systemic response.

Phagocytic cells such as monocytes and macrophages then bind the SAP, CRP, or PTX3, and engulf the debris or other material the pentraxin has bound (Bharadwaj et al, 2001). CRP and PTX3 can similarly bind a variety of debris molecules (Du Clos, 2013). Proteins with strong similarity to SAP (and CRP and PTX3) are present in the hemolymph of horseshoe crabs (Iwanaga, 2002) The Pepys group found a small molecule compound that causes two human SAP pentamers to stick to each other, and this complex is then quickly cleared from the circulation (Pepys et al, 2002). In SAP knockout mice expressing human SAP, the compound decreased serum SAP levels but did not reduce the severity of experimentally-induced amyloidosis. Adding anti-SAP antibodies to this treatment however did reduce experimentally-induced amyloidosis, suggesting that reducing SAP levels is a possible therapeutic for amyloidosis (Bodin et al, 2010).

This question is especially important in relation to the activity of membrane proteins, because losing the activity of such systems will ultimately lead to malfunction or death of the cell. In this study, the interactions of SAA and SAA protofibrils with different cells of the mouse are dealt with in detail to study the binding of SAA protofibrils in various conditions. Specifically, interaction of amyloid A fibrils with a cell surface binding site/receptor might alter the local environment to cause cellular dysfunction and to be more favorable for amyloid formation. Already RAGE (receptor for advanced glycation endproducts) a polyvalent receptor in the immuglulobulin superfamily has been implicated in binding with the isoform of SAA (SAA1.1) which has the highest fibirillogenic property.

MATERIALS AND METHODS

Isolation of SAA Protein:

HDL was isolated from the pooled plasma by sequential centrifugation (Lindhorst et al 1997). Briefly, the density of the plasma was adjusted to 1.063 g/l with solid KBr, followed by centrifugation at 10°C for 16 hrs at 75000g in a Beckman 50 Ti rotor. The top quarter, which contained VLDL and LDL, was aspirated and discarded. The pooled infranatants, adjusted to solvent density 1.21 g/l by addition of KBr, were re-centrifuged for 48 hrs at 106,000g in 50 Ti rotor. The top layers (HDL) were aspirated, pooled and concentrated

with 0.15 M NaCl, 0.1% EDTA, pH 6.4 for 2 hrs at 40°C and then with 10% formic acid for 2 hrs using FILTRON. (GELMAN Sciences. India).

Gel Chromatography of HDL-SAA Protein

The concentrated HDL preparation was fractionated on a column 2.5 cm × 100 cm of Sephacryl S-200 (Pharmacia Biotech, Uppsala, Sweden) previously equilibrated with 10% (v/v) formic acid at a flow rate of 25 mL/hr. 5 mL fractions were collected using FPLC (Fast Protein Liquid Chromatography) Gradifrac™ system (Pharmacia Biotech, Uppsala, Sweden). Hiload pump p-1 fractions were monitored at 280 nm. Fractions were pooled and concentrated using FILTRON (GELMAN Sciences, India) and freeze-dried.

Anion Exchange Chromatography

The freeze dried sample obtained from the gel filtration step was dissolved in 10 mM tris (pH 8.2/8 M urea and was applied to a column (1 cm × 30 cm) of DEAE cellulose (Whatman DE-52) previously equilibrated with the same buffer. After loading, the column was washed further with 80 mL of initial buffer, using FPLC Gradifrac™ system (Pharmacia Biotech, Uppsala, Sweden). The column was then eluted with a linear gradient of 150 mL each at 10mM Tris (pH 8.2)/8 M urea and 150 mM Tris (pH 8.2)/8 M urea. The flow rate was 12 mL/hr and 3 mL fractions were collected. Fractions were pooled and concentrated using FILTRON (GELMAN Sciences, India). At the end of the gradient, the column was further washed with 7.5 mL of 1 M Tris (pH 8.2)/8 M urea. Protein was determined by the method of Lowry et al (Lowry et al 1951). Fractions were pooled and concentrated using FILTRON (GELMAN Sciences, India) and freeze-dried.

Characterization of SAA Protein

Column fractions were analyzed for their potential component on 15% (w/v) polyacrylamide gel in SDS system as described by Laemmli (1970) and 10-20% native-PAGE gradient gels system as described by Layfield et al (1996).

RP-HPLC. The RP-HPLC equipment (Kaplan et al 1999) consisted of a Waters RP-HPLC pump 515 A and B (Waters, Milford, MA, USA) solvent delivery system with automated gradient controller coupled to a Shimadzu. SPD 10A UV-Visible detector (Shimadzu, Kyoto, Japan) with an 8 µl cell, a waters 746 data module. A Waters Spherisorb ODS 2 analytical column LC_{18} 25 cm × 4.6 mm (Waters, Milford, MA, USA) with 20 µl sample loop. The samples of amyloid protein were suspended in aqueous 0.1% TFA in water and filtered in 0.2 µ nylon membrane filter. RP-HPLC was performed over 45 min with a linear gradient at 77% acetonitrile in 0.1% TFA. The effluent was monitored at 220 nm.

Size Exclusion Chromatography (SEC)

Size exclusion HPLC columns were used to purify the amyloid protein, monitored with Shimadzu UV-Visible detector (Shimadzu, Kyoto, Japan) Ultrahydrogel 250™, Ultrahydrogel500™ 7.8 × 300 mm column (Waters, Japan). Eluents consist of 20% acetonitrile 0.1% TFA solution and applied to the sample loop (20 µl per run). The elution was monitored at 220 nm; with isocratic flow rate at 0.8 mL/min. Signals were collected from Waters 746 data module (Waters, Milford, MA, USA).

Generation of SAA fibrils

Swiss white mice by producing a small sterile dorsal subcutaneously injecting 0.5 mL of 10% (w/v) Vitamin free Casein (ICN Pharmaceuticals, Cleveland, OH, USA) solution under the skin of the back (Botto et al 1997; **Benson et al 1977).** After 24 hrs the mice were sacrificed and transferred to chilled plate.

Fluorescence Spectroscopy

Purified SAA and SAA protofibrils (400 µg/400 µl 0.001% NH_4OH, pH 9.0) were labeled with fluorescein isothiocyanate (FITC) (Fluka, USA) by dialysis through a 10000 Da cutoff membrane. FITC (0.4 mg) was dissolved in 40 µl DMSO and added to 40 mL of PBS adjusted to pH 9.0 with NH_4OH. After 12 hrs at 4°C the SAA-FITC and SAA protofibrils-FITC was dialyzed against milli-Q water, at 4°C (Linke et al 1991). 1.25-6.25 µM SAA-FITC and SAA protofibrils-FITC assayed for fluorescence intensity (FL, at λ_{max} 530±15 nm) (PERKIN-Elmer Chemiluminescence, USA).

Single Photon Counting

1.25-6.25 µM SAA-FITC and SAA protofibrils-FITC assayed for lifetime measurements (FL, at λ_{max} 530±15 nm) (Spectra physics, IBH Consultants, Scotland, UK).

Induced Circular Dichroic Spectroscopic Studies

1.25-6.25 μM SAA-FITC and SAA protofibrils-FITC assayed for induced CD measurements (Jasco J-715 Spectropolarimeter, Tokyo, Japan).

FACScan Analysis

BC3H1 cells were incubated with 1.25-6.25 μM SAA-FITC and SAA protofibrils-FITC assayed for 24 hrs at 4°C, washed in PBS, fixed immediately in 1% paraformaldehyde in PBS, and assayed for cellular fluorescence (FL, at λ_{max} 530±15 nm), forward scatter (FSC), and side scatter (SSC). Binding was analyzed on a FACScan flow cytometer using FACScan analysis software WINMDI (Becton Dickinson, San Jose, CA, USA).

MTT Assay

1.25-6.25 μM SAA-FITC and SAA protofibrils-FITC assayed incubation with BC3H1 cells for 24 hrs. Added of 200 μl fresh medium at the end of the growth period and add 50 μl of MTT to all wells in columns 1 to 6. Wrap plates in aluminum foil and incubate for 4 hrs in a humidified atmosphere at 37°C. This is a minimum incubation time and plates can be left for up to 8 hrs. Remove the medium and MTT from the wells and dissolve the remaining MTT-formazan crystals, adding 200 μl of DMSO to all wells in columns 1 to 6. Add glycine buffer (25 μl per well) to all wells containing the DMSO. Record absorbance at 570 nm immediately, since the product is unstable. The wells in column 1, which contain medium, MTT, but no cells, are used to blank the plate reader.

RESULTS AND DISCUSSIONS

RESULTS

Deposition of amyloid A fibrils in tissues resulting in the displacement of normal structure and cellular dysfunction is the characteristic feature of systemic amyloidosis. This work was initiated to explore whether SAA monomeric and protofibrillar aggregates would bind to the different cell lines from mouse brain BC3H1 (smooth muscle cells). This was selected as model system for parenchymal cerebral cortex system where localized amyloid seems to be the maximum effect. The use of parenchymal cells for the study of SAA gains importance as the SAA expression is observed in the parenchymal cells in the inflammatory condition. It has shown in under the systemic amyloid condition the serum amyloid protofibrils pass the blood brain-barrier and bind widely in the parenchymal region (Asokan 2014). It is also observed that the crossing of BBB is accompanied by the expression of Aβ peptides 20 times more than the control indicating the importance of the SAA interaction to the cells of parenchymal origin. Further, since SAA the value dramatically increased in the systemic circulation during the infection condition their interaction with the cell lines gains importance (Yamada 1999). Binding SAA of protein and AA protofibirls seemingly target the membranes (Yan et al 2000). Immediately after synthesis, the SAA is released from the cell and binds with HDL and is never found in the free form. In the circulation it is rapidly cleared: its half-life in mice is 30-50 min faster than apolipoprotein (Benson et al 1995; Kluve-Beckerman et al 1997). As the amyloid toxicity depends upon the assembled state of the protein in this study measured the cell-protein interaction in both monomer and assembled form. To determine the interaction in this study conjugated the proteins with FITC, which could be identified by fluorescence and absorption, distinctly.

Fluorescence spectroscopy

SAA-FITC binding to each other was studied using fluorescence intensity changes. The binding activity of protein was detected using fluorescence spectral change. The principle of the method is based on the reported phenomenon of fluorescence quenching in the protein abundantly labeled by fluorophores (this phenomenon is caused by an interaction of aromatic rings of different fluorophore molecules and interaction of disulfide bonds with fluorophores) and the fluorescence increases greatly after polypeptide chain breaks occur. The signal change in FITC fluorescence is applied here to study the nature of interaction of serum amyloid components with each other. The SAA is covalently linked to FITC. Typical increase in fluorescence spectra was observed on increasing the SAA-FITC + SAP ($CaCl_2$) 0.12-1.2 nM concentration. At higher SAA-FITC concentration, the self-quenching of fluorescence was observed (Fig. 1a). Linear increase in FITC fluorescence was observed during addition of SAA protofibril-FITC + SAP ($CaCl_2$) 0.12-1.2 nM. This linear increase was diminished after addition of SAA-FITC protofibril at a concentration greater than 2.5 μM. The deviation of fluorescence curve may be associated with aggregation of SAA protofibril resulted in interaction of aromatic side chains with each other near the fluorophore (Fig. 1b).

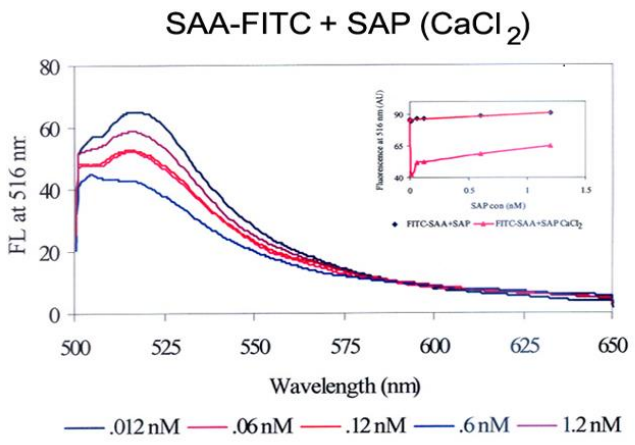


Fig. 1a. Fluorescence spectra of SAA-FITC + SAP ($CaCl_2$) (0.12-1.2 nM) (a) SAA-FITC (2.5µM) with increasing concentrations of SAP ($CaCl_2$) (0.12-1.2 nM) and insert plot shows ((0.12-1.2 nM) concentration Vs Fluorescence

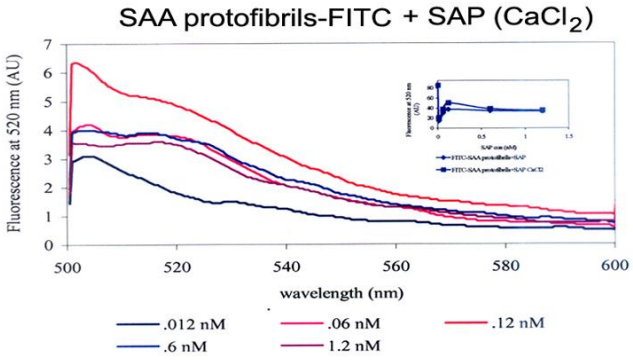


Fig. 1b. Fluorescence spectra of SAA protofibrils-FITC + SAP ($CaCl_2$) SAP ($CaCl_2$) (0.12-1.2 nM) (a) SAA protofibrils -FITC (2.5µM) with increasing concentrations of (0.12-1.2 nM) SAA protofibrils-FITC + SAP ($CaCl_2$) and insert plot shows SAP ($CaCl_2$) (0.12-1.2 nM) concentration Vs Fluorescence

Fluorescence Lifetime Measurements

The fluorescence life of a fluorophore depends strongly on the properties of the molecules surrounding the probe. A decrease in fluorescence intensity followed by a decrease of the lifetime suggests an increase in excitation energy transfer, a photochemical reaction or other quenching processes. One of the most widely used fluorophores to label proteins for structural and conformational studies is fluorescein isothiocyante (FITC). Fluorescence dyes differ in their ability to quench. The more hydrophobic the dye the lower ratio of dye: protein where quenching will occur.

The effect of SAA concentration on lifetime of FITC + SAP ($CaCl_2$) 0.12-1.2 nM is indicated in Fig.2a. The average fluorescence lifetime increased from 2.5 to 3.3 ns indicating that the fluorophore is in highly polar environment than in the previous state. In high SAA concentration (2.5 µM), the association of SAA results in exposure of more FITC to the polar environment. The amplitude for short lifetime (τ_1) was decreased significantly from 45% to 30% during addition of SAA protofibrils FITC+ SAP ($CaCl_2$) 0.12-1.2 nM (Fig. 2b). However, long-lived species increased from 38 to 47% during further SAA incubation.

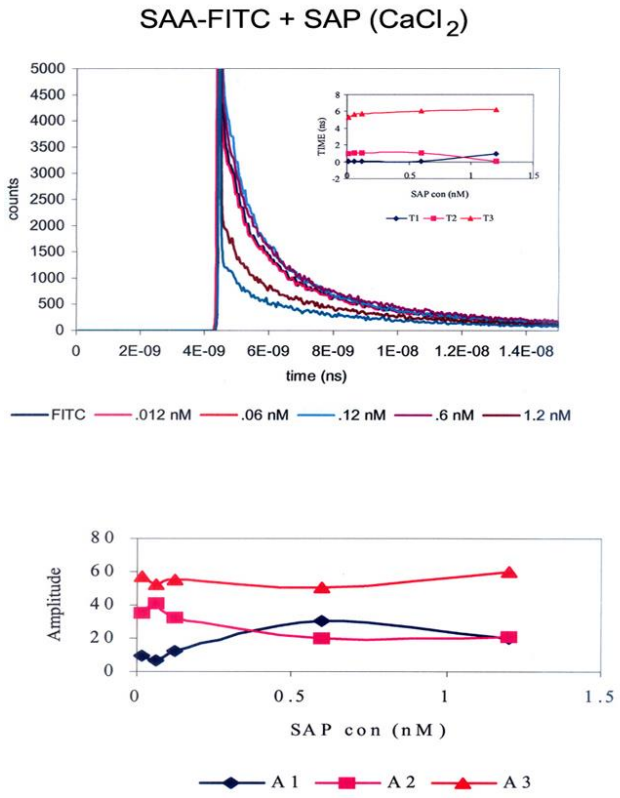


Fig. 2a. Life time measurements of fluorescence decay of SAA-FITC (2.5µM) with increasing concentration of (0.12-1.2 nM) SAA-FITC + SAP ($CaCl_2$), (b) increasing concentration of SAP ($CaCl_2$) (0.12-1.2 nM) SAP ($CaCl_2$) Vs Amplitude and insert plot shows SAP ($CaCl_2$) (0.12-1.2 nM) concentration Vs time.

Induced CD Measurements

The CD spectrum of SAA-FITC + SAP ($CaCl_2$) 0.12-1.2 nM, which was concentration-dependent, is shown in Fig. 3a. The association SAA at different concentrations altered the CD band at 450 and 505nm and became maximal at 1.25µM of SAA. Further addition of SAA did not alter the Molar

ellipticity. The SAA-FITC protofibrils + SAP ($CaCl_2$) 0.12-1.2 nM addition decreased the positive band at 450 nm and increased the negative band 505 nm (Fig. 3b). There was no significant change in the CD band at 450 nm indicated in plot.

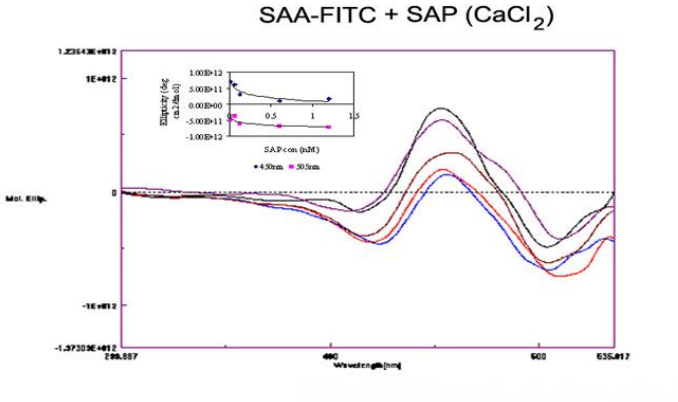


Fig. 3a. CD spectra of SAA-FITC + SAP ($CaCl_2$) SAP ($CaCl_2$) (0.12-1.2 nM) (a) SAA-FITC with increasing concentration of SAP ($CaCl_2$) (0.12-1.2 nM) SAP ($CaCl_2$) and insert plot shows SAP ($CaCl_2$) (0.12-1.2 nM) SAP ($CaCl_2$) concentration Vs elipticity at 450 and 505 nm

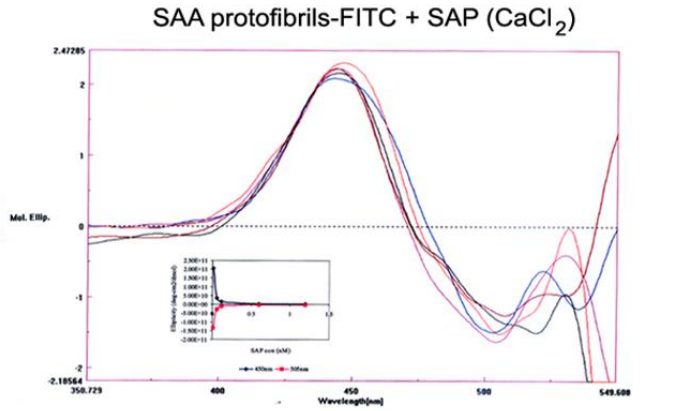


Fig. 3b. CD spectra of SAA protofibrils-FITC + SAP ($CaCl_2$) SAP ($CaCl_2$) (0.12-1.2 nM) (b) SAA protofibrils-FITC with increasing concentration of SAP ($CaCl_2$) (0.12-1.2 nM) SAP ($CaCl_2$) and insert plot shows SAP ($CaCl_2$) (0.12-1.2 nM) SAP ($CaCl_2$) concentration Vs elipticity at 450 and 505 nm

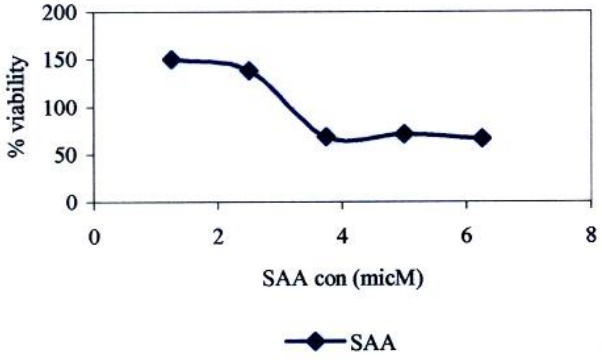
Binding Study of Monomeric and Protofibrils of SAA to BC3H1 Cells of Mouse

Viability test for the SAA protein - FITC + SAP ($CaCl_2$) 0.12-1.2 nM (Fig. 4a) BC3H1 cells (Fig. 4b) Viability test for the SAA protofibrils -FITC+ SAP ($CaCl_2$) 0.12-1.2 nM. SAA-FITC binding to BC3H1 cells was analyzed by flow cytometry and the respective histogram is shown in Fig . 5 e, f, g, h and m, n, o, p, q. The shifting of cell population towards higher fluorescence side in the histogram reveals the binding of monomeric SAA-FITC + SAP ($CaCl_2$) 0.12-1.2 nM to cell populations. The forward and side scatter is not changed significantly indicating that volume and granularity is not affected during serum amyloid binding to the respective cell lines. SAA protofibril-FITC + SAP ($CaCl_2$) 0.12-1.2 nM also binds to BC3H1 cells similar to monomeric SAA as shown in cytometry histogram (Fig. 5 m, n, o, p, q).

Table: 1.Determination of affinity constant (Kd)

Cell type	Monomeric SAA (μM)	Protofibrillar SAA (μM)
BC3H1	0.55	0.65

Affinity constants (Wang et al 1996) for monomeric and fibrillar SAA-FITC were calculated from the flow cytometry experiments given in table 1. The affinity constants for SAA-FITC in monomeric and fibrillar form remains same in all the cell lines indicating the presence of high affinity binding sites for serum amyloid in both the forms. Concentration dependent toxicity was observed in SAA binding. (Fig. 4a and 4b), show the changes in cell viability induced by exposure to 1.25-6.25 μM SAA for 24 hrs caused significant cell death. Incubation of varying concentrations of SAA resulted in BC3H1, only 50% viable BC3H1 cells at 4–6 μM with an LD_{50} of 3.5 μM.



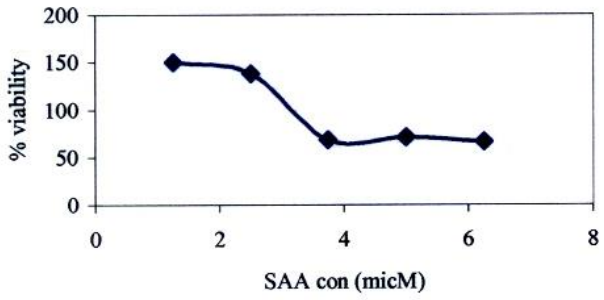
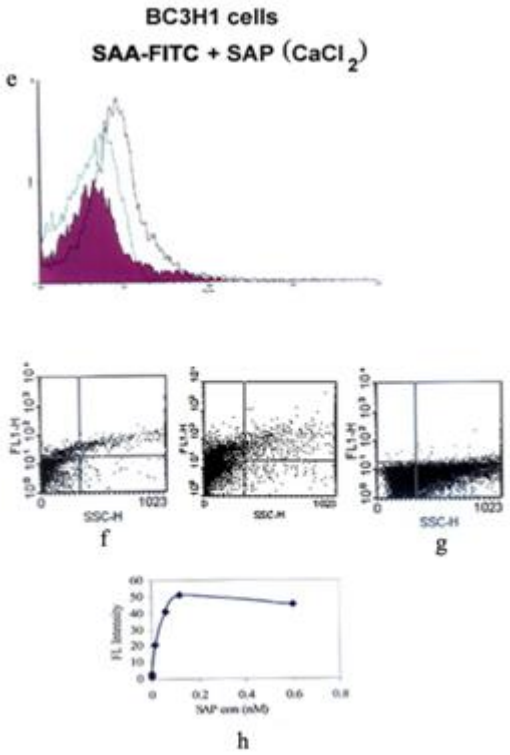


Fig. 4. Viability test for the SAA protein (1.25-6.25 µM) (a) BC3H1 cells (b) Viability test for the SAA protofibrils (1.25-6.25 µM).



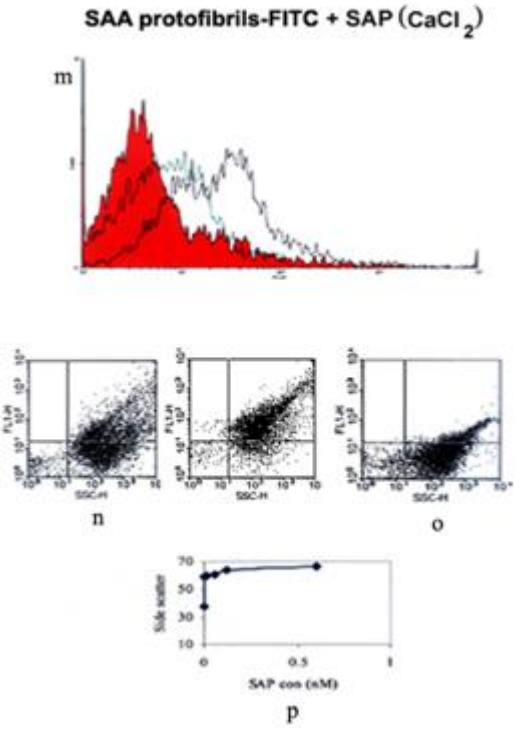


Fig. 5. Fluorescence histogram of SAA-FITC + SAP ($CaCl_2$) SAP ($CaCl_2$) (0.12-1.2 nM) and SAA protofibrils-FITC + SAP ($CaCl_2$) SAP ($CaCl_2$) (0.12-1.2 nM) in mouse BC3H1 cells (e) histogram shows filled curve mouse BC3H1 control cells, black curve shows SAA-FITC (6.25µM), green curve shows SAP ($CaCl_2$) (0.25 nM) with SAA-FITC (2.5 µM) (f) side scatter of control cells and side scatter of SAA-FITC (6.25µM), (g) side scatter of SAA-FITC with SAP $CaCl_2$ (0.25µM,), (h) plot shows increasing concentration(0.12 -1.2 nM) of SAP ($CaCl_2$) with SAA-FITC ((2.5 µM) Vs side scatter (i) histogram shows filled curve mouse BC3H1 control cells, black curve shows SAA protofibrils-FITC (6.25µM), green curve shows SAP ($CaCl_2$) (0.25nM) with SAA protofibrils-FITC (2.5µM), (i) side scatter of control cells and side scatter of SAA protofibrils-FITC (6.25µM), (K) side scatter of SAA protofibrils-FITC (2.5µM) with SAP ($CaCl_2$ (0.25nM), (l) plot shows increasing concentration (0-1.2nM) of SAP ($CalCl_2$) with SAA protofibrils-FITC (2.5µM) Vs side scatter (m) histogram shows filled curve mouse BC3H1 control cells, black curve shows SAA protofibrils-FITC (6.25µM), green curve shows SAP ($CaCl_2$) (0.25nM) with SAA protofibrils-FITC (2.5µM), (n) side scatter of control cells and side scatter of SAA protofibrils-FITC (6.25µM), (o) side scatte of SAA protofibrils=FITC (2.5µM) with SAP ($CaCl_2$) (0.25nM), (p) plot shows increasing concentration (0.12-1.2nM) of SAP ($CaCl_2$) with SAA protofibrils-FITC (2.5µM) Vs side scatter.

DISCUSSION

The multiexponential decay of SAA-FITC is possibly due to heterogeneity of conformational isomers of the protein, time dependent relaxation around the excited state, or the intrinsic heterogeneity of the FITC around the SAA protein. SAA is showing heterogeneity because it contains three additional lysine molecules labeled FITC together with amino terminal region on the protein molecule.

For many fluorophores, intermolecular interactions and energy transfer between molecules in close proximity to one another result in self-quenching. Molecular association can be assessed, if during this process, the molecular environment changes around the fluorescent probes used to label the desired protein. Using polarity changes near the probe, accessibility of the binding site of the protein by various molecules of interest can be evaluated. Conformational change of the fluorophore (FITC) induced by binding of proteins can cause a change in the fluorescence intensity. Time resolved fluorescence spectroscopy enables the independent observation of different populations of a fluorophore (free or protein bound) in contrast to the steady state fluorescence spectroscopy.

Flow cytometric analysis suggested that interaction of SAA with its receptor on BC3H1 was stronger. Moreover, FITC labeled SAA was found to bind, in a saturable manner, to all the cells, reaching a binding affinity of ~10^{-9} M. Although the chemical structure of SAA is defined, presence of its putative receptor on various target cells is unknown. Previous research work has shown that SAA acts as a chemo attractant and regulator of the migration of monocytes, polymorphonuclear cells, and lymphocytes through its avid interaction. The human SAA binds to T lymphocytes with a dissociation constant Kd=10^{-9} M.

The principal role of SAA during the acute-phase reaction appears to be the association with HDL-particles and subsequent changes of apolipoprotein composition and metabolic properties of its physiological carrier. Because SAA may displace apolipoprotein A-I, the major apolipoprotein of HDL, it is hypothesized that SAA could alter the protective function of HDL during 'reverse cholesterol transport. SAA binding acute phase reaction was studied with macrophages (representative peripheral cells) revealed that the binding affinity for SAA is enhanced. Earlier findings suggest that extracellular matrix proteins appear to serve as a temporary anchorage sites for SAA and amyloid A. (Preciado-Patt et al 1996). Though SAA exists predominantly with HDL, its physiological function is unknown. From the side scatter and forward scatter values of SAA treated cells, it is clear that SAA binding does not alter the size and granularity of cells. Moreover, SAA had no effect on either protein synthesis or DNA synthesis, suggesting that SAA specifically altered lipid synthesis. Earlier findings suggest that acute-phase SAA down-regulates lipid biosynthesis in cultured aortic smooth muscle cells in a time and dose-dependent manner.

For SAA, the situation is different since SAA, an acute phase reactant, rapidly integrates into HDL. Since HDL are potent LPS neutralizing entities by themselves. Studying the LPS-SAA interaction in acute phase sera will help to elucidate the exact role for SAA. In a clinical study the exact ratio of free and HDL-associated SAA will be determined in septic patients.

Amyloidosis is a disease where misfolded proteins aggregate and form large deposits in a tissue, leading to organ dysfunction (Sipe, et al, 2014). SAP was originally isolated as a serum-derived protein found in all types of amyloid deposits (hence the serum amyloid part of its name) (Cathcart et al, 1967). SAP was found to be a pentameric protein, hence the P part of its name (Pepys, 2006). SAP is also easily purified by incubating serum with certain types of agarose in the presence of calcium, washing unbound protein off, and then eluting fairly pure bound SAP with a calcium chelator (Painter et al, 1982). One possibility is that the SAP in the amyloid deposits binds to the misfolded proteins in an attempt to opsonize them for phagocytosis, but cannot pull proteins out of the deposit, and the SAP then gets stuck in the deposits. SAP knockout mice have reduced severity of experimentally-induced amyloidosis, suggesting that the stuck SAP exacerbates the amyloid deposit formation and/or hinders the ability of other opsins to pull the amyloid complexes apart (Botto et al, 1997). The Pepys group found a small molecule compound that causes two human SAP pentamers to stick to each other, and this complex is then quickly cleared from the circulation. In SAP knockout mice expressing human SAP, the compound decreased serum SAP levels but did not reduce the severity of experimentally-induced amyloidosis (Pepys et al, 2002). Adding anti-SAP antibodies to this treatment however did reduce experimentally-induced amyloidosis, suggesting that reducing SAP levels is a possible therapeutic for amyloidosis (Richards et al, 2018).

In the present study, we sought to define the region of acute phase apoSAA involved in cholesterol binding and to investigate the ability of constitutive apoSAA4 to bind cholesterol. Binding of [3H] cholesterol to apoSAAp was inhibited by unlabeled cholesterol (1-100 nM), but not significantly by vitamin D and estradiol. Direct binding of acute phase, but not constitutive, apoSAA to the surfaces of polystyrene microtiter wells was strongly diminished in the presence of cholesterol. The ability of apoSAA to bind cholesterol was inhibited by antibodies to human apoSAA1 and to peptide 1-18 of apoSAA1. There was only slight inhibition of cholesterol binding by antibodies to peptide 40-63, and no inhibition by antibodies to peptides spanning regions containing amino acid residues 14-44 and 59-104. [3H] Cholesterol uptake by neonatal rabbit aortic smooth muscle and

HepG2 cells was enhanced by a synthetic peptide corresponding to amino acids 1-18 of hSAA1, but not by peptides corresponding to amino acids 1-18 of hSAA4. [3H] Cholesterol uptake by HepG2 cells was slightly increased by a peptide corresponding to amino acids 40-63 of hSAA1. These findings suggest that apoSAA modulates the local flux of cholesterol between cells and lipoproteins during inflammation and atherosclerosis (Liang and Sipe 1995)

The this study data reported here demonstrate that SAA and amyloidotic AA bind with cholesterol rich region of brain cells, smooth muscle (BC3H1) cells of the brain. Previous workers have observed that acute-phase SAA binds and transports cholesterol into aortic smooth muscle and HepG2 cells (Liang and Sipe 1995; Liang et al 1996) suggesting that SAA might have a role in cholesterol homeostasis during chronic inflammation in diseases including atherosclerosis.

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Research Article

Partial Purification of Beta Galactosidase Enzyme from Dairy Effluent *Bacillus Species*

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ABSTRACT

Thermostable beta-galactosidases producing *Bacillus* sp D4 can be used as a probiotic food for lactose intolerant people. In the present study, the *Bacillus* sp was isolated from the diary industry effluent in Chennai and screened with X-Gal media. The *Bacillus* sp. strain D4 was characterized by biochemical test. The strain D4 was assessed for its probiotic nature using antibiotic markers. Maximum production of enzyme was obtained when the medium was incubated for 48 hours at 37°C and maintained at pH 7. The addition of various carbon, nitrogen, aminoacid sources, metal ions and natural substrates to the medium were studied at concentration 1% m/v. Xylose, Yeast extract, L-phenylalanine, Mg^{2+} ion, Mn^{2+} ion and wheat bran increased the production of beta galactosidase. The enzyme was partially purified by acetone and ammonium sulphate precipitation and characterized using SDS PAGE anaylsis.

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INTRODUCTION

Most enzyme reaction rates are millions of times faster than those of comparable un-catalyzed reactions. As with all catalysts, enzymes are not consumed by the reactions they catalyze, nor do they alter the equilibrium of these reactions. However, enzymes do differ from most other catalysts by being much more specific. Enzymes are known to catalyze about 4,000 biochemical reactions. Enzymes work by lowering the activation energy for a reaction, thus increasing the rate of the reaction.

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As a result, products are formed faster and reactions reach their equilibrium state more rapidly. A few RNA molecules called ribozymes also catalyze reactions, with an important example being some parts of the ribosome.

β-galactosidase (E.C 3.2.1.23) is a hydrolase enzyme that catalyses the hydrolysis of β-galactosides (e.g. lactose) into monosaccharides (e.g. galactose and glucose), where the glycosidic group on the non-reducing β-D-galactose residue is replaced by a hydroxyl group (an acceptor group). β-galactosidase is also known to catalyze the transglycosylation of sugars, i.e. when a sugar moiety is an acceptor instead of the water molecule. This then leads to the synthesis of new oligosaccharides (Yamamoto et al, 2004). β-galactosidase hydrolyses the β-1,4-D-galactosidic linkage of lactose, as well as those of related chromogens, o-nitro-phenyl-β-D-

galactopyranoside (ONPG), p-nitrophenyl-β-Dgalactopyranoside (PNPG) and 6-bromo-2-naphthyl-galacto-pyranoside (BNG). This enzyme is widely distributed in nature, being found in various types of microorganisms, plant and animal tissues (Ahn and Kim, 1977).

MATERIALS AND METHODS

The enzyme β-Galactosidase was produced by *Bacillus* sp using the following steps:

Sample Collection: The diary effluent were collected from the Heritage Food India LTD, Ambattur, Chennai and brought to laboratory under aseptic condition in a small container. *Bacillus* sp has been present in the diary effluent which has been proved to be probiotic and produces more amount of β-Galactosidase (Liu, et al, 2008).

Isolation of Beta-Galactosidase producing *Bacillus* species: From the sample collected from the diary effluent industry, β-galactosidase producing *Bacillus* sp was first isolated. Nutrient agar, X-gal media, Petri plates, Test tubes, 0.8% saline water. ***Principle of the Beta-Galactosidase screening medium (Xgal.)*** Beta-Galactosidase cleaves X-Gal to yield galactose and 5-bromo-4-chloro-3-hydroxyindole. The latter spontaneously dimerizes and is oxidized into 5, 5´- dibromo-4, 4´- dichloro- indigo, an intensely blue product which is insoluble. X-Gal is colourless and the presence of blue colored product can be used to test the presence of an active Beta-Galactosidase (Fatima, 2007). One gram of the diary effluent was serially diluted upto 10^{-7} times using sterile 0.8% saline water as a blank. The diluted samples were then plated on a nutrient agar plate infused with X-Gal (5-bromo-4-chloro-3-indole β-D Galactopyranoside) to detect the presence of Beta-Galactosidase. 20 mg of X-Gal in 1 ml of Dimethyl sulfoxide(DMSO) was used. The plates were then incubated at room temperature for 48 hours. After 48 hours, blue colonies were observed on the plate indicating the presence of Beta-galactosidase (**Sreekumar**, 2010).

Sporulation: Sporulation is a process of release of spores into the external environment. The spores can be situated terminally or at the centre. ***Sporulation process:*** Spore septum begins to isolate newly replicated DNA and a small portion of cytoplasm. Plasma membrane starts to surround DNA, cytoplasm, and membranes. Spore septum surrounds isolated portion, forming forespore. Peptidoglycan layer forms between membranes. Spore coat forms and the endospore is freed from cell. Each blue colony was separated and plated on a nutrient agar medium as a pure culture. Inorder to isolate spore forming bacteria, one loopful of isolates were inoculated in 50 ml of Difco Sporulation Medium (DSM). The plates were then incubated at 37° C for 48 hours (Nicholson and Setlow, 1990).

The spores were purified by treating the spores with lysosyme and then washed with salt and water. They were used to remove the extraneous layer surrounding the spore coat. The purified spores were resuspended in sterile deionized water, heat shocked (80° C for 15 minutes) and stored at 4° C in a glass tube (Kasthuri Venkateswaran et al, 2003)

Spore staining: The spore formations were checked microscopically using Schaeffer-Fulton staining technique (Harley and Prescott, 2002; Momoh et al, 2009). The smear of the culture was prepared using a sterile loop and air dried. The smear was covered with malachite green and steam was passed for stain penetration for five minutes. The slide was allowed to cool for five minutes and then washed with distilled water for 30 seconds. The slide was then counterstained with safranin for 1 minute. The slide was again washed with distilled water for 30 seconds, air dried and examined under oil immersion. Those spores which stained green indicated the presence of endospores

Biochemical test for the characterization of the strain D4: For the confirmation of the strain D4, a number of biochemical test such as Gram staining, Motility test, Oxidase test and Catalase test were carried out.

Gram staining method: Gram staining method is one of the most important biochemical test carried out to determine whether the species is gram positive or gram negative (Gram staining, Sridhar Rao). The smear was placed on a glass slide and treated with Gentian violet (a mixture of methyl violet and crystal violet) and exposed for one minute. After exposure, the smear was washed with water and drops of Grams Iodine was added and allowed to act for one minute. This results in the formation of a dye- iodine complex and thus served as a mordant. The slide was then washed with water and finally the smear was decolorized with acetone: ethyl alcohol for not more than 30 seconds. The slide was then washed with water and counterstained using safranin. If purple color develops, it indicates that the isolated species is Gram Positive. If pink color develops, it indicates that the isoltd species is Gram Negative.

Catalase test: This biochemical test is carried out to determine whether the isolated species was aerobic, anaerobic or microaerophilic (Karen Reiner, 2010). This test was carried out by adding 4-5 drops of 3% hydrogen peroxide to the tube. A small amount of the organism was isolated from an 18-24 hour

culture and placed into it. The appearance of bubble formation indicates that the species is aerobic otherwise the species is anaerobic.

Motility test: Prior to the process of sporulation, the cells might become motile by producing flagella. This can be identified by Hanging drop method (National Standard Method, Identification of Bacillus Species). A loopfull of culture is placed at the centre of the coverslip which has been dabbed with Vaseline at its corners. Place the coverslip over the slide such that the coverslip is on the top and the drop can be observed banging from the coverslip. Place the preparation in the microscope slide holder and necessary adjustments were done to view the edge of the drop as an irregular line crossing the field. Focus the edge of the drop carefully such that the cells will look either dark or slightly greenish, very small rods or spheres. The morphology and grouping of cells were observed under microscope. If motility was observed, then the cells are motile.

Oxidase test: This test is conducted to determine whether the bacterium produces Cytochrome C oxidases. It uses disks impregnated with reagents such as N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) or N,N-dimethyl-p-phenylenediamine (DMPD), which is a redox indicator. This reagent is dark blue to maroon color when oxidized and colourless when reduced. Wet each disk with about four inoculating loops of deionized water. Use a loop to aseptically transfer a large mass of pure culture to the disk. Observe the disk for up to three minutes. If the area of inoculation turns dark-blue to maroon, then the result is positive. If a color change does not occur within three minutes, the result is negative.

Antibiotic susceptibility marker to detect the Probiotic nature of D4: The antibiotic susceptibility of the strain D4 was analyzed by using standard single disc-diffusion method [Sreekumar, 2010; Wilkins, 1972]/ The overnight culture of the test was seeded on the nutrient agar plate using a swab. The various antibiotic-impregnated discs containing amoxicillin (25 µg), penicillin (10 µg), ciprofloxacin (5 µg), gentamycin (10 µg), cotrimoxazole (25 µg), chloramphenicol (30 µg), bacitracin (8 µg), tetracycline (30 µg), kanamycin (30 µg), erythromycin (15 µg), vancomycin (30 µg), ampicillin (10 µg), streptomycin (10 µg), Clindamycin (2 µg), Methicillin (5 µg) were placed on the seeded plate. The zone of growth inhibition was analyzed after 24 hr.

Growth medium for the production of β-Galactosidase: Preparation of Inoculum: The inoculum for the production of enzyme was prepared using Luria-Bertani (LB) broth.The pure culture was inoculated into the sterile inoculum broth and was incubated at 37℃ in a rotary shaker overnight. The fresh overnight culture was used for the production of enzyme **(**Robert et al, 2006).

Enzyme production: *The production medium*: Production medium is a medium which allows the enzyme to grow at a faster rate provided proper aeration, incubation time, temperature and pH is given. The medium was prepared according to Nehad, 2011. For effective production, it was carried out in a shaker with an aeration rate of 200 rpm under the volume 50/200 v/v at 37℃ for 24 hours. The samples were then centrifuged at 10,000 rpm for 10 min at 4° C (Vijay Anand et al 2010, Akcan, 2011, Panesar, 2006). The supernatant was then used for optimization studies.

Chemical Assays for Quantifying Beta Galactosidase: *ONPG assay (O- nitro phenyl –β-D galactopyranoside)*: The enzyme activity was measured by the method of Nehad and Enas, 2011. The filtrate was centrifuged at 8000 rpm for 15 minutes. The enzyme was assayed by using ONPG as a substrate prepared by dissolving 2.5 mg/ml of ONPG in 0.1M sodium acetate buffer (pH-5). 0.2 ml of the culture filtrate was added to 1 ml of the substrate solution (20mM of ONPG: 0.05 g of o-Nitro phenol β-D-Galactopyranoside dissolved in 10ml of Phosphate buffer at pH 7) and incubated at 55˚C for 20 minutes. The reaction was then stopped by adding 1 ml of 10% sodium carbonate. The absorbance was read at 420 nm and the amount of ONP was calculated using standard curve. One unit of enzyme activity was defined as the amount of enzyme that liberates 1 µM of ONP per min at 55° C.

Estimation of Total Protein content: *Bradford's method:* It was used for determining the protein content of cell fractions and assessed to determine the protein concentrations for gel electrophoresis. The principle is the based on the interaction of CBB with proteins. The unbound dye has absorbance maxima of 465 nm. When the dye binds with the proteins, the dye turns blue and the absorbance maximum is displaced to 595 nm. The Bradfords reagent was prepared by dissolving 100mg of Commassie Brilliant Blue G -250 in 50ml 95% ethanol and then 100 ml 85 % phosphoric acid is added. Dilute to 1 litre and when the dye is completely dissolved, filter the solution through Whattman No: 1 paper prior to use. 1 ml of the culture filtrate was taken and 5 ml of Bradfords reagent was added. It was gently mixed by tilting. The blank was prepared by mixing 1 ml of distilled water with 5 ml of Bradfords reagent. Then the absorbance was determined by UV – VIS spectrophotometer at 595 nm. The protein concentration was determined by using a standard graph prepared using Bovine Serum Albumin (BSA).

Partial Purification of the Enzyme: *Bacillus subtilis* D4 strain was cultured by optimizing the parameters for the maximum production of Beta-galactosidse. The enzyme was partially purified by precipitation methods. The following were performed for the maximum purification of the enzyme (Zoltaet al, 2000).

Separation of enzyme for the culture medium: The enzyme was separated from the production medium by centrifugation technique. The medium was centrifuged at 10,000 rpm for 30 minutes at 4℃. The supernatant was then filtered through 0.4 millipore filter and was estimated for its enzyme activity and protein content.

Precipitation: Acetone precipitation: Organic solvents cause the precipitation of proteins by largely changing the salvation of water. The reaction was carried out at low temperature to prevent the denaturation. The most suitable solvent used for the precipitation of protein was acetone. The optimum amount to precipitate the proteins was determined by adding acetone at a concentration of about 65%. Pre-cooled acetone was slowly added to prevent the high concentration of the organic solvent. The temperature of the solvent was kept below 0℃. When the addition of the solvent was completed, the mixture was allowed to stand for 15 minutes for equilibration. The mixture is then centrifuged at 7,000 rpm for 15 minutes at 0℃. The supernatant was decanted and the pellets were dissolved in ice-cold water and stored at -20℃.

Ammonium sulfate precipitation: Ammonium sulfate precipitation is a method of protein purification by altering solubility of protein. It is a specific case of a more general technique known as salting out. Ammonium sulfate is commonly used as its solubility is so high that salt solutions with high ionic strength are allowed. The solubility of proteins varies according to the ionic strength of the solution, and hence according to the salt concentration. Two distinct effects are observed: at low salt concentrations, the solubility of the protein increases with increasing salt concentration (i.e. increasing ionic strength), an effect termed salting in. As the salt concentration (ionic strength) is increased further, the solubility of the protein begins to decrease. At sufficiently high ionic strength, the protein will be almost completely precipitated from the solution (salting out).

Since proteins differ markedly in their solubilities at high ionic strength, salting-out is a very useful procedure to assist in the purification of a given protein. The commonly used salt is ammonium sulfate, as it is very water soluble, forms two ions high in the Hofmeister series and has no adverse effects upon enzyme activity. It is generally used as a saturated aqueous solution which is diluted to the required concentration, expressed as a percentage concentration of the saturated solution (a 100% solution). The aim is to find the ammonium sulfate concentration which will precipitate the maximum proportion of undesired protein, whilst leaving most of the desired protein still in solution.

The precipitated protein is then removed by centrifugation and then the ammonium sulfate concentration is increased to a value that will precipitate most of the protein of interest whilst leaving the maximum amount of protein contaminants still in solution. The precipitated protein of interest is recovered by centrifugation and dissolved in fresh buffer for the next stage of purification. Extracellular β-galactosidase from bacteria was purified using 55% ammonium sulphate precipitation and dialysed against 0.1mM Tris-Hcl buffer (pH 5.0) [Shalini Agarwal, 2006]

Dialysis: Dialysis Tubing is a type of semi or partially permeable membrane tubing_made from regenerated cellulose or cellophane. It is used for diffusion or more accurately osmosis.It allows the passage of small molecules but not larger ones. It is used in clinical circumstances to ensure a filtered flow of molecules, preventing the flow of larger solute molecules. Small molecules can be 'washed' out of a solution which is pumped through the tubing into a solvent, usually water which surrounds it and in which they can be flushed away. Since the dialysis membrane consists of a spongy matrix of cross linked polymers, the pore rating referred to as Molecular Weight Cut off (MWCO), is an indirect measure of the retention performance. More precisely, the membrane MWCO is determined as the solute size that is retained by at least 90%. However, since a solute's permeability is also dependent upon molecular shape, degree of hydration, ionic charge and polarity, it is recommended to select a MWCO that is half the size of the MW of the species to be retained and/or twice the size of the MW of the species.

SDS- PAGE Analysis: It is done to separate the given mixture of proteins into individual bands. It is based on the polymerization of acrylamide monomers with N, N' – bisacrylamide that in turn forms a gel [Laemelli, 1979]. The two glass plates were cleaned with ethanol. Two glass plates were separated using spacers and the plates were held upright with the help of clamps using the stand. The resolving gel is poured into the plates and allowed to polymerize. The stacking gel is then poured over the resolving gel and the combs are placed immediately over it and the gel is allowed to polymerize. Mean while, 30µl of the sample and 20µl of the loading dye were mixed and denatured at 75℃ for 5 mins. After polymerization, the combs are removed and the wells are carefully cleaned to remove the gel smeared in it. The plates

are placed in the electrode buffer which runs at a constant voltage of 50V. The samples and a known molecular weight marker are loaded into the wells. After a while, when the bands separate and reach at a certain point, staining with CBB takes place overnight and then the gel is destained.

RESULTS AND DISCUSSIONS

The species was isolated and conditions necessary for the optimization of the medium and the characterization of the enzyme were carried out and they are as follows:

Isolation and Screening

In this study, the bacterial strains were isolated from dairy industry effluent collected from the Heritage dairy industry, Ambattur, Chennai. After serial dilution and nutrient agar plating, 18 bacterial strains producing β-Galactosidase were isolated. Among these 18 strains, the best 8 strains were selected and plated on a new nutrient agar media and the best culture giving the better result were streaked to isolate the pure colonies as shown in Fig 1, 2, 3.

A pyschrophilic bacterial isolate, *Bacillus* sp. was isolated from blue green algal mats collected from Schirmacher Oasis of Antartica (Ram Kumar et al, 2004) was screened for β-galactosidase production. In contrast, thermophilic organisms isolated from Ta Pai hot spring, Maehongson, Thailand (Somyos and Phimchanok, 2010) produced β-galactosidase. Commercial lactases are produced from both yeasts, such as Kluyveromyces lactis and Kluyveromyces fragilis, and moulds such as Aspergillus niger and Aspergillus oryzae (Mahoney et al 1985. Even fungal cultures like Aspergillus, Trichoderma, Penicillium, Rhizopus and Fusarium sp. were known to produce β-galactosidase at promising level (Isil Seyis et al, 2004). The bacterial strain present in dairy industry effluent has been proved to be probiotic and has a greater ability to produce Beta-galactosidase (Sreekumar, 2010).

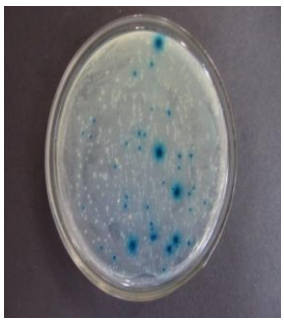

Fig 1: screening plate for beta-galactosidase infused with X-gal

Fig 2: The best culture was selected

Fig 3: Pure colonies isolated after streaking

Sporulation

The 8 best strains isolated were cultured in a sporulation media to detect the presence of spore forming bacteria. Six strains with spore forming ability were observed. Among the 6 strains, the best strain was selected and preceded for further procedure. These spores were then stained with Safranin using Schaeffer-Fulton Technique and the position of the spores was centralized (Fig: 4).

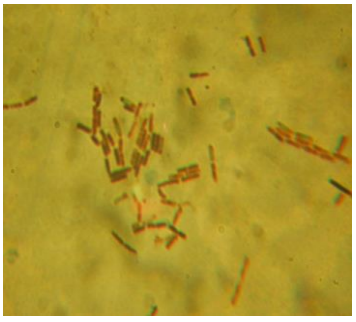

Fig 4: The presence of centralized spores in Bacillus sp.

In 2003, a report showed that out of 6 spore forming isolates, those spores which resisted the treatment of UV irradiation, γ radiation and desiccation were selected for further studies (Kasthuri Venkateshwaren et al, 2003).

Identification of the strain D4

The strain D4 was subjected to Gram Staining test, Catalase test, Oxidase test, Motility test and the strain was Gram positive, catalase positive, motile, rod shaped, and produces Cytochrome C. These characteristics confirmed that the strain D4 was identified as *Bacillus species*. Further confirmation was done by sequencing the 16S rRNA gene and compared with the GenBank databases using the BLASTN program. The 16S rRNA sequence of the isolate revealed a

close relatedness to *Bacillus subtilis* with 99-100% similarity. Hence the strain was confirmed as *Bacillus subtilis*. The phylogenetic tree established the evolutionary relationship of the species.

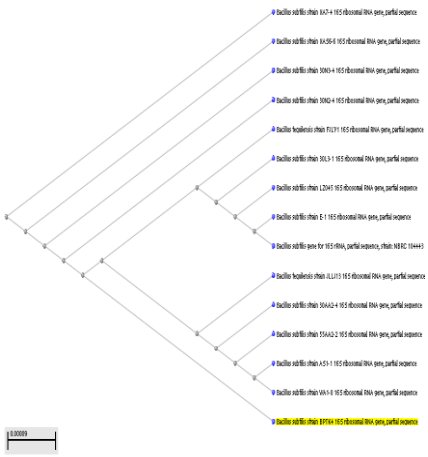

Fig 5: Phylogenetic relationship of the strain D4

Antibiotic susceptibility of the *Bacillus* species

The isolate D4 was inhibited by most of the antibiotics like Amoxillin, Ciprofloxacin, Gentamycin, Cotrimoxazole, Chloramphenicol, Bacitracin, Tetracycline, Kanamycin, Erythromycin, Vancomycin, Ampicillin, Streptomycin, Clindamycin, Methicillin but showed resistance only to Penicillin. This makes the isolate to be used as a probiotic for lactose intolerance people and also in dairy industry (Miller, 1995). David et al, 1999 reported the different strains of *Bacillus* which are commercially available now like biosubtyl and Enterogemina as probiotic preparations by using antibiotic markers. Ammini et al, 2011 reported that *Bacillus pumilus* causes skin infections in immune compromised individuals. So, further studies would be performed to understand the effect of micro organisms on human health, their genetic basis and resistance to antimicrobials.

Table 1: the reaction of strain BPTK 4 towards a number of antibiotics of different concentration

S.No	Antibiotics	Stimulatory	Resistance
1	Amoxillin	+	-
2	Ciprofloxacin	+	-
3	Gentamycin	+	-
4	Cotrimoxazole	+	-
5	Chloramphenicol	+	-
6	Bacitracin	+	-
7	Tetracycline	+	-
8	Kanamycin	+	-
9	Erythromycin	+	-
10	Vancomycin	+	-
11	Ampicillin	+	-
12	Streptomycin	+	-
13	Clindamycin	+	-
14	Methicillin	+	-
15	Penicillin	-	+

Partial Purification of the enzyme

After the optimization of media, the enzyme was partially purified by acetone followed by ammonium sulfate precipitation.

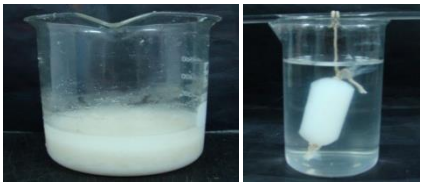

Fig 6: precipitation of proteins by 65% acetone

SDS PAGE analysis

Finally, the protein profile was analyzed using SDS-PAGE; it showed the presence of multiple bands because in all the cases the total protein content of the samples is moderately high. So other proteins (other than enzyme) may be present and form bands. But the presence of protein band nearing the molecular weight around 80-110 KDa confirms the presence of the enzyme β-Galactosidase (Fig: 16). Generally, the molecular weight of β-Galactosidase from *Bacillus sp.* is 70-90 kDa. The molecular weight of the denatured enzyme was estimated to be 86,000 by SDS-PAGE after the sample had been boiled in the presence of SDS and 2-mercaptoethanol. The *L. plantarum* β-galactosidase is a heterodimeric enzyme of a molecular mass of approximately 107 kDa, consisting of a larger subunit of approximately 72 kDa and a smaller subunit of ~35 kDa as estimated under denaturing conditions of SDS–PAGE

Activity of β-galactosidase was measured by incubating 20 µl of suitably diluted enzyme with 480 µl of 22 mM o-nitrophenyl-β-Dgalactopyranoside (ONPG) in phosphate buffer pH 6.5 at 40°C. After 15 min, 0.4 M Na_2CO_3 was added to the reaction mixture to stop the reaction (Phimchanok et al, 2008). β-phosphogalactosidase activity was routinely estimated at 37ºC in reaction mixtures containing 1 µmol of ONPG-6-P (Calbiochem), 50 µmol of sodium phosphate buffer, pH 7.0, 1 µmol of DTT (Calbiochem), and enzyme in a total volume of 1.0 ml. Reactions were terminated

by the addition of 1.0 ml of 0.5 M Na2CO3 [McDonald et al,1974]. In this experiment, slight changes were made in terms of substrate concentration 20mM ONPG in phosphate buffer pH 7.0. 0.2 M of sodium carbonate solution was used to stop the reaction.

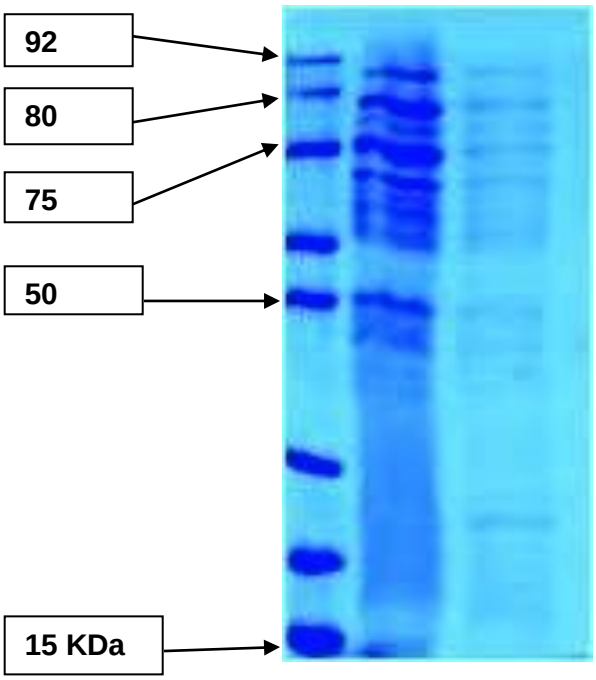

Fig 7: Various bands showed after each precipitation

(Lane 1: Marker, Lane 2: Dialyzed sample)

SUMMARY

The *Bacillus subtilis* strain D4 was isolated from dairy industry effluent collected from the Heritage food India Ltd, Ambattur, Chennai. The isolated strain was screened for the production of β-Galactosidase enzyme by using X-Gal as a substrate. The strain D4 was characterized based on Grams staining, Oxidase test, Motility Test and the presence of endospores. The genomic DNA extraction was performed for further use in 16Sr RNA Sequencing. The systematic position of bacterial culture was determined and BLAST homology analysis revealed that the sequence of *Bacillus subtilis* strain D4 showed 100% sequence identity with *Bacillus subtilis* from the database.

Furthermore, the strainD4 has been tested for its antibiotic susceptibility to determine the probiotic nature of the isolate which can be used in dairy industry for lactose intolerance people. The medium was optimized for the maximum production of β-Galactosidase based on various parameters like incubation time, temperature, pH and the effect of the suplementation of carbon, nitrogen, and aminoacid sources to the medium and the assay was performed using ONPG as substrate where the release of o-Nitro phenol produced a yellow colour read at 420 nm. Results showed that pH 7 and temperature 35ºC is an optimum environmental parameter for the growth of the strain and for its better production. In addition to this, xylose was found to be better carbon source and yeast extract as better nitrogen source for better production of β-Galactosidase.

The enzyme was purified by acetone and ammonium sulphate precipitation & dialysis. The protein profile in SDS-PAGE shows the presence of β-Galactosidase in partially purified culture crude by showing the suitable bands. Finally, the partially purified enzyme was characterized based on the temperature and pH using ONPG as a substrate. Results showed that the enzyme was active at pH 7 and showed 90% activity and when incubated at 55˚ C retained 100% of its activity. The future work focuses on the formulation of β-galactosidase tablets to reduce the lactose in milk. This would definitely be a boon to lactose intolerant people in future.

CONCLUSION

The *Bacillus subtilis strain* D4 was isolated from the dairy industry effluent and exhibited an antagonist property which proved the probiotic nature of the isolated strain. The enzyme β-Galactosidase was isolated, screened and characterized. This shows that it is an ideal candidate for hydrolysis of lactose in milk which can be used for lactose intolerant people.

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Research Article

Phytochemical Analysis of Pesticide in Grapes (*Vitis Vinifera*)

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ABSTRACT

Phytochemicals (from the Greek word phyto, meaning plant) are biologically active, naturally occurring chemical compounds found in plants, which provide health benefits for humans further than those attributed to macronutrients and micronutrients. They protect plants from disease and damage and contribute to the plant's color, aroma and flavor. In general, the plant chemicals that protect plant cells from environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attack are called as phytochemicals. Recently, it is clearlyknown that they have roles in the protection of human health, when their dietary intake is significant. More than 4,000 phytochemicals have been cataloged and are classified by protective function, physical characteristics and chemical characteristics and About 150 phytochemicals have been studied in detail. Phytochemicals are known as secondary plant metabolites and have biological properties such as antioxidant activity, effect, modulation of detoxification enzymes, stimulation of the immune system, decrease of platelet aggregation and modulation of hormone metabolism and anticancer property. There are more than thousand known and many unknown phytochemicals. In this study, its concluding that plants produce these chemicals to protect themselves and demonstrated that many phytochemicals can also protect human against diseases.

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INTRODUCTION

Dietary phytochemicals are found in fruits, vegetables, legumes, whole grains, nuts, seeds, fungi, herbs and spices (Mathai, 2000). Broccoli, cabbage, carrots, onions, garlic, whole wheat bread, tomatoes, grapes, cherries, strawberries, raspberries, beans, legumes, and soy foods are common sources (Moorachian, 2000). Phytochemicals accumulate in different parts of the plants, such as in the roots, stems, leaves, leaves, fruits or seeds (Costa *et al*., 1999). Many phytochemicals, particularly the pigment molecules, are often concentrated in the outer layers of the various plant tissues. Levels vary from plant to plant depending upon the variety, processing, cooking and growing conditions (King and Young, 1999). Phytochemicals are also available in supplementary forms, but evidence is lacking

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that they provide the same health benefits as dietary phytochemicals

Phytochemicals are not essential nutrients and are not required by the human body for sustaining life, but have important properties to prevent or to fight some common diseases. Many of these benefits suggest a possible role for phytochemicals in the prevention and treatment of disease, Because of this property; many researchers have been performed to reveal the beneficial health effects of phytochemicals. The purpose of the present review is to provide an overview of the extremely diverse phytochemicals presents in medicinal plants.

The exact classification of phytochemicals could have not been performed so far, because of the wide variety of them. In resent year Phytochemicals are classified as primary or secondary constituents, depending on their role in plant metabolism. Primary constituents include the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophyll‟s etc. Secondary constituents are the remaining plant chemicals such as alkaloids, terpenes, flavonoids, lignans, plant steroids, curcumines, saponins, phenolics, flavonoids and glucosides (Hahn, 1998). Literature survey indicate that phenolics are the most numerous and structurally diverse plant phytocontituents.

The phytochemicals present in plants are responsible for preventing disease and promoting health have been studied extensively to establish their efficacy and to understand the underlying mechanism of their action. Such studies have included identification and isolation of the chemical components, establishment of their biological potency both by in vitro and in vivo studies in experimental animals and through epidemiological and clinical- case control studies in man. Study findings suggest that phytochemicals may reduce the risk of coronary heart disease by preventing the oxidation of low-density lipoprotein (LDL) cholesterol, reducing the synthesis or absorption of cholesterol, normalizing blood pressure and clotting, and improving arterial elasticity. Phytochemicals may detoxify substances that cause cancer Dai and Mumper 2010). They appear to neutralize free radicals, inhibit enzymes that activate carcinogens, and activate enzymes that detoxify carcinogens. For example, according to data summarized by Meagher and Thomson, genistein prevents the formation of new capillaries that are needed for tumor growth and metastasis. The physiologic properties of relatively few phytochemicals are well understood and more many research has focused on their possible role in preventing or treating cancer and heart disease. Phytochemicals have also been promoted for the prevention and treatment of diabetes, high blood pressure, and macular degeneration. While Phytochemicals are classified by function, an individual compound may have more than one biological function serving as both an antioxidant and antibacterial agent. Bioactive and Disease-preventing Phytochemicals present in plant (Serrano et al, 2009).

Purification and isolation of bioactive compounds from plants is a technique that has undergone new development in recent years (Narasinga Rao (2003). This modern technique offers the ability to parallel the development and availability of many advanced bioassays on the one h other. The goal when searching for bioactive compounds is to find an appropriate method that can screen the source material for bioactivity such as antioxidant, antibacterial, or cytotoxicity, combined with simplicity, specificity, and speed Narasinga Rao(2003).. In vitro methods are usually more desirable than in vivo assays because animal experiments are expensive, take more time, and are prone to ethical controversies. There are some factors that make it impossible to find final procedures or protocols to isolate and characterize certain bioactive molecules. This could be due to different parts (tissues) in a plant, many of which will produce quite different compounds, in addition to the diverse chemical structures and physicochemical properties of the bioactive phytochemicals Lafay and Gil-Izquierdo (2008). both the selection and the collection of plant materials are considered primary steps to isolate and characterize a bioactive phytochemical. The next step involves a retrieval of ethno-botanical information to discern possible bioactive molecules. Extracts can then be made with various solvents to isolate and purify the active compounds that are responsible for the bioactivity. Column chromatographic techniques can be used for the isolation and purification of the bioactive compounds. Developed instruments such as High Pressure Liquid Chromatography (HPLC) accelerate the process of purification of the bioactive molecule. Different varieties of spectroscopic techniques like UV-visible, Infrared (IR), Nuclear Magnetic Resonance (NMR), and mass spectroscopy can identify the purified compounds Narasinga Rao(2003).

Many bioactive molecules have been isolated and purified by using paper thin- layer and column chromatographic methods. Column chromatography and thin-layer chromatography (TLC) are still mostly used due to their convenience, economy, and availability in various stationary phases (Zhang et al., 2015). Silica, alumina, cellulose, and polyamide exhibit the most value for separating the phytochemicals. Plant materials include high amounts of complex phytochemicals, which make a good separation difficult. Therefore, increasing polarity using multiple mobile phases is useful for highly valued separations. Thin- layer chromatography has always been used to analyze the fractions of compounds by column chromatography. Silica gel column chromatography and thin-

layer chromatography (TLC) have been used for separation of bioactive molecules with some analytical tools Narasinga Rao (2003). Determination of the structure of certain molecules uses data from a wide range of spectroscopic techniques such as UV-visible, Infrared (IR), Nuclear Magnetic Resonance (NMR), and mass spectroscopy. The basic principle of spectroscopy is passing electromagnetic radiation through an organic molecule that absorbs some of the radiation, but not all. By measuring the amount of absorption of electromagnetic radiation, a spectrum canbe produced. The spectra are specific to certain bonds in a molecule. Depending on these spectra, the structure of the organic molecule can be identified. Scientists mainly use spectra produced from either three or four regions Ultraviolet (UV), Visible, Infrared (IR), radio frequency, and electron beam (Ghasemzadeh, et al, 2010) for structural clarification.

Organic molecules are bombarded with either electrons or lasers in mass spectrometry and thereby converted to charged ions, which are highly energetic. A mass spectrum is a plot of the relative abundance of a fragmented ion against the ratio of mass/charge of these ions. Using mass spectrometry, relative molecular mass (molecular weight) can be determined with high accuracy and an exact molecular formula can be determined with a knowledge of places where the molecule has been fragmented (Ghasemzadeh, et al, 2010). In previous work, bioactive molecules from pith were isolated and purified by bioactivity-guided solvent extraction, column chromatography, and HPLC. Vitis vinifera, Family: Vitaceae, Genus: Vitis, Species: V. vinifera Vernacular Name: English:Grapes, Hindu:Angoor Malayalam:Munthiri Telungu:Draksa, Tamil:Drakshai, Part Used: fruit peel.

Anti-sclerotic effects: lipoprotein oxidation of low density (LDL) by free radicals is associated with initiation of atherosclerosis. The active ingredients of the grape reduce formation of this lipoprotein. Leaves have venotonic, vasoprotective, astringent and diuretic effects. The fruits are vitamins, tonics, anticancer, hepatoprotective, promote hair growth and prevent ischemic processes. The seeds oil: Hypolipidemic, prevents the increase of vascular permeability.

Plants have provided man with all his needs in terms of shelter, clothing, food, flavours and fragrances as not the least, medicines. Plants have formed the basis of sophisticated Traditional Medicine (TM) systems that have been in existence for thousands of years and continue to provide mankind with new remedies. Some of the oldest known medicinal systems of the world such as Ayurveda of the Indus civilization, Arabian medicine of Mesopotamia, Chinese and Tibetan medicine of the Yellow River civilization of China and Kempo of the Japanese are all based mostly on plants. The ancient cultures are known for their systematic collection of information on herbs and their rich and well defined herbal pharmacopoeias. Although some of the therapeutic properties attributed to plants have proven to be erroneous, medicinal plant therapy is based on the empirical findings of hundreds and thousands of years Palavy and Priscilla 2006)

A trend in phytomedicine is the use of new plant origin bioactive compounds with the potential for chemical modification, which will broaden phytomedical importance. Molecular biology is also being used in this process and the pharmacological profiles of these compounds are screened using new research equipment and new technology Narasinga Rao 2003). Palavy and Priscilla 2006) investigates the phytochemicals, antioxidant and antifungal activity of Pithecellobium dulce leaves" extract. Phytochemical analysis of the extract revealed the presence of alkaloids, flavonoids, saponins, coumarin, tannins, anthocyanin and triterpenoids.

Palavy and Priscilla 2006) have been evaluated the bioactive components of *Psidium guajava* leaves by qualitative and quantitatively. The qualitative analysis of ethanolic and aqueous extract of *Psidium guajava* leaves showed that tannin, phlobatannins, saponin, flavonoids, steroids, terpenoids, triterpenoids, carbohydrate, polyphenol and glycoside present in both extract. Palavy and Priscilla (2006) compared the percentage value of *Mentha Piperita, Mentha longifolia* and *Osimum basilicum* extracts by using different solvent and extraction methods, phytochemical screening of constituents present in dry leaves, also to study fluorescent characteristics of leaves powder which are responsible for the medicinal properties of the plant using various standard tests. Phytochemical analysis revealed the presence of alkaloids, saponins, phenols, flavonoids, terpenoids, cardioactive glycosides, tannins, carbohydrates in dry leaves extracts. Presence of alkaloids and phenolic acid was determined in high quantity in *Mentha longifolia*, while saponins present in high quantity in *Mentha piperita* and flavonoids in *Osimum basilicum.* The fluorescent characteristics of leaves powder with various chemical reagents were noted under visible and UV light.

MATERIALS AND METHODS

Collection of plant materials: The fruit peels of *Vitis vinifera* were collected in January 2022 from New Bus stand, Gudiyattam, Vellore, Tamil Nadu, India from a single herb. The collected *Vitis vinifera fruit* peels were washed several times with distilled water to remove the traces of impurities from the peel. Peel was spread out in a plain paper and shade dried at room temperature for about 10 days and makes a fine

powder using grinder mixture. The powder materials were used of anticorrosive studies.

Preparation of plant extract: 2 gram of the powder of *Vitis vinifera* fruit peels were transferred in to different conical flask (250ml). The conical flask containing 100ml of different solution (methanol and water). The conical flask containing *Vitis vinifera* fruit peels were shake it well for 30 minutes by free hand. After 24 hrs, the extracts were filtered using whatman filter paper No.1 was transfer in to china dish. The supernatant was completely removed by keeping the china dish over water bath at 45ºC. The obtained extract was used for phytochemical analysis.

Phytochemical screening Chemical tests were carried out on the alcoholic extract and on the powdered specimens using standard procedures to identify the constituents as described by Sofowara (1993.

Test for Tannins: About 1ml of sample is boiled in 20 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride is added and observed for brownish green or a blue-black colouration.

Test for Phlobatannins: Deposition of a red precipitate when an extract of each plant sample (1ml) is boiled with 1% aqueous hydrochloric acid is taken as evidence for the presence of Phlobatannins.

Test for Saponin: About 2 ml of sample is boiled in 20 ml of distilled water in a water bath and filtered. 10ml of the filtrate is mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The frothing is mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of emulsion.

Test for Flavonoids: 5 ml of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract followed by addition of concentrated H_2S0_4. A yellow colouration observed in each extract indicated the presence of flavonoids. The yellow colouration disappeared on standing.

Test for Steroids: 2 ml of acetic anhydride is added to 1ml of extract of each sample with 2 ml H_2SO_4. The colour changed from violet to blue or green in some samples indicating the presence of steroids.

Test for Terpenoids (Salkowski test): 5 ml of each extract is mixed in 2 ml of chloroform, and concentrated H_2SO_4 (3 ml) is carefully added to form a layer. A reddish brown colouration of the interface is formed to show positive results for the presence of terpenoids.

Test for triterpenoids: 1ml of the extract is added in 1 ml of chloroform, 1 ml of acetic anhydride is added following the addition of 2 ml of concentrated H_2 SO_4. Formation of reddish violet colour indicates the presence of triterpenoids.

Test for alkaloids: Mayer‟s test : To a few (one) ml of the extract, a drop of Mayer‟s reagent is added by the side of the test tube. A creamy or white precipitate indicates the test is positive.

Test for carbohydrates: Benedict‟s test: To 0.5 ml of the filtrate, 0.5 ml of Benedict‟s reagent was added. The mixture was heated on boiling water bath for 2 min. A characteristic red colored precipitate indicates the presence of sugar.

Test for Proteins: 1ml of plant sample was taken and added 1ml of 40% Sodium hydroxide and added slowly in the sides of test tubes of few drops of copper sulphate. Appearance of violet or pink colour indicates that the presence of protein.

Test for anthraquinones: Five ml of the extract solution is hydrolysed with diluted concentrated H_2SO_4 extracted with benzene. 1 ml of dilute ammonia is added to it. Rose pink coloration suggested the positive response for anthraquinones.

Test for Polyphenols: Ethanol (4 ml) is added to each extracts (1ml) and the resulting solution is transferred in test tubes and warmed in a water bath (15 minutes). Three drops of freshly prepared ferric cyanide solution were added to the extract solution. Formation of a blue green colour indicated the presence of polyphenols.

Test for Cardiac glycosides (Keller-Killani test): 5 ml of each extracts is treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This is underlayed with 1 ml of concentrated H_2 SO_4. A brown ring of the interface indicates a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown

ring, while in the acetic acid layer, a greenish ring may form just gradually throughout thin layer.

Determination of total phenols by spectrophotometric method: The fat free sample was boiled with 50 ml of ether for the extraction of the phenolic component for 15 min. 5 ml of the extract was pipetted into a 50 ml flask, then 10 ml of distilled water was added. 2 ml of ammonium hydroxide solution and 5 ml of concentrated amylalcohol were also added. The samples were made up to mark and left to react for 30 min for colour development. This was measured at 505 nm.

Determination of Flavonoid: Flavonoid determine by the method of Bohm and Kocipai-Abyazan (1994).. 80% aqueous methanol 10 g of the plant sample was extracted repeatedly with 100 ml of 80% aqueous methanol at room temperature. The whole solution was filtered through what man filter paper No 42 (125 mm). The filtrate was later transferred into a crucible and evaporated into dryness over a water bath and weighed to a constant weight.

Histochemical tests: The powder of *Vitis vinifera* fruit peels were treated with specific chemicals and reagents. The treated plant powder further analysed in light microscope. The *Vitis vinifera* fruit peels powder treated with phloroglucinol and diluted HCl gave red colour indicates lignin, treated with diluted ammonia and H_2SO_4 gave yellow color indicates flavonoids and treated with Dragant draft reagent gave reddish brown color indicates alkaloids.

GC-MS Analysis: GC-MS analysis was carried out on Shimadzu 2010 plus comprising a AOC- 20i auto sampler and gas chromatograph interfaced to a mass spectrometer instrument employing the following conditions: column RTX 5Ms (Column diameter is 0..32 mm, column length is 30 m, column thickness 0.50 µm), operating in electron impact mode at 70 eV; Helium gas(99.99 %) was used as carrier gas at a constant flow of 1.73 ml /min and an injection volume of 5 µI was employed (split ratio of 10:1), injector temperature 270 ºC; ion source temperature 200 ºC. The oven temperature was programmed from 40 ºC (isothermal for 2 min), with an increase of 8 ºC/min, to 150 ºC, then 8 ºC/min to 250 ºC, ending with a 20 min isothermal at 280 ºC. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 40 to 450 Da. Total GC running time is 51.25min. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a TurboMass Ver 5.2.0.

RESULTS AND DISCUSSIONS

The secondary metabolites formed also are an important trait for our food plants (taste, colour, scent, etc.) and ornamental plants. Moreover, numerous plant secondary metabolites such as flavonoids, alkaloids, tannins, saponins, steroids, anthocyanins, terpenoids, rotenoids etc. have found commercial application as drug, dye, flavour, fragrance, insecticide, etc. Such fine chemicals are extracted and purified from plant materials (Bohlmann et al, 1998).

In the present study was carried out on the plant sample revealed the presence of medicinally active constituents. The phytochemical characters of the *Vitis vinifera* fruit peels investigated and summarized in Table-1 and Fig2. The phytochemical screening *Vitis vinifera* fruit peels showed that the presence of saponins, steroids, flavonoids, terpenoids carbohydrate, anthroquinones, polyphenol and glycosides while tannin, phlopatannins, triterpenoids, alkaloids and protein was absent in methanol extracts.

Table.1 Qualitative analysis of Phytochemicals in *Vitis vinifera* fruit peels

S.No	Test analysis	Result
1	Tannin	-
2	Phlobatannin	-
3	Saponin	+
4	Flavanoids	++
5	Steroids	+
6	Terpenoids	++
7	Triterpnoids	-
8	Alkaloid	-
9	Carbohydrate	++
10	Protein	-
11	Anthroquinone	+
12	Polyphenol	++
13	Glycoside	++

(-) Indicates Absence; (+) Indicates Presence; (++) Moderately present

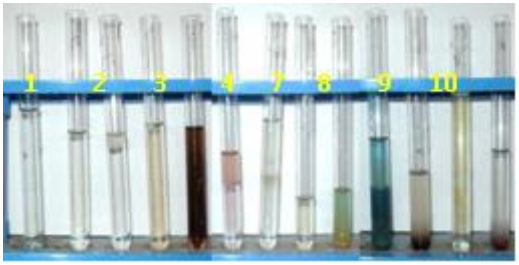


Fig. 2: Qualitative analysis of Phytochemicals in *Vitis vinifera* fruit peels

Balasundram et al, (2006) analyzed preliminary phytochemicals in some of the important medicinal and aromatic plants. The leaves and fruits of *Pedalium murex* were experimented to evaluate the phytochemical components. Preliminary phytochemical screening of petroleum ether and alcohol extract of *Symplocos recemosa* was carried out. The phytochemicals minerals and vitamins A and C compositions of *Spondias mombin* leaves were determined by evaluated the phytochemical constituents and bioactivities of the extracts of *Cassia nigricans*.

Reena Ganesan *et al*., (2013) aimed to carry out preliminary phytochemical of six different solvents extracts from leaf and leaf derived callus of *Sebastiania chamaelea*. The preliminary phytochemical analysis reflects the presence of phenolic compounds, carbohydrate, alkaloids, phytosterols, fats and oils, terpenoids. The result highlights among two extracts, leaf extract show negligible activity than callus extracts Kumar *et al*., (2013) investigated the preliminary phytochemical screening of the leaves of the plant *Lasia spinosa* (Lour) Thwaites. The phytochemical screening showed that the methanol and aqueous extracts contained alkaloid, the carbohydrates and the phenolic compounds were present in all of the solvent extract except petroleum ether extract. The chloroform, ethyl acetate and the aqueous extract contained glycosides whereas the saponins present in methanol and aqueous extract. The ethyl acetate extract contain only the flavonoids.

Quantitative analysis revealed that the plant has phenols, alkaloids, tannin and saponin. Significant amount of total phenols (233.32mg/gm) and flavonoids (100mg/gm) were presented (Table 2). The above phytoconstituents were tested as per the standard methods.

Table 2 Quantitative phytochemical analysis of methanolic peel extract of *Vitis vinifera*

S.No	Secondary Metabolites	Result (mg/gm)
1	Flavonoids	100±6.35
2	Total phenol	233.32±13.74

Values are expressed as mean ± SD for triplicates

Flavonoids are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action. Flavonoids are 15 carbon compounds generally distributed throughout the plant kingdoms. Some isoflavones widely used in insecticides. They might also play a role in disease resistance. Some flavonoids such as quercetin and rutin, are known to support human health by serving anti- inflammatory, anti-histaminic and anti-viral agents (Takechi et al, 1999). Flavonoid compounds exhibit inhibitory effects against multiple viruses. Numerous studies have documented the effectiveness of flavonoids, such as glycyrrhizin and chrysin against HIV. Flavonoids are potent water soluble antioxidants and free radical scavengers which prevent oxidative cell damage and have strong anticancer activity. Flavonoids have been referred to as nature‟s biological response modifiers, because of inherent ability to modify the body‟s reaction to allergies and virus and the

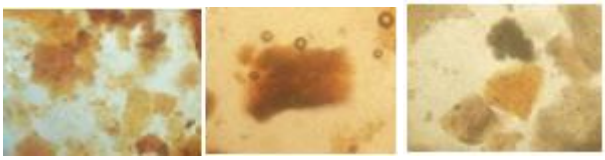
showed their anti-allergic, anti- inflammatory, anti-microbial and anti-cancer activities (Duraipandiyan *et al.*, 2006).

Histochemical analysis of leaves powder of *Vitis vinifera* fruit peels

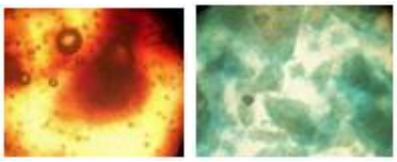
The powder of *Vitis vinifera* fruit peels were treated with specific chemicals and reagents. The *Vitis vinifera* fruit peels powder treated with phloroglucinol and diluted HCl gave red colour indicates lignin, treated with diluted ammonia and H_2SO_4 gave yellow colour indicates flavonoids and treated with dragant draft reagent gave brown colour indicates alkaloids. The treated plant powder further analysed in light microscope (Table 3 and Fig3**).**

S.No	Charecterisation	Observation	Result
1	Flavonoids	Yellow	++
2	Glycoside	Red	++
3	Terpenoids	Orange	++
4	Saponin	Yellow	+
5	Polyphenol	Blue Green/red	++

Table 3 Histochemical analysis of leaves powder of *Vitis vinifera fruit* peels.



Flavonoids Glycoside Terpenoids



Saponin Polyphenol

Fig. 3: Histochemical analysis of leaves powder of *Vitis viniferous fruit* peels

Histochemical techniques have been employed to characterize structure and development and to study time course of deposition and distribution of major storage compounds such as proteins, lipids, starch, phytin and minerals like calcium, potassium and iron (Mishra, 1989). The importance of histochemistry in solving critical biosystematic problems is as popular as the use of other markers. According to botanical literatures, the use of histochemical characters in taxonomic conclusions is now a common practice.

Histochemical analyses of the plant were carried out using light microscopy and fluorescence study was analyzed by UV lamp. Results of histochemical tests showed positive reaction to phenol compounds, polyphenol and tannin in the thallus. Fine powder and different solvent extracts of *Turbinaria ornata* obtained using petroleumether, benzene, chloroform, acetone, ethanol and aqueous were examined under visible and UV light. The powdered materials were also treated with various reagents such as 50 % nitric acid, 50 % sulphuric acid, 1N HCl, 1N NaOH and changes in colour were recorded. He concluded that the histochemical and the fluorescence analysis could be used for rapid identification of potential medicinal plants and bioactive compounds which is present in the particular plant.

Identification of compounds in *Vitis vinifera fruit* peels extract by GC MS analysis

The term „pesticide" encompasses a wide range of organic compounds used to control weeds (herbicide), fungi and moulds (fungicide), insects (insecticide) and pests (pesticide). Traces of these compounds may enter rivers and surface water as run-off from agriculturalland (where they are used to protect crops) or via non- agricultural use (for example, the use of herbicides to control weeds along motorway verges). They may also percolate through soil into ground water.

Sensitive and simultaneous analysis of multiple compounds is possible using GC/MS, the method is being widely used for analysis of pesticides for environmental specimens such as water, contaminated food and soil. Among main pesticides which hadbeen reported causative for poisoning cases more than 30 kinds of pesticides have been picked up from organophosphorus, organochlorine, carbamate and triazine pesticides, and a method for simultaneous analysis of many pesticides by GC/MS is presented **(Traore et al, 2000).** Twenty compounds were identified by GCMS. The GC-MS analysis revealed the presence of various compounds like 2-Acetylbenzoic acid, Acetamide, 2- dimethylamino-N-

phenyl-2-thio and Imidazole in the methanolic extract of *Vitis vinifera* fruit peels. The presence of this compounds justifies the application of pesticide to grapes. This

compounds are insecticide, herbicide and fungicides (Fig 6, Table 4 and 5).

Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area%	Height%	A/H	Mark	Name
1	11.548	11.500	11.617	5.14	7.86	2.59		SILANE, TETRAMETHYL-
2	13.954	13.908	14.000	3.32	6.49	2.03		1,3-DIOXOLANE
3	15.697	15.592	15.717	11.62	7.80	5.90		2-Acetylbenzoic acid
4	15.767	15.717	15.792	3.60	3.15	4.53	V	Benzene, (1,2,2-trimethoxyethyl)-
5	18.252	18.208	18.342	6.86	6.57	4.14		Cyclopentane, 1,2-dimethyl-3-(1-methylethen
6	19.817	19.750	19.917	3.19	2.97	4.25		1,3-Dioxolane, 2-tert-butyl-2-phenyl-
7	19.950	19.917	19.983	4.09	5.00	3.23		OCTADECANOIC ACID, ETHYL ESTER
8	19.999	19.983	20.042	2.43	4.79	2.01	V	PENTANOIC ACID
9	21.017	20.867	21.033	2.42	1.88	5.10		2-Propanol, 1,1'-oxybis-
10	21.236	21.167	21.283	9.75	8.56	4.52		DECANE, 1,2-EPOXY-
11	21.350	21.283	21.367	5.19	3.90	5.27	V	L-Serine, N-methyl-
12	21.974	21.817	21.992	4.65	1.99	9.27	V	Chlorocyclohexyldimethylsilane
13	22.517	22.492	22.717	2.63	1.15	9.09		Acetamide, 2-dimethylamino- N-phenyl-2-thio
14	23.878	23.858	23.983	3.45	3.18	4.31	V	Imidazole, 2-[[(.beta.-carboxyvinyl]amin
15	28.178	28.033	28.217	4.50	3.11	5.74		1,1,3,3,5,5,7,7,9,9,11,11,13,13-TETRADECA
16	28.292	28.217	28.375	2.81	2.25	4.96	V	3-METHOXY-4-[(TRIMETHYLSILYL)OX
17	28.700	28.642	28.717	7.55	10.05	2.97	V	9-Phosphabicyclo[4.2.1]nona-2,4,7-trien-9-an
18	28.734	28.717	28.758	5.58	9.92	2.23	V	dl-Homoserine
19	28.792	28.758	28.833	6.05	5.95	4.03	V	3-FURYLMETHYL 4-(4-MORPHOLINYL(
20	28.917	28.833	28.958	5.18	3.45	5.94	V	Cyclohexene,3-(2-propenyl)-
				100.00	100.00			

Table 4: Identification of phytocompounds in the peels of *Vitis vinifera* fruit using GCMS

S.No.	R. Time	Area %	Name of the compounds	Nature of Pesticide
1.	15.59	11.6	2-Acetylbenzoic acid	Insecticidal
2.	22.517	2.63	Acetamide, 2-dimethylamino- N-phenyl-2-thio	Herbicides
3.	23.878	3.45	Imidazole	Fungicide

Table 5: Identification of pesticide compounds present in the peels of *Vitis vinifera*

CONCLUSION

Agricultural chemicals include not only pesticides for protecting plants, such as insecticides, germicides, herbicides and rodenticides, but also fertilizers and growth regulating substances being used in agricultural production and horticulture. In poisoning cases with agricultural chemicals, the causative poisons are largely the pesticides. They are many cases, in which the poisoning due to exposure to an organophosphorus pesticide is obvious with clinical symptoms. However, there are more than 5,000 agricultural chemicals registered and commercially available in Indian markets. It is essential to identify a causative chemical to make the final clinical diagnosis in a poisoning-suspected case. Therefore, the present study was to investigate the phytochemical screening and identification of pesticides of *Vitis vinifera* fruit peels using GCMS analysis.

The phytochemical screening *Vitis vinifera* fruit peels showed that the presence of saponins, steroids, flavonoids, terpenoids carbohydrate, anthroquinones, polyphenol and glycosides while tannin, phlopatannins, triterpenoids, alkaloids and protein was absent in methanol extracts. Quantitative analysis revealed that the plant has phenols, alkaloids, tannin and saponin. Significant amount of total phenols(233.32mg/gm) and flavonoids (100mg/gm) were presented (Table 4.3). The above phytoconstituents were tested as per the standard methods. The histochemical analysis further confirmed the flavonoids, polyphenol, terpenoids glycoside and saponin. The GC-MS analysis revealed the presence of pesticide compounds like 2-Acetylbenzoic acid, Acetamide, 2-dimethylamino-N-phenyl-2- thio and Imidazole in the methanolic extract of *Vitis vinifera* fruit peels.

Overall, it can be concluded from the present study that *Vitis vinifera* fruit peels contains rich source of phytochemicals confirmed by qualitative and quantitative analysis. GC-MS analysis of *Vitis vinifera* fruit peels showed the presence of pesticide compounds like 2-Acetylbenzoic acid, Acetamide, 2- dimethylamino-N-phenyl-2-thio and Imidazole. This study is the first scientific report that provides contamination of pesticides in the *Vitis vinifera* fruit peels, thus providing scientific validity may be a toxicity for consumption by the local populace of south India.

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Research Article

Antioxidant activity of β – Sitosterol isolated from *Dodoneoa viscosa* leaf extract on lead induced oxidative stress in rats

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ABSTRACT

Oxidative stress is considered a possible molecular mechanism involved in Lead (Pb) toxicity. Considering the vulnerability of the developing heart, liver and brain to Pb toxicity, this study was carried out to investigate the free radical scavenging activity of β – Sitosterol from methanol extract of D*odonoea viscosa linn* leaf on Pb exposure on the heart, liver and regions of the brain. Adult male albino rats Wister strain were exposed to 200ppm of Pb, as Pb acetate in their drinking. The activities of antioxidant enzymes such as superoxide dismutase (SOD), Glutathione peroxidase (GPx) and Catalase (CAT) were determined in the heart and liver of male adult rats. Superoxide dismutase (SOD), gluthione peroxidase (GPx) and Glutathione reductase (GRed) were also determined in the brain of rats. In addition levels of non-enzymatic antioxidants namely vitamin E, vitamin C from plasma along with total reduced glutathione from liver and heart were also determined. In the Pb-exposed rats, the diminished activity of super oxide dismutase, Glutathione reductase and glutathione peroxidase in the brain of plant extract treated rats was significantly enhanced. The diminished activity of SOD, CAT, GPx in the heart and liver was increased. The decreased activity of vitamin E and vitamin C was normalized in the plasma of plant treated. There was a significant effect of the plant extract on the enzyme activity and the three regions of the brain evaluated in pb exposed rated. Based on the present results, it seems that oxidative stress due to decreased antioxidant function is normalized in methanol extract of *Dodonoea viscosa linn* solution treated rats. Toxicity in the organs tested and neurotoxicity in the brain is due to Pb exposure. And Spectral analysis of the methanol extract of *Dodoneoa viscoasa lin* was carried out.

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INTRODUCTION

Lead (Pb) is one of the common non-essential toxic heavy metal widely distributed in the environment Chronic exposure to low levels of Pb has been a matter of public health concern in many countries. Pb poisoning exerts its most severe consequences in the developing cardiovascular system (Milton, R 1979), blood (Haeger-Aronen,B 1966), kidney (Cramer, K1974) , liver (Scoppa,P 1973) and brain(Burdette and Goldstein, 1986) due to the intense cellular proliferation, differentiation and synaptogenesis. Moreover, the developing organism presents a five fold greater absorption of Pb (Lockitch, 1993) and lacks a functional blood brain barrier (Goyer, 1990).

Absorption of inorganic lead can lead to certain biochemical and metabolic toxicities (Murthy, G.K1971). The effect of lead toxicity may be due to its impact on the release of free radicals like hydroxyl (Ding, Y.2000). Therefore, a need for a reliable antioxidant which must be capable of scavenging the free radicals when a system is exposed to lead arises. Free radical attack is indicated by change in the levels of some biomolecules in the body. The main products of lipid peroxidation are Malondialdehyde (MDA), conjugated dienes and Hydroperoxides (HYPDX). Moreover, lead is also reported to release free radicals (hydroxy) thereby stimulating the process of lipid peroxidation. Lipids, particularly polyunsaturated fatty acids (PUFA), are the major class of biomolecules susceptible to oxidative damage by free radicals (lipid peroxidation), (Ray, S. 1992 and Akocyznska,A.1993). Lipid peroxides have been shown to impair tissue membranes, which is a risk factor in many diseases. Lead is reported to have an inhibitory action on the membrane bound enzymes such as Na+-K+-ATPase and Mg2+-ATPase in various vital organs (Siegel, G.J. 1977).

Perinatal exposure to low levels of Pb has been involved in behavioural and neuro chemical alterations detected in both suckling and adult rats (Lasley and Lane, 1988; Moreira et al., 2001;Moresco et al., 1988; Rodrigues et al., 1996; Widzowski et al., 1994). One possible molecular mechanism involved in the Pb neurotoxicity is the disruption of the pro oxidant/antioxidant balance (Adonaylo and Oteiza, 1999a; Hermes-Lima etal., 1991; Sandhir et al., 1994), which can lead to heart, liver, kidney and brain injury via oxidative damage to critical biomolecules, such as lipids, proteins and DNA.. To date, the studies that have evaluated oxidative stress in heart, liver and brain following Pb exposure were carried out in adult animals (Sandhir et al., 1994; Adonaylo and Oteiza, 1999b; Rehman, 1984) or in newborns exposed post natally to high doses of Pb (Hsu, 1981; Valenzuela et al., 1989). Based on the above considerations, this study was carried out to investigate the effects of Pb exposure on the antioxidant system of heart, liver and brain. Albino rats were exposed to lead acetate through drinking water for 30 days to induce oxidative stress. Anti oxidant levels in heart, liver,blood and brain (Moreira, et.al.,2001) of rats exposed to Pb were evaluated. The non-enzymatic and enzymatic antioxidants evaluated were: Reduced Glutathione (SGH), Vitamin C, VitaminE, superoxide dismutase (SOD), glutathione peroxidase (GPx) and, Catalase (CAT). The levels of Iron, Phospho lipids and Malondialdehyde (MDA) were also analysed.

MATERIALS AND METHODS

Plant material

The Leaves of *Dodonoea viscosa linn* were collected during the month of December – January in India. The collected plant material (leaf) was washed thoroughly with fresh water and dried under shade. The shade dried leaf material was powdered and sieved through muslin cloth.

Methanol Extract preparation

The root extract was prepared first by refluxing it with petroleum ether (50–60 ◦C for 72 h) and then with methanol (60–80 ◦C for 72 h) in a Soxhlet apparatus. The extract was then concentrated in vacuo, kept in a dessicator at room temperature and expressed in terms of dry weight. Before use, the extracted material was dissolved (10 mg/ml) in 0.9% saline and centrifuged at 2000 rpm×10 min at room temperature.

Isolation and purification of active plant constituent

Silica gel column chromatography

The methanolic leaf extract (100 g) of *Dodonoea viscosa* was dissolved in methanol, centrifuged (2000 rpm ×30 min). The suspension was added to silica gel and evaporated to dryness. The residue was placed on top of the silica gel (60–120 mesh) column (85 cm ×18 cm) and was eluted with petroleum ether:chloroform (9:1, 4:1, 2:1, 1:1, 0:1, 500 ml ×10 fractions each), chloroform:methanol (98:02, 95:05, 90:10, 500 ml ×10 fractions each). The fraction elutes were evaporated to dryness and tested for (i) homogeneity through TLC and (ii) venom neutralization in experimental animals. For thin layer chromatography, a mixture of silica gel G in water was degassed and poured on TLC plates (20 cm ×10 cm ×1 cm). The plates were activated at 110 .C for 2 h. The eluted fractions were tested for the homogeneity of the compounds. Spots were developed in iodine chamber and *R* f calculated. Each fraction was then tested for venom neutralization.

The extract of *Dodoneoa viscosa* (100 g) fractionated over silica gel (60–120 mesh) column chromatography (Chatterjee et al.2006) gave an active fraction, which was

eluted with chloroform: methanol (95:05, v/v). This active fraction when further rechromatographed on silica gel (100–200 mesh) column chromatography resolved into a pure compound eluted by chloroform: methanol (50:50, v/v). On TLC, this compound produced a single spot at *R* f 0.60 using benzene: ethyl acetate (70:30, v/v). Yield of the active compound was 0.2%. Chemical characterization by various spectroscopy viz. IR spectra, NMR spectra and mass spectrum indicated the active compound to be β – Sitosterol.

Spectroscopic analysis

Spectroscopic analysis was performed by Ultraviolet–visible (UV), Infrared (IR) and Nuclear Magnetic Resonance (NMR). UV absorption spectra were obtained in 1.0% H20 solution using a UV–vis U-2010 Spectrophotometer (Shimadzu). IR spectra were recorded in a Mattson Instruments FTIR spectrophotometer, using KBr disks and 4 cm−1 resolution. 1D and 2D (HSQC) NMR spectra were obtained in D20, taking dioxane or acetone as internal reference, using a Bruker DRX 400 AVANCE spectrometer. The NMR analyses were carried out at the "Sophisticated Analytical instrument facility, Department of chemistry, Indian Institute of Technology(IIT), Madras, India.

Animal and tissue preparation

Adult male albino rats Wistar strain weighing 100-120g and free from the pathogens were purchased from the Indian Institute of Sciences, Bangalore with the permission of Animal Ethics Committee. The rats were divided into five groups and housed in rat cages, which were exposed to sunlight. The drinking water given to them was adulterated with 200ppm Pb, as Pb acetate. To prevent the formation of Pb precipitate, 0.5ml of glacial acetic acid was added while stirring to prepare 1000ml of Pb acetate solution. The treatment lasted throughout the period. The Pb exposure regimen in brain regions was chosen based on a previous study (Moreira et al., 2001).

The rats were randomly divided into 5 groups with each group containing 8 rats. All the animals were allowed to drink deionised water ad libitum throughout the study period (30days). (Group1) Normal animals received deionised water alone. (Group2) Control animals had free access to lead acetate in deionised water in a concentration of 200ppm for 30 days. (Group3) Animals were treated with the crude powder of *Dodonoea viscosa* linn leaf in physiological saline at a concentration of 50mg\100g body weight once daily for 30 days. They had free access to deionised water. (Group4) Drug Positive control; Animals were treated with crude powder of *Dodonoea viscosa linn* leaf in physiological saline at a concentration of 50mg\100g body weight once daily for 30 days and had free access to deionised water. (Group5) Lead exposed animals as per group2 were treated with the standard drug vitamin E in Sunflower oil at a concentration of 50 IU\kg.

At the end of 30 days the animals were anesthetized with pentobarbital sodium (60 mg/kg, intraperitoneally), killed under mild anesthesia and blood collected from the jugular vein in heparinised tubes. The body was cut open and tissues were dissected out and rinsed in ice-cold saline. After perfusion through the ascending aorta with 140 mM phosphate buffer saline (PBS) pH 7.4, the brain was removed and dissected using the method of Glowinski and Iversen (1966) into the three regions of interest: hypothalamus, hippocampus and striatum. Due to the small amount of tissue, tissue of three littermates was pooled. The tissue was weighed and homogenized (1 mg tissue/4 ml buffer) in ice-cold 140 mM PBS using 10 strokes in a Teflon/glass homogenizer. The tissue was maintained at 0-4°C throughout the dissection and homogenization procedures. The homogenate was centrifuged for 15 min at 1000*g* at 4 °C and the supernatant centrifuged again at 18 000*g* for 15 min at 4 °C. The organs such as liver, heart and brain were per fused with physiological saline and made into a 10% homogenate with tris sucrose buffer.

Enzyme assays

The final supernatant from brain tissue homogenate was used for the evaluation of the activity of SOD (McCord and Fridovich, 1969), GPx (Sies et al., 1979) and GRed (Sies et al., 1979). Enzymatic antioxidants such as Superoxide dismutase (SOD), Glutathione Peroxidase (GPx), Catalase (CAT) present were determined from the liver and heart tissue homogenates. Non enzymatic antioxidants namely vitamin E and vitamin C from plasma along with total reduced Glutathione from liver and heart were estimated. The excised liver and heart tissues of albino rats were rinsed in ice-cold physiological saline and then homogenized in Tris_HCL buffer (pH7.4). The tissue homogenates and plasma were used for the following estimations. (1) Thiobarbituric acid reactive substances (TBARS) (lipidperoxide) were estimated according to the method of Nichans and Samuelson (1968). (2) Estimation of Serum Iron by the method of Ramsay et al., ((1958). (3) Phospholipids were estimated by the method of Zilversmith and Davis methods (1950). (4) Alpha-Tocopherol was determined according to the method of Baker and Frank (1980). (5) Ascorbic acid was estimated by the method of Roe and Kuether (1943). (6) Reduced glutathione was estimated by the method of Ellman (1959). (7) The activity of superoxide dismutase (SOD) in heart and liver was assayed by the method of Beauchamp and Fridovich, (1971). (8) Catalase (CAT) activity was assayed by the method of Sinha (1972). (9) The activity of glutathione peroxidase (GPx) was assayed according to the method Rotruck et al., (1973) and SOD, GPX

and GRed in the brain were assayed by the method of Morreia et. al.,(2001).

Protein content was determined by the biuret method (Kit Proti A/G, Celm, Brazil). SOD result is expressed as one unit U/mg of protein and GPx and GRed results as one thousand units mU/ mg of protein. All chemicals were obtained from Sigma (St. Louis, MO).

Blood Pb determination

Pb concentration was determined in blood of albino rats by atomic absorption spectrophotometry with a Zeeman-corrected graphite furnace (Model Spectra AA-220Z, Varian, Australia), as described by (Moreira et al., 2001).

Statistics

Data are expressed as mean ± SD. The Mann Whitney non-parametric tests and paramertric student's t-test (SAS.1985) were used for tests of significance of differences between groups. A probability of less than 0.05 was accepted as significant. Statistical calculations were performed using SPSS (version 9) software.

RESULTS AND DISCUSSIONS

Identification of different compounds of *Dodoneoa viscosa leaf*

The fraction E on TLC plate showed 5 bands and designated as E1, E2, E3, E4 and E5 with R_F values of 0.66, 0.60, 0.55, 0.48 and 0.44 cm, respectively. After elution, the Fraction E2 with anti snake venom activity showed single band in TLC plate with the R_F value 0.61 cm.

Spectral analysis of the compound from *Dodoneoa viscosa*

The UV–vis spectrum of methanol extract in 1.0% aqueous solution shows bands at 206.0 nm (1% 0.60) Uv λ_{max} (MeOH): 206 nm . The UV–vis spectrum of Methanol extract showed bands characteristic of phenol group (Okuda, 1999 The IR spectrum of methanol extract shows absorption bands (KBr disks) at:IRυcm^{1}3423,2956.2937.2866,1647,1461,1311,1261,1056,1022,960,789 (Fig.3.6) The 1 H and 13 C NMR of methanol extract was obtained in D2O. Table 2 shows the 1 H NMR and 13 C NMR chemical shifts in ppm(δ): ^{1}H NMR ($CDCl_3$, 300 MHz, δ ppm) values: H- 2(1.48,2H,m); H-3(1.99,2H,m); H-(1.82,2H,m); H-5,(3.53,1H,m); H-6(2.27,1H,m); H-7(1.82,2H,m); H-8(1.99,1H,m); H-9(5.36,1H,m); H-10(1.48,1H,m); H-11(1.48,1H,m); H-12(1.11,2H,m); H-13(1.48,1H,m); H-14(1.148,1H,m); H-15(1.48,2H,m); H-16(1.98,1H,m); H-17(1.23,2H,m); H-18(1.11,2H,m); H-19(1.82,1H,m); H-20(1.48,1H,m); H-21(0.82,3H,s); H-221'(0.82,3H,s); H-22(1.08,2H,m); H-23(0.93,3H,s); H-24(0.93,3H,s); H-25(1.00,3H,s); H-26(0.67,3H,s) and ^{13}C NMR ($CDCl_3$, 300MHz, δ ppm) values: C-1,36.46;2,37.19; C-2',140.70, C-3,42.25; C-4,31.60; C-5,71.77; C-6,31.85; C-8,50.05; C-9,121.71; C-10,56.70; C-11,39.70; C-11',42.25; C-12,21.03; C-13,55.97; C-14,28.22; C-15,24.27; C-16,36.11; C-17,33.65; C-18,25.93; C-19,45.75; C-20,33.87; C-21,18.98; C-21',18.98; C-22,22.99; C-23,12.37; C-24,19.38; C-25,19.81; C-26,12. Finally, the NMR data showed that the aliphatic resonances predominate, indicating the higher proportion of the carbohydrate moieties in methanol extract constituents. Furthermore, the NMR data confirm the tentative structure for this compound as given β – Sitosterol (Fig.1.).

Antioxidant enzymes activity in Brain

Hypothalamus

The effect of Pb exposure during on the antioxidant enzymes activity of hypothalamus is shown in Table 1. The students t-test showed that SOD, GPx and GRed were decreased (P<0.05) in the Pb-exposed group. Plant treated animals showed statistically significant enhancement of the activity of SOD,GPx (P<0.05) and GRed (P<0.05) .

Groups	SOD (U/mg protein)	GPx (mU/mg protein)	GRed (mU/mg protein)
Normal	32.0±1.3	38.6±1.4	26.0±1.5
Control	24.3±1.8*	26.0±3.1*	22.8±2.5*
Leaf+Pb	27.0±1.4 *	35.0±1.2	25.0±1.0
Leaf	33.0±1.4 *	39.0±1*	25.0±1.7*
Standard Vit.E	33.0±1.4 *	36.0±1.2	24.6±1.4

Table.1. Levels of SOD,GPx and GRed in hypothalamus
Data are presented as mean±SE (Eight rats in each groups). Statistical significance student's t-test * (P <0.05).

Hippocampus

The effect of Pb exposure during feeding on the antioxidant enzymes activity of hippocampus is shown in Table2. The students t-test showed that SOD, GPx and GRed were decreased (P<0.05) in the Pb-exposed group. Plant treated animals showed statistically significant enhancement in the activity of SOD (P<0.05), GPx (P<0.05) and GRed (P<0.05).

Groups	SOD (U/mg protein)	GPx (mU/mg protein)	GRed (mU/mg protein)
Normal	20.7±1.4	35.6±1.4	45.0±1.5
Control	17.3±1.8*	30.0±1.5*	41.8±1.7*
Leaf+Pb	19.0±1.4	35.0±1.2*	43.0±1.0
Leaf	21.0±1.4*	34.0±1.2*	43.0±1.7*
Standard Vit.E	21.0±1.4	36.0±1.2	44.6±1.4

Table.2. Levels of SOD,GPx and GRed in Hippocampus

Data are presented as mean±SE (Eight rats in each groups). Statistical significance student's t-test * (P <0.05).

Striatum

The effect of Pb exposure during feeding on the antioxidant enzymes activity of striatum is shown in Table 3. The students t-test indicated a significant increase in the activities of SOD, GPx and GRed (P< 0.05) when compared to Pb - exposed rats.

Enzyme assays

Significant differences in enzyme activities were found between the Pb exposed and plant treated liver, heart, plasma and brain of albino rats. As lipid per oxidation increased there was a significant increase in the level of MDA in plasma, liver, and heart of rats exposed to lead (Table 4). This MDA level was normalized in rats treated with *Dodonoea viscosa linn* leaf. The level of vitamin E, Vitamin C (Table 5) and Iron (Table 4) was significantly reduced (P□0.05) in all Pb exposed rat plasma samples when compared with normal and plant treated rat plasma samples.

The level of total reduced glutathione was significantly reduced (P<0.05) in all-Pb exposed rat tissues when compared with normal and plant treated rat tissues (Table 5). The activity of superoxide dismutase was significantly (P<0.05) reduced in all the Pb exposed tissues of rats, while it was highest in heart and liver of normal and plant treated rats. (Table 6), Catalase activity was higher in the heart and liver of normal rats. In Pb exposed rats there was a significant decrease (P<0.05) in catalase activity in heart and liver (Table 6). Glutathione peroxidase activity was highest in the heart and liver of normal rats while it was significantly lower (P<0.05) in all Pb exposed tissues of heart, liver and brain (Table 7). Overall, activity of antioxidants was significantly lower in all the tissues of Pb exposed rats when compared to equivalent tissues of normal and plant treated rats.

Blood Pb Levels in rats

The blood Pb levels in the rats were 57.6±4.7μg/dl for the Pb-exposed group. It was not detectable (i.e. 0.1μ g/dl) for the normal groups and plant treated goups.

Discussions

Oxidative stress is a term denoting an imbalance between the production of oxidants and the respective defense system of an organism. Oxidants encompass reactive oxygen species, sulfur centered radicals and various others. Oxidative damage may be involved in aging and pathogenesis of major diseases such as cancer, otherosclerosis and certain neurological disorders.

In the present study, a significant increase in the level of MDA in plasma, liver and heart of rats exposed to Lead indicates enhanced lipid per-oxidations. This is due to non-essential toxic heavy metal lead that releases free radicals (hydroxy). The elevated level of MDA in plasma clearly shows lipid per-oxidation in the polymeric fatty acid present within membrane phospholipids of myocardial and hepatic cells. Myocardial and hepatic MDA levels were found to be normalized in rats treated with *Dodonoea viscosa linn* leaves.

The antioxidant enzymes SOD, CAT and GPx play an important role in scavenging toxic intermediates of reactive oxygen species. In the control group, there was a significant reduction in SOD, CAT, GPx in liver and heart. The antioxidant enzymes SOD, GPx and GRed play an important role in scavenging toxic intermediates of reactive oxygen species. In the control group, there was a significant reduction in SOD, GPx and GRed activity. In plant treated groups there was an increase in their antioxidant levels.

We have examined the antioxidant system status and the effect of *Dodoneoa viscosa linn* treatment on three regions of the brain (Hypothalamus,hippocampus and striatum) exposed to Pb. We have chosen to evaluate brain regions instead of whole brain because different regions may respond differently to oxidative stress (Sandhir et al., 1994; Shukla et al., 1988).

In male albino rats the only statistically significant effect that Pb exposure induced was decrease in the activity of SOD ,GPx and GRed in the hypothalamus. The diminished activity of SOD,GPX and Gred were normalized in *Dodoneoa viscosa linn* treated rats.

In adult rats, no effects of treatment were observed in any of the regions evaluated. One could imply that the lack of effects on adults is to be expected since Pb would no more be detected in the blood of these rats at the end thirty days. However, after (Moreira et al.,2001) have reported that Pb is

still present in the brain tissue which could have a long term effect of Pb on the antioxidant enzymes.

Oxidative stress has been suggested as one possible mechanism for Pb neurotoxicity. Pb-induced oxidative stress damage could result from: (1) the inhibition of 5-aminolevulinic acid (ALA) dehydratase by Pb leading to the accumulation of ALA, a potential endogenous source of free radicals; (2) direct interaction of Pb with biological membranes, inducing lipid peroxidation; (3) increase of intracellular levels of calcium, impairing mitochondrial functions; and (4) Pb-induced decrease on free radical scavenging enzymes and glutathione (Hermes-Lima et al., 1991; Sandhir et al., 1994). The latter is mainly attributed to the high affinity of Pb for sulfhydryl groups or metal cofactor in these enzymes and molecules (Sandhir et al., 1994).

Based on the present results, it has been confirmed that oxidative stress due to decreased antioxidant function in pb exposed group has been normalized by treating with *Dodoneoa viscosa linn* leaf extract The active compound of the extract was identified as β - Sitosterol which was confirmed by NMR Data fig.1. Moreira et al., 2001) have observed neurochemical alterations in different brain regions in adult rats exposed to a Pb exposure regimen similar to that employed in this study (Moreira et al., 2001). We have observed a behavioral alteration in our study. Oxidative stress is the major mechanism involved in the toxicity and neurotoxicity induced by Pb exposure in rats. The result of the present study suggests that *Dodonoea viscosa* leaf offers protection to myocardial cells, hepatic cells and brain tissues either by quenching free radicals or chelating iron enzymes.

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Research Article

Clinical Assessment of Gout Arthritis and Rheumatoid Arthritis

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Anti-streptolysin O

ABSTRACT

Rheumatoid Arthritis is generally a chronic, autoimmune inflammatory condition, while Gout Arthritis is an inflammatory condition, both affecting the joints, causing pain and inflammation with varying severity among patients. The risk factors include age, gender, genetics, and environmental exposure. Data's on age and sex of the subjects are obtained from the interviews conducted to the subjects Uric acid of the subjects were measured in serum samples by using spectrophotometrically. Anti-streptolysinO and Rheumatoid factors levels were also measured by UV spectroscopy. C-reactive protein is significantly highly increases in the case of Gout Arthritis,and Rheumatoid factor and Anti-streptolysinO are slightly increased. In the case of Rheumatoid arthritis, C-reactive protein are significantly elevated.

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INTRODUCTION

Many complications can follow, such as permanent joint damage requiring arthroplasty, rheumatoid vasculitis, and Felty syndrome requiring splenectomy if it remains unaddressed. Often, the bone and cartilage of joints are destroyed, and tendons and ligaments weaken (Lee JE, Kim IJ, Cho MS, Lee J. et al, 2017).

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All this damage to the joints causes deformities and bone erosion, usually very painful for a patient. Common symptoms of RA include morning stiffness of the affected joints for > 40 min, fatigue, fever, weight loss, joints that are tender, swollen and warm, and rheumatoid nodules under the skin. The onset of this disease is usually from the age of 40 to 60 years, with remission and exacerbation. It can also afflict young children even before the age of 16 years, referred to as juvenile RA (JRA), which is similar to RA except that rheumatoid factor is not found (McInnes IB, Schett G. et al 2011)(Chaudhari K, Rizvi S. et al, 2016). In the West, the prevalence of RA is believed to be 1–2% (Alamanos Y, Voulgari PV, Drosos AA. et al, 2006) and 1% worldwide (Chopra A, Abdel-Nasser A.et al, 2008). As there is no cure for Arthritis, the treatment goals are to reduce the pain and slow further damage. Here, we present a brief summary of various past and present treatment modalities to address the complications associated with Arthritis. Clinically, the diagnosis of RA can be differentiated from osteoarthritis (OA) as the affected areas in RA are the proximal interphalangeal (PIP) and metacarpophalangeal (MP)

joints; OA typically affects the distal interphalangeal (DIP) joint. OA is the most common type of arthritis and is caused by wear and tear rather than an autoimmune condition. It has no effects on the lungs, heart, or immune system. In addition, OA typically affects only one side of the body, as opposed to the symmetrical nature of RA. Another differentiating factor is that RA patients suffer from persistent morning stiffness for at least ≥1 h. Patients with OA may have morning stiffness, but this typically resolves or decreases within 30–40 min (McGonagle D, Hermann KG, Tan AL. et al, 2015) (Piyarulli D, Koolaee RM. et al, 2016).

The goals of treatment for RA are to reduce joint inflammation and pain, maximize joint function, and prevent joint destruction and deformity. Treatment regimens consist of combinations of pharmaceuticals, weight-bearing exercise, educating patients about the disease, and rest. Treatments are generally customized to a patient's needs and depend on their overall health. This includes factors such as disease progression, the joints involved, age, overall health, occupation, compliance, and education about the disease (Staheli LT, Hall JG, Jaffe KM, Paholke DO. et al, 1998). This review briefly highlights the classic and current treatment options available to address the discomfort/complications of RA. An exhaustive review was recently published by (Smolen JS, Aletaha D, Barton A, Burmester GR, Emery P, Firestein GS, et al, 2018)

Gout is a picturesque presentation of uric acid disturbance. It is the most well understood and described type of arthritis. Its epidemiology is studied. New insights into the pathophysiology of hyperuricemia and gouty arthritis; acute and chronic allow for an even better understanding of the disease. The role of genetic predisposition is becoming more evident. The clinical picture of gout is divided into asymptomatic hyperuricemia, acute gouty arthritis, intercritical period, and chronic tophaceous gout. Diagnosis is based on laboratory and radiological features. (Roddy E, Doherty M. et al, 2010)

Gout distinguished itself in the history of Homo sapiens since time immemorial. It appeared in medical records very early in the history of medical writing, and was also mentioned in the biographies of many famous names. It was depicted as the fate of a life of affluence as much as the challenge to a physician's skill, and truly it was. Modern ages witnessed remarkable progress in managing gout. More recently, thanks to quantum leaps in molecular biology, diagnostic modalities, and pharmacotherapy, we enjoy deeper understanding of the disease and a more sophisticated armamentarium.(Corrao S, Argano C, Calvo L, Pistone G. et al, 2015) (Agudelo CA, Turner RA, Panetti M, Pisko E. et al, 1984).

Gout is a systemic disease that results from the deposition of monosodium urate crystals (MSU) in tissues. Increased serum uric acid (SUA) above a specific threshold is a requirement for the formation of uric acid crystals. Despite the fact that hyperuricemia is the main pathogenic defect in gout, many people with hyperuricemia do not develop gout or even form UA crystals. In fact, only 5% of people with hyperuriceamia above 9 mg/dL develop gout. Accordingly, it is thought that other factors such as genetic predisposition share in the incidence of gout (Dalbeth N., Merriman T.R. et al, 2016).(Emmerson B.T. 1996).

MSU crystals can be deposited in all tissues mainly in and around the joints forming tophi. Gout is mainly diagnosed by identification of the pathognomonic MSU crystals by joint fluid aspiration or in tophi aspirate. Early presentation of gout is an acute joint inflammation that is quickly relieved by NSAIDs or colchicine. Renal stones and tophi are late presentations. Lowering SUA levels below deposition threshold either by dietary modification and using serum uric acid lowering drugs is the main goal in management of gout. This results in dissolution of MSU crystals preventing further attacks (Pascual E., Sivera F. et al, 2007) (Singh J.A. 2014).

MATERIALS AND METHODS

Uric acid

The quantitative invitro enzymatic determination of uric acid in serum or plasma at 520 nm

Uric acid + O_2+2H2O Uricase Allation + Co2+H2O2

Hydrogen per oxide reacts with 3,5dichloro 2 hydroxybenzenesulfonate and 4 – amino antipyrine in a reaction catalysied by horseradish peroxidase to give aquinomiemine. The intensity of the colour of the solution of this dye is proportional to the concentration of uric acid in the sample. The assay is carried out at 520nm.The freshly reconstituted reagent as an absorbance at 520nm over 0.300 discard the reagent. A light pink colouration in a stored reagent is however normal. Another indication of reagent deterioration is when the reagent fails to recover stated values in control sera. If the reagent develops turbidity this indicates contamination.

Procedure'

Wavelength :520 nm

Test : 1ml reagent +25 µl sample

Standard 1ml reagent+ 25 µl sample

Blank 1ml reagent

Incubate 10 Minutes at 30ºC and 37ºC

Calculation:

Cst = Value of the standard in mg/IDL uric acid

A sample X Cst – uric acid in sample in mg/dl

A standard

Normalvalue :

Men : 3.6 – 7.7 mg/dl

Women : 2.5 – 6.8 mg/dl

C – reactive protein

This reagent is intended for invitro quantitative determination of C – reactive protein in serum.The reagent (C – reactive protein – Turbilates agglutination assay is a quantitative furbidimetric assay for measurement of C – reactive protein in human serum. Particles coated with specific human anti C – reactive protein are agglutinated when mixed with sample containing C- reactive protein. The agglutination causes an absorbance change depending upon C – reactive protein contents of the patients sample, that can be quantified by comparison from a calibrated known as C – reactive protein concentration.

Mix and read the absorbance immediately (A1) and after 2 minutes (A2) of the sample additions

Calculation:

C – reactive protein concentration in mg/l

(A2-A1) sample x Calibrator concentration

(A1-A2) sample

Rheumatoid factor

Diagnostic reagent for qualitative measurement of RF (Rheumatoid factor)The RF – latex is an slide agglutination test for the qualitative and semi quantitative detection of RF in human serum. Latex particles coated with human gamma globulin are agglutinated when mixed with sample containing RF.

Qualitative method:

Allow reagents and sample to reach room temperature. The sensitivity of the test may be reduced at low temperature.Place 50 µl of the sample and one drop of each positive and negative control into separate circles on the slide test.Shake the RF latex reagent gently before using and add a drop of this reagent next to the sample to be tested. Mix both drop with a stirrer, spreading them over the entire surface of the circle, use different stirrers for each sample. Rotate the slide with the mechanical rotator at 82 – 100 rpm for 2 minutes false positive results could appear if the test is readable later 2 minutes.

Semi – quantitative methods:

Make series two field dilution of the sample in 9 g/l saline solution.Process for each dilution as in the qualitative method.

Calculation:

The approximate RF concentration in the patient sample is the calculated as follows 8 x RF Titer = 1 U/ml

Reference value :Upto 8 IU / ml

Anti – streptolysin O (Aso – Titre)

Diagnostic reagent of qualitative measurement of Anti – streptolysinO.The Aso latex is an slide agglutination test for the qualitative and semi – qualitative detection of anti – streptolysinO antibodies.

Qualitative method:

Allow reagents and sample to reach temperature. The sensitivity of the test may be reduced at low temperature.Place 50 µl of the sample and one drop of each positive and negative control into separate circles on the slide test.Shake the Aso – latex reagent gently before using and add a drop of this reagent next to the sample to be tested.Mix both drops with a stirrer, spreading them over the entire surface of the circle, use different stirrers for each sample. Rotate the slide with a mechanical rotator at 80 – 100 rpm for 2 minutes false positive results could appear if the test is readable later that 2 minutes.

Semi – quantitative method:

Makes series two field dilutions of the sample in 9 – g/l saline solution.Proceed for each dilution as in the qualitative method.

Calculation:

The approximate Aso concentration in the patient sample is calculated as foolows 200 x Aso – Titer = IU/ml

Reference value:

Up to 200 IU/ml (Adults and 100 IU/ml children)

RESULTS AND DISCUSSIONS

The data's of the study are discussed under following headings,

1. Demographic profile
2. Biochemical analysis

Demographic profile :

a) Age distribution

S No.	Age and distribution	Control	Gout Arthritis Subject	Rheumatoid Arthritis Subject
1.	40 – 50 years	14 (70%)	14 (70%)	14 (70%)
2.	50 – 60 years	4 (20%)	4 (20%)	4 (20%)
3.	60 – 70 years	2 (10%)	2 (10%)	2 (10%)

Number in parentheses indicate number of subjects. Data of the age distribution of the subject are given in the table.Regarding age, among the subjects under Control, Gout arthritis and Rheumatoid arthritis, about 70% were in the age of 40 – 50 years, 20% in the age of 50 – 60 years and 10% in the age of 60 -70 years respectively.

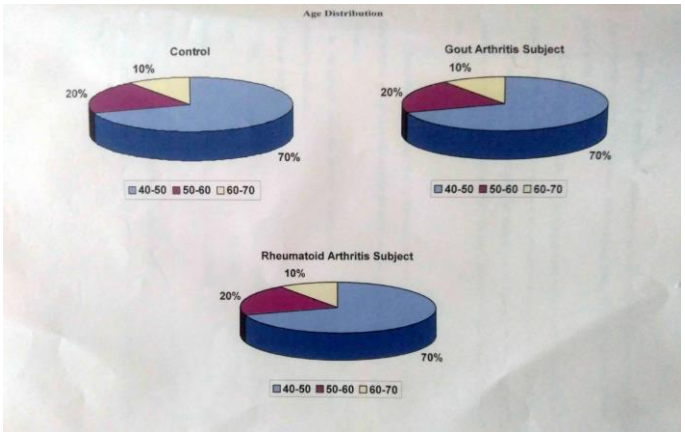

Fig. 1.

According to De Filippis L. et al, 2004.Gout arthritis and Rheumatoid arthritis are mostly affects patients in the age group of 40 – 60 years. (Jebakumar AJ, Udayakumar PD, Crowson CS, Matteson EL. et al, 2013)

In our present study the patients ranging from 40 – 60 years of age are mostly affected by both arthritis.

a) Sex of the subjects

Sex	Control	Gout Arthritis Subject	Rheumatoid Arthritis Subject
Male	10 (50%)	10 (50%)	10 (50%)
Female	10 (50%)	10 (50%)	10 (50%)

Table 1.

Number in parentheses indicate number of subjects. The sex of the studies shows that the control subjects are same 50% either.In comparison of all subjects control, Gout arthritis and Rheumatoid arthritis had equal percentage of subjects.According to Arbaelovic MC Alindon. Tenure Rhematic Rep, 2005 Mar : 7(1) 29 – 35 both sex are equally affected in our present studies.

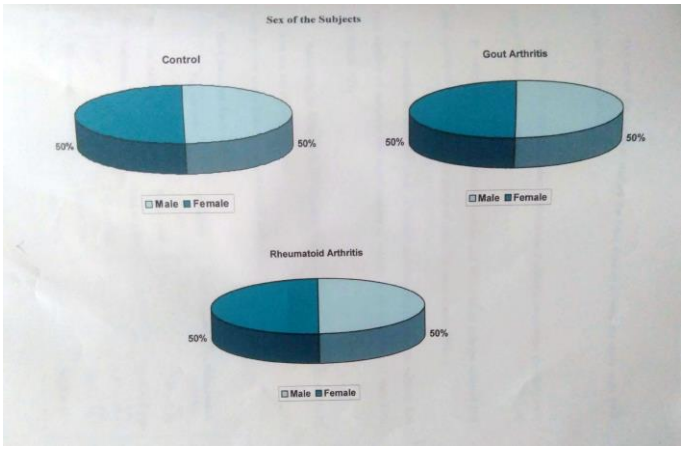

Fig. 2.

II Bio chemical Analysis:

S No.	Parameters	Control	Gout Arthritis	Rheumatoid Arthritis
1.	Uric Acid	9.4 ± 3.08	11.1 ± 2.44**	11.84 ± 2.20**
2.	C-reactive protein	8.02 ± 1.642	15.94 ± 0.884**	30.8 ± 14.12**
3.	Rheumatoid Factor	20.6 ± 7.94	15.0 ± 5.38**	126.6 ± 48.2**
4.	Anti-streptolysinO	102.8 ± 55.4	76.8 ± 49.8**	102.8 ± 55.4**

Table 2.

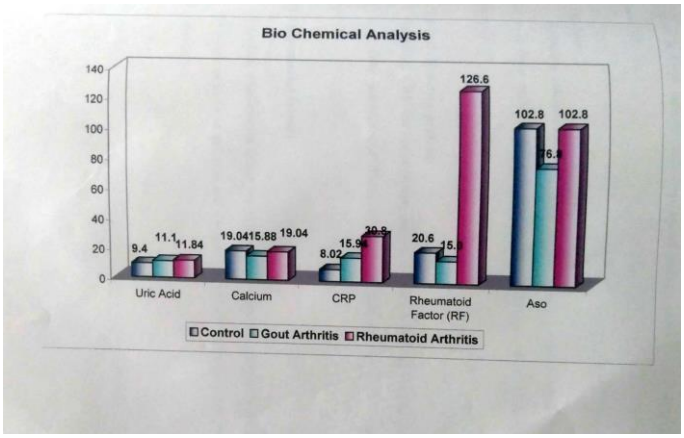

Fig. 3.

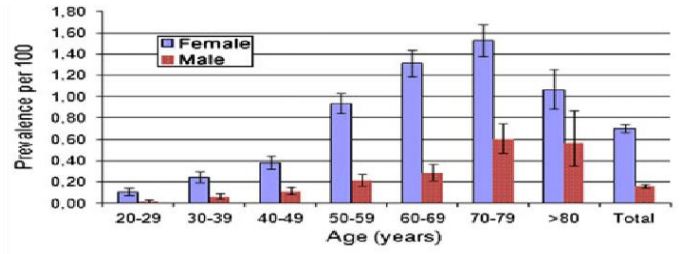

Fig. 4.

In comparison between controls and Gout arthritis the following changes were noted among the biochemical parameters. The level of uric acid was found significant increase in the result and the C – reactive protein were significantly very high increase in the result but the level of Anti-streptolysinO and Rheumatoid factor was found to be slightly increased.

In comparison between controls and Rheumatoid arthritis the following changes were noted among the biochemical parameters. The level of uric acid was found slightly increased in the result and the Anti-streptolysinO tire value way normal when compare to the control. But there was a very high significant change in the C-reactive protein and Rheumatoid factor they found to be very highly significantly increased.

According to Altman R, Appelrouth D, et al (1990), the comparison of body lead stores between Gout arthritis and Rheumatoid arthritis. The levels of uric acid were found to be increased both in Gout and Rheumatoid Arthritis. The level of calcium was highly decreases in Gout arthritis, but there was no significant change in the case of Rheumatoid Arthritis.

Regarding C – reactive protein and rheumatoid factor the C – reactive protein was found to be highly increased both in Gout arthritis and Rheumatoid arthritis. The Rheumatoid arthritis was found to be slightly decreased in case of Gout arthritis and highly increased incase of Rheumatoid arthritis. The Aso level was found to be normal incase of Rheumatoid factor bt these was an elevated change in case of Gout arthritis.

According to Struck R (2000) Gout easy to wrongly diagnoses. In Gout arthritis the uric acid levels will be increased where the calcium levels are found to be decreased. In Rheumatoid arthritis the Rheumatoid factor levels increased where the uric acid and calcium levels are found to be normal.

CONCLUSION

The present study summarizes the level of uric acid in patients with RA (either seropositive or seronegative arthritis). The data concluded that serum uric acid did not show association with RA as revealed by the lack of association with specific markers. The inflammatory marker, hsCRP was significantly higher among patients as compared to controls. The higher hsCRP showed a high grade of systemic inflammation in RA patients. C-reactive protein was elevated in rheumatoid factor positive patients and patients with high BMI value. Additionally, the result of our study showed that different socio-economic factors had an association with disease activity of RA.

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Research Article

Antimicrobial activity of Solanum Nigrum Silver nanoparticle

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ABSTRACT

Solanum nigrum is a species in the *Solanum* genus, native to Eurasia and introduced in the Americas, Australasia and South Africa. Parts of this plant can be highly toxic to livestock and humans, and it's considered a weed. Solanum nigrum, are mentioned and often illustrated in all of the early Herbals, with Dioscorides being one of the first to record their medicinal effects in every county in which the taxon is found. Anti microbial of silver nano particle along with water extract of solanum nigrum plant dried powder to derived silver nanoparticle pellet were used for the evaluation of antimicrobial activity. Extract of solanum nigrum were streaked separately on mullerhinton agar and nutrient agar to check the purity. after24hours of incubation the plants were used for bioassay. The antimicrobial activity of the silver nanoparticle and solanum nigrum were investigated with different bacterial and fungi strains. Synthesis of nanoparticles solution with leaf extract may be easily observed by ultraviolet spectrophotometer. FTIR measurement were carried out to identify the possible biomolecules responsible for capping and efficient stabilization of the metal nanoparticle synthesized by leaf extract. Hence, solanum nigrum can be employed as a source of natural antimiCcrobials that can serve as an alternative to conventional medicines.

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INTRODUCTION

Solanum nigrum is a species in the *Solanum* genus, native to Eurasia and introduced in the Americas, Australasia and South Africa.

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Parts of this plant can be highly toxic to livestock and humans, and it's considered a weed. Nonetheless, ripe berries and cooked leaves of edible strains are used as food in some locales; and plant parts are used as a traditional medicine.

Various part of many of the species belonging to the section solanum are widely used medicinally throughout the world. Their use as such is recorded from the earliest times and various species, especially Solanum nigrum, are mentioned and often illustrated in all of the early Herbals, with Dioscorides being one of the first to record their medicinal

effects in every county in which the taxon is found. Among the great British herbals, Gerard's Herbal of 1636 reported that the "Nightshade is used for those infirmities that need cooling and binding" and that is was good against 'St.Anthonies fire, the shingles ,panic of the head ,heart burning or heat of the stomache ". Later in Culpeper's Herbal of 1649, the black nightshade was described as a "cold Saturnine plant", which was commonly used to cool hot inflammations either externally or taken internally. Among the soothing effects of the clarified juice of this plant he mentioned inflamed throats, eye inflammations, shingles, ringworm, running ulcers, testicular swelling, gout and ear pain. In Europe, 'S.nigrum' has been used as a remedy for convulsions ,and has been administered as a soporific in Germany-especially for children, with leaves being placed in babies cradles to promote sleep in "Bohemia"(? Czech Republic) (Grieve 1931). The bruised fresh leaves used externally are reputed to ease pain and reduce inflammation; they are applied to burns and ulcers by the Arabs, Leaf juice has also been used for ringworm, gout and earache, while it is also reputed to be a good gargle and mouthwash when mixed with vinegar (Grieve 1931). In North America, the Houmas Indians use an infusion made from boiled roots of this 'species to administer to babies with worms, and crushed green leaves mixed with a grease to make poultices for sores, while the Rappahannock used a weak infusion to cure insomnia (vogel 1990). There are relatively few reports of these species being used medicinally in South America, an exception being the moderate narcotic action attributed to flowers and leaves resulting in their use to calm fever and combat the effects of alcoholic excesses in southern Ecuador (Heiser 1963).

In India, the 'plant is noted for its antiseptic and ant dysenteric properties and is given internally for cardialgia and gripe. An infusion of the plant is used as an enema for infants with abdominal upsets. The plants also a household treatment for anthrax pustules when I it is applied locally it is further reported to have emollient, diuretic and laxative properties and its decoction is regarded as both antispasmodic and narcotic. Freshly prepared extracts of the plants are apparently effective in the treatment of cirrhosis of the liver and also serve as an antidote to opium poisoning. An alcoholic extract of leaves is active against staphylococcus aureus and Escherichia coli. Infusion or decoctions of the plant, after transient stimulation, are reported to depress the central nervous system and the reflexes of the spinal cord. small doses increase cardiac activity while large doses decrease it. Extracts also reduce blood pressure. Berries apparently possess tonic, diuretic and cathartic properties and are also useful in heart disease and as a domestic treatment for fevers, diarrhoea, ulcers and eye troubles (Anon.1965a, 1965b). The seeds are reportedly used to treat gonorrhoea and dysuria (Jain and Brothakur 1986). In Pakistan Akhtar and Muhammad (1989) showed that a power from the aerial parts of the plant could be "antiulcerogenic".

In China leaves are used as a febrifugal or detoxicant drug . Medicinally used preparations consist of dries aerial parts of plants which are used as a diuretic ,antihypertensive and anticancer agent for infections of the urinary system,hypertension and cancer of the digestive system chilling et al,1992). Fresh leaves are also used to treat wounds.

In Japan Saijo et al. (1982) observed that immature fruits of 'S.nigrum contain steroidal glycosides which show considerable anticancer activity. These glycosides could be solasonine ,solamargine ,diosgenin and solasodine. In Hawaii plants conspecific with S. Americanum are used in disorders of the respiratory tract, skin eruptions, cuts, wounds and trachoma, while in the Mauritius, a poultice of the plant is used to relive abdominal pain and inflammation of the urinary bladder (Watt and Breyer-Brandwijk 1962). In the Orient Tandon and Rao (1974) reported that the fruits and juices of 'S.nigrum' are used to cure stomach ailments ,fevers and blood impurities and young shoots to cure skin disease. In the Philippines, leaf extracts are apparently used to restore body skin pigments. In East Africa the raw fruit is chewed and swallowed for treatment of stomach ulcers or for general abdominal upsets which leads to continued stomach-ache. Infusion of leaves and seeds are rubbed onto the gums of children who have developed crooked teeth. pounded leaves are soaked in water, fermented and used for the treatment of boils ulcers and swollen glands. Unripe berries are used to treat ring worms. Various parts of the plants are also believed to cure malaria, black fever, dysentery and urinary infection (Kokwaro 1972).

Several studies have been conducted to investigate the nutritive value of the 'vegetable black nightshades. The leaves can provide appreciable amounts of protein and amino acids, minerals including calcium, iron and phosphorous, vitamin A and C, fat and fibre, as well as appreciable amount of methionine, an amount of methionine, an amino acid scare in other vegetables (Fortuin and omta 1980; FAO 198.) Moreover, the berries can apparently yield high mounts of vitamins C and carotene. The seeds too contain vitamin C and carotene. The nutrient values may however, vary with soil fertility, plantage and type (i.e, variant or species) (Chweya 1997) .Imbamba (1973) ,for example, found that the leaf protein contents of 'S.nigrum was dependent on the age of the plant .Moreover the application of nitrogen increase the amount of ascorbic acid and protein while decreasing the calcium content in the leaves (Chweya 1997). The values of available ascorbic acid depend on the method of cooking. Mathooko and Imungi (1994) observed that ascorbic acid

content decreased with both an increase in the cooking time and in the volume of water used for cooking. This loss could reach as much as 75-89% when boiling the vegetable for as long as 20 minutes. However, leaves boiled with six volumes of water for 15 minutes resulted in the loss of approximately 70% of ascorbic acid. Similar reductions in the levels of vitamins A and C through excessive boiling have been reported for various other Nitrates are harmful to humans when consumed and converted into nitrites which oxidize ferrous ions of the blood hemoglobin, resulting in reduced oxygen-carrying capacity of the blood (Lee 1970; Maynard et al. 1976; Mengel 1979). Oxalates indicate the presence of oxalic acid in plant material. When ingested by humans, the acid combines with calcium to form an insoluble salt which the body cannot absorb (Buck # al. 1966. Robinson 1973). This renders the calcium unavailable to the body. Phenolics bind proteins, hence interfering with the assimilation of proteins into the body (Haslam 1974; Singleton 1981).

The present study is aimed to focus on antimicrobial activity with the effect of metal ion of plant sample Solanum nigrum. The plant extracts were prepared in five solvents (methanol, acetone, ethyl acetate, hot water and normal water) to check the antibacterial and anti-fungal activity against bacterial pathogens (P. aeruginosa, S. aureus and & col) and fungal pathogens (M. canis, C. albicans, T. rubrum and A. niger) by agar well diffusion method the best activity against S. aureus and E. coli with maximum zone of inhibition ranging from 12 mm to 16.5mm. The activity of all parts of S. nigrum was enhanced in the presence of metal ion. The lowest concentration (highest dilution) of the extract was regarded as MIC. Among the different types of tested S. nigrum parts extract the fruit extract with different solvents showed the least MIC value at concentration ranging from 2.7µg/ml to57µg/ml. A lowest MIC value of fruit (0.14 OD at conc. of 57µg/ml) was obtained against S. aureus. The acetonic extract of leaves also showed the lowest MIC value (0.12 OD at conc. of 96µg/ml) against S.aureus. ThephytochemicalanalysisofS.nigrumpartsrevealedthepresence ofsecondarymetabolites were penoids, sterols, flavonoid sannins, phenols in leaves and fruits and terpenoids, sterols, saponin and flavonoids in stem.

MATERIALS AND METHODS

The present investigation was made to evaluate the antimicrobial of silver nano particle along with water extract of solanum nigrum. The study was carried out in the royal bio research center velacher TY, Chennai, tamilnadu, Duringthe period of one month (January) 2014.test.Glass wares and culture media were sterilized in an autoclave at 121ºC for15mintues at 15lbs/inch²

Collection of samples

The various clinical specimen for culture was obtained from royal bio research invelacherry, Chennai.The organisms were isolated from the clinical sample by performing the following test.

Collection of plant

The plant solanum nigrum was collected from Collected plants were carefully examined and dent fixed with the help of regional flora.

Preparation of power

The leaves of plants were collected and dried under shape these dried materials were prepared by using motor and pestle and Sture in a air right container the powdered materials were used for furthered process

Preparation of extract cold extraction technique

23g of dried plant powder of solanum nigrum mixed with 150ml of water for the extract.It was in maintained at room temperature for 24hours. The extract was filtered with watt man no:1 filter paper and clear filtrate was concentrated in room temperature.The concentrated extract was termed as total water extract. This total water extract was used for finding the antimicrobial activity

Synthesis of silver nano particles

23 gm of solanum nigrum leaf powder mixed in I 50ml of distilled water.It was incubated for 24hours.The mixer was filtered with watt man no: l filter paperThe silver nitrate solution was prepared by using 1 mill molar (1mm)(0.0l69g) of silver nitrate in l00ml of distilled H2O.The 10ml of filtered plant extract solution was mixed with 90ml of silver nitrate solutionIt was incubated at room temperature for 72hours.The colour change from yellow to brown colour was observed, after the 72hours of incubation.The Solution was centrifuged at 10,000rpm for 10mintues, the supernatant wasDischarged the pellet was collected.The derived silver nanoparticle pellet were used for the evaluation of antimicrobial activity.

Disc preparation

Wattman no:l filter paper disc (5mm0 were prepared the disc's were sterilized by Autoclave at 121°c after sterilization the moisture disc were rinsed 2Omnl of the silver nanoparticles of solanum nigrum and allowed to dry at room temperature.

Sterility check

The silver nanoparticle of solanun nigrum and the water extract of solanum nigrum were streaked separately on mullerhinton agar and nutrient agar to check the purity. After 24hours of incubation the plants were used for bioassay.Nutrient agar and mullerhinton agar was prepared and poured in Petri plates. The silver nanoparticle of solanum nigrum and water extract of solanum nigrum were Shaked into the agar plates and incubate at 37°c for 24 hours. After incubation the plastics were observed for any contaminationThe contaminated silver nanoparticle of solanum nigrum and water extract of solanum nigrum were discarded.

Microorganisms used

The antimicrobial activity of the silver nanoparticle and solanum nigrum were investigated with different bacterial and fungi strains.

Bacterial strain

Eschenchiacoli(E. coli), Klebsiella pneumonia, Staphylococcus aureus, Salmonalla typhi, Pseudomonas

fungal strain.

Tricoderma, Candida albicans, Aspergillus flavus, Aspergillus niger, Aspergillus fumigates

Characterisation of The Synthesized Silver Nanoparticles.

UV-VIS spectrophotometer analysis, Synthesis of nanoparticles solution with leaf extract may be easily observed by ultraviolet spectrophotometer. The UV-vis spectrophotometer analysis were carried out as a function of a time of the reaction at room temperature at an interval of one hour' s once in 5 hour's (1.2,3,4,5,) in 400-680nm range.

SEM-Analysis for the silver nanoparticles (anna university in biotechnology dep)

Scanning Electron Microscope (SEM) analysis was done using Hitachis-4500, SEM machine thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of sample on the grid, extra solution was removed using a botting paper and 1 then the film on the SEM grid were allowed to dry by putting in under a mercury lamp tor5 minutes

FTIR- Analysis of silver Nanoparticles

To synthesis silver nanoparticles solution was centrifuged at 10,000 rpm for30 minutes. The pellet was washed thrice with 5ml of de ionized water to grid of the free protein or enzymes that are not capping the silver nanoparticles. The pellet was dried by using vacuumdrier. This was analyzed by FTIR.

Characteristics of the medium. The main characteristics of the medium (muller hinton and agar medium) is to

support the growth of the organisms normally tested and it should not show any antimicrobial activity.

Agar Disc Diffusion Method: Preparation of Inoculum:

Stock cultures were maintained at 4°c on slant of nutrient agar. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of nutrient broth for bacteria that were incubated at 24hrs at 37°C. The Assay was performed by agar disc diffusion method.

Antibacterial Activity:

Antibacterial activity of plant sample was determined by disc diffusion method on Muller Hinton agar (MHA) medium. The Muller Hinton Agar medium was weighed as 3.8gms and dissolved in 100ml of distilled water and add 1gm of agar. Then the medium is kept for sterilization, after sterilization the media was poured in to sterile Petri plates, these Petri plates were allowed to solidify for twenty minutes. After the medium was solidified, the inoculums were spread on the solid plates with sterile swabmoistered with the bacterial suspension. The disc were placed in MHA plate and add 20ul of sample [concentration: 100Oug, 500 ug, 250 ug. 125 ug, 62.5 ug]. The plates were incubated for 24 hrs., at 37°c. Then the microbial growth was determined by measuring the diameter of zone of inhibition.

Antifungal Activity Assay;

Preparation of Potato Dextrose Broth (PDB): Antifungal activity of plant sample was determined by antifungal susceptibilitytest. Prepare PDB Broth and inoculate the culture. Then it is kept in shaker for a day. Thepotato dextrose agar was weighed as 3.9gms and dissolved in 100ml of distilled

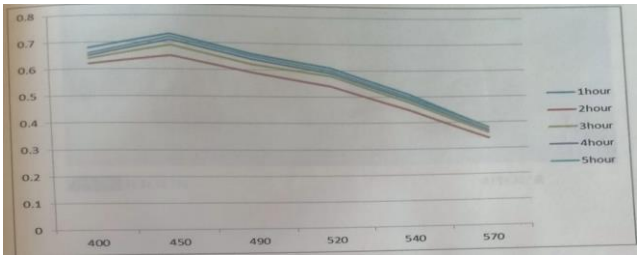
waterand add1gm of agar. Then the medium is kept for sterilization. After sterilization themedia was poured in to sterile Petri plates, these Petri plates were allowed to solidify fortwenty minutes. After solidification, the inoculums were spread on the solid plates withsterile swab moistered with the fungal suspension. The disc was placed in PDA plateand add 20 µlof sample [concentration: 1000ug, 500 ug, 250 ug, 125 ug, 62.5 ug]. Theplates were kept it at Room Temperature. Then the microbial growth was determined bymeasuring the diameter of zone of inhibition.

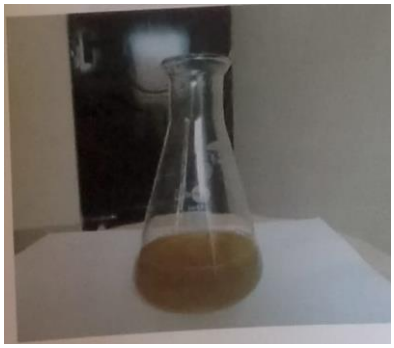
RESULTS AND DISCUSSIONS

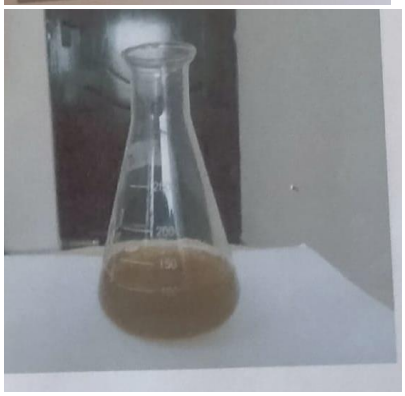
Nanometer Reading of UV Visible - Spectrometer Characterization For Synthesized Silver Nanoparticle

Nano meter (nm)	Reading taken on different time intervals				
	1 Hour	2 Hour	3 Hour	4 Hour	5 Hour
400nm	0.68	0.62	0.64	0.65	0.66
450nm	0.73	0.65	0.69	0.71	0.72
490nm	0.66	0.59	0.62	0.64	0.65
520nm	0.61	0.54	0.58	0.59	0.60
540nm	0.51	0.45	0.48	0.49	0.50
570nm	0.38	0.34	0.36	0.37	0.38
620nm	0.32	0.27	0.30	0.31	0.32
680nm	0.30	0.26	0.28	0.29	0.30

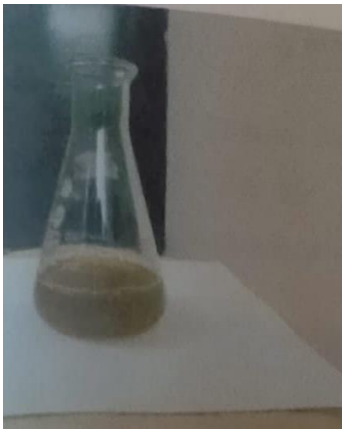
UV Visible -Spectrophotometer Chart







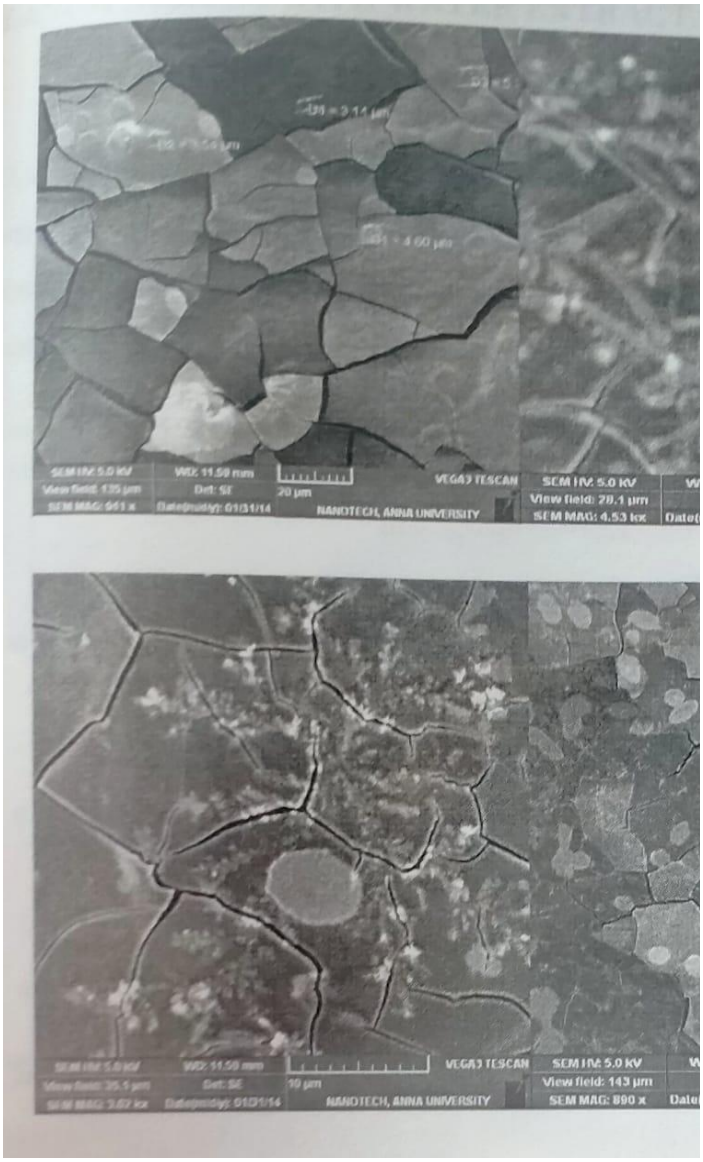
1 Hour 2 Hour



3 Hour 4 Hour

5 Hour

The silver nanoparticle synthesized by solanum nigrum extract leaf were scanned using SEM from which its concluded that the average mean size of silver nanoparticles was 72nm and seems to be spherical in morphology as shown in figure.

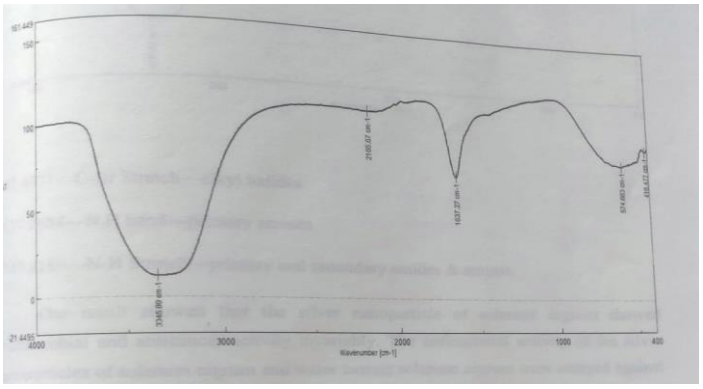


SEM ANALYSIS

Ftir Spectrum

Solanum Nigrum with Water Extract

FTIR spectrum of AG nanoparticle synthesized from solanum nigrum extract were shown in (Chart). FTIR measurement were carried out to identify the possible biomolecules responsible for capping and efficient stabilization of the metal nanoparticle synthesized by leaf extract.

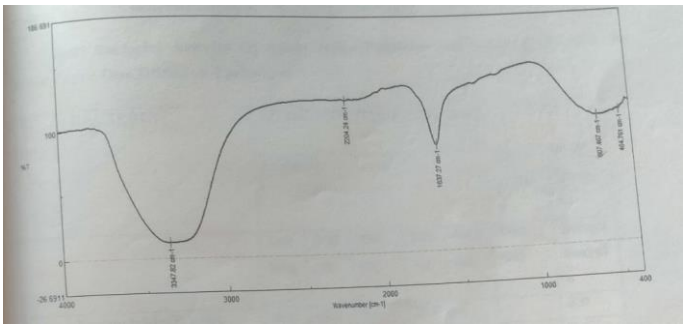


586.254---C-Br stretch ---alkyl halides

1637.2684--- N-H bend ---primary amines

3345.8901--- NH stretch ----primary and secondary amines and amides,

Solanum Nigrum and Silver Nitrate Extracts



607.4671---C-Br Stretch -alkyl halides

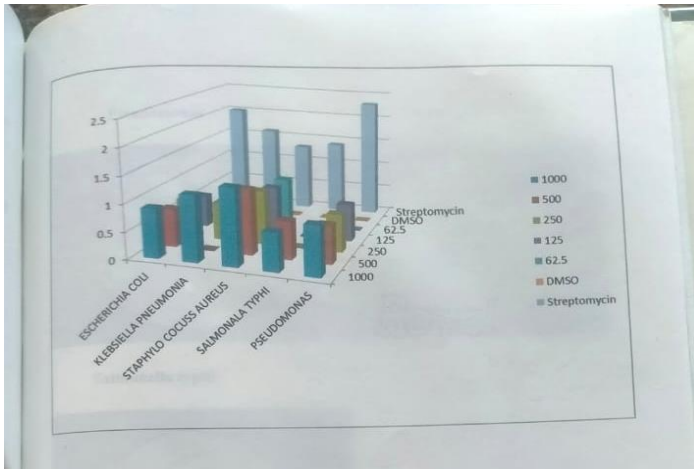
1637.2684---N-H bend ---primary amines

3347.8186---- N-H stretch ----primary and secondary amines &amides.

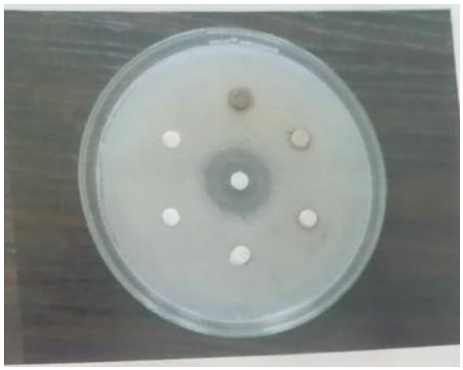
Our result showed that the silver nanoparticle of solanum nigrum showed antimicrobial and anticancer activity invariably. The antibacterial activity of the silver nanoparticles of solanum nigrum and water extract solanum nigrum were assayed against the test organisms by agar diffusion using filter paper disk method. The mean zone of inhibition obtained were between 6mm to 22mm and 5mm to 27mm. The antibiotic streptomycin was used as antibacterial control indicates that the antibacterial activity of the silver nanoparticles. In this the silver nanoparticles of solanum nigrum showed least antibacterial activity on the filter paper disk method. The Control streptomycin produced zone of inhibition between 22mm to 25mm (Figure and Chart).

Anti-Bacterial Activity of Silver Nano Particles and Solanum Nigrum By Filter Paper Disc Diffusion Technique

S No	BACTERIA	ZONE OF INHIBITION (mm)						
		Dilution					Negative	Positive
		1000 µg	500 µg	250 µg	125 µg	62.5 µg	DMSO	Streptomycin
1.	Escherichia (E.Coli)	0.9	0.7	0.7	0.6	-	-	2.0
2.	Klebsiella Pneumonia	1.2	T	0.7	0.7	0.6	-	1.6
3.	Staphylococcus aureus	1.4	1.2	1.0	0.9	0.9	-	1.3
4.	Salmonella typhi	0.7	0.7	T	T	T	-	1.4
5.	Pseudomonas	0.9	0.7	0.7	0.7	-	-	2.2

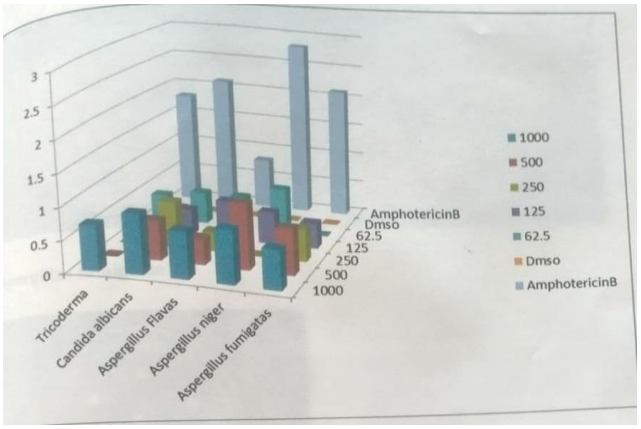


Escherichia (E.coli) Klebsiella pneumonia



Pseudomonas Staphylococcus aureus

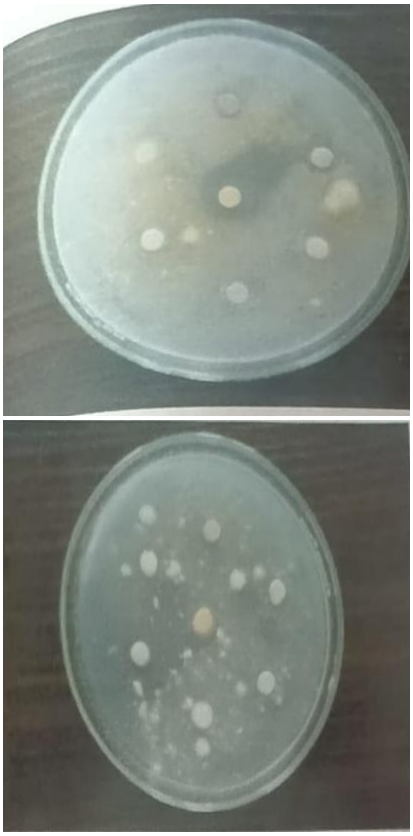
Salmonella typhi



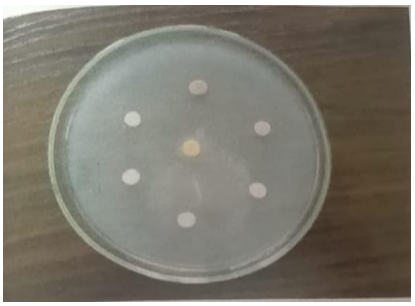
Anti-Fungal Activity of Silver Nano Particle of Solanum Nigrum by Filter Paper DISC diffusion Technique

Tricoderma Candida albicans

SNo FUNGAI		**ZONE OF INHIBITION (mm)**						
		Dilution					**Negative**	**Positive**
		1000µg	500 µg	250 µg	125 µg	62.5 µg	DMSO	AmphotericinB
1.	Tricoderma	0.7	-	T	T	0.4	-	1.8
2.	Candidaalbicans	0.9	0.6	0.7	0.4	0.5	-	2.1
3.	Aspergillus Flavas	0.7	0.4	0.3	0.6	0.5	-	0.8
4.	Aspergillus niger	0.8	1.0	T	0.5	0.7	-	2.8
5.	Aspergillus Fumigatas	0.6	0.7	0.5	0.4	T	-	2.1



Aspergillus Flavas Aspergillus niger



Aspergillus fumigates

DISCUSSION

Botanicals or phytomedicines have been a major part of traditional medical healing in developing countries, which have also been an integral part of their history and culture with deep concern and relevance to Indian medicinal plant and sense of realization about its medicinal value the present research work is undertaken.

In the recent years, research on medicinal plant has attracted a lot of attention globally large body of evidence has accumulated the promising potential of medicinal plant used in Various traditional, complementary and alternate systems of human diseases Plants are Tich in a wide variety of secondary metabolites such as tannins terpenoids alkaloids flavonoid, ect, which have been found in vitro to have anti-microbial properties

This present investigation was carried out to analyse the antimicrobial activity anticancer activity of silver nanoparticle from solanum nigrum and water extract ofSolanum nigrum against Bacteria, Fungi.

The synthesis of nanoparticles is in the lime light in modern nanotechnology Biosynthesis of nanoparticles by plant extract is currently under exploitation The development of inspired experimental processes for the synthesis of nanoparticles is evolving into an important branch of nanotechnology the present study deals with the synthesis of silver nanoparticles using leaves extract of solanum nigrum and aqueous Ag+ ions Comparative experiments were carried out to be cost effective alternative to conventional methods of assembling silver nanoparticle.

UV-VIS Spectra analysis

In this study as the solanum nigrum leaf extract was mixed with solution of the silver nitrate it started to change the colour from water to brown due to reduction of silver ion which indicates the formation of silver nanoparticle. similar to our study it is generally recognized that UV-Vis spectroscopy could be used to examine size and shape-controlled nanoparticle in aqueous suspension.

SEM

The representative SEM images recorded from drop-coated films of the silver nanoparticle synthesized by treating silver nitrate solution with solanum nigrum leaf extract-The silver nanoparticle formed were predominantly cubical with uniform shape. It is known that the shape of metal nanoparticle considerably changes their optical and electronic properties (XuH,Kall,2002) .The SEM image showed relatively spherical shape nanoparticle formed with diameter range 72 nm. similar phenomenon was reported by chandran et.al.,2006

FTIR

FTIR spectrum of silver nanoparticle synthesized from solanum nigrum extracts FTIR measurements were carried to identified the possible biomolecules responsible for capping and efficient stabilization of the metal nanoparticles synthesis by leaf. broth The peaks near 3440cm-1,2924cm-1 and 2854-1 assigned to OH stretching and aldehydic C-H stretching respectively. The weaker band at l629cm-I crosses ponds to C=C stretching (non conjugated) The peak near 833cm-1 assigned to -C= CH2. The peak near677cm-1 of plane bending vibrations are substituted and 651.96 cm-1 assigned to CH out ethylene system -CH-CH(cis).

Antimicrobial Study

In this study the antibacterial activity of the silver nanoparticle of solanum nigrum Were screened against Gram Positive and Gram-Negative bacteria Escherichia coli which

have the maximum level of zone of inhibition is similarly to klebsiella Pneumonia, staphylo coccus aureus, salmonella typhi, and pseudomonas which have the medium level of zone of inhibition of zone of inhibition The report suggest that the effect was more pronounced against gram-negative organisms than gram-positive ones.

Regarding anti-fungal activity of silver nanoparticle of solanum nigrum the maximum level of inhibition towards the fungus candida albicans and Fusarium The medium level of inhibition toward Aspergillus niger and Trichoderma. The minimum level of inhibition toward Trichophyton rubrum and Alternaria. Similar to our study sereema spun.et.al.,2008). The maximum toxicity was observed in silver nanoparticles treated cells than theAgNo3 and the plant extract the reason could be the smaller size of the particles which lead to increase membrane permeability and cell destruction. Silver nanoparticle have an ability to interfere with metabolic pathway by modulating tyrosine phosphorylation of putative peptide substrate critical synthesis from leaves of solanum nigrum are toxic to multi drug resistant microorganism. It shows that they have great potential in biomedical application.

SUMMARY

Solanum nigrum was collected from Gudiyattam.,vellore. Dis. Tamilnadu. The leaves of solanum nigrum were used to produce the silver nanoparticle and water extract. l0 ml of filter distilled water was mixed with the 100ml silver nitrate solution. The reduction of Ag+ ions are identified by the colour change yellow to brown colour and it indicates that the synthesis were taken place. The silver nanoparticle were characterized by using UV-Vis spectroscopy, FTIR analysis and SEM analysis.

The preliminary antimicrobial activity of silver nanoparticle from solanum nigrum and water extract from solanum nigrum was screened using filter papered method. The Zone of inhibition for bacterial strains is about 6mm to 13mm.The zone of inhibition for fungal strain is about 8mm to28mm for water extract. The zone of inhibition for bacteria is highly susceptible to silver nanoparticle from solanum nigrum and water extract and there is also high zone of inhibition toward salmonella typhi when compare to the other Bacterial strains. The zone of inhibition for fungi nearly equal to the standard antibiotic Amphotericin B. The zone of inhibition tor bacteria were less to the standard antibiotic Streptomycin.

CONCLSION

The present investigation concludes that the solanum nigrum can acts as anti-microbial agent The solanum nigrum were found to be effective almost all tested bacterial strains and We have developed a biological method to synthesize silver nanoparticle using the aqueous leaves extract of solanum nigrum and also it does not cause any side effect when compare to the chemical. Drug. Hence, solanum nigrum can be employed as a source of natural antimicrobials that can serve as an alternative to conventional medicines. From the present study it is concluded that the solanum nigrum could be uscd for the treatment of bacterial, fungal infections

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Research Article

Phytochemical Analysis and Nutritional Significance of *Catharanthus Roseus*

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Phytochemical

ABSTRACT

Herbal medicine is the oldest form of healthcare known to mankind. Herbal Medicine sometimes referred to as Herbalism of Botanical Medicine, is the use of herbs for their therapeutic or medicinal value. Phytochemical screening (qualitative) detection of alkalolds, mayer's test, Test for phytosterol, Salkowskis. Detection of Carbohydrates and glycosides, Benedicts Test and Borntragers test, detection of Saponins Foam test Detection of proteins and amino acids Biuret test Detection of phenol compound Ferric chloride test. The plant extractive studied could be an answer to the people seeking for better therapeutic agents from natural sources which is believed to be more efficient with little or no side effects when compared to the commonly used synthetic chemotherapeutic agents.

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INTRODUCTION

Plants are not just used as food. Plant products have a variety of other applications as well. All human nutrition depends on plants directly or indirectly. Most parts of the plants are edible in nature. Fruits and vegetables, flower, stems and leaves are also used for providing energy.

Plants provide a variety of taste enhancers as well. Spices and herbs are chief among them which are traditionally used in the culinary of different cultures. Plants also provide products such as sugar, tea, coffee, beverages etc., this being the chief use of plants, there are other areas as well where the plants are equally used. Wood from plants is used for building furniture, as a source of paper, cardboard and for the manufacture of various other equipment. Plane also provide fossil fuel in the form of coal, petroleum and natural gas. Thousands of plants are cultivated to beautify the environment, provide shade, reduce pollution. abate noise and wind speed and to prevent soil erosion.

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Herbal medicine is the oldest form of healthcare known to mankind. Herbal Medicine sometimes referred to as Herbalism of Botanical Medicine, is the use of herbs for their therapeutic or medicinal value. An herb is a plant of plant part valued for its medicinal, aromatic or savory qualities. Herb plants produce and contain a variety of chemical substances that act upon the body. Herbs also contain minerals, vitamins, volatile oils, glycosides, bioflavonoid, and Other substances that are important in supporting a particular herb's medicinal properties. These elements act as supporters for are efficiency of the active compound. Herbalists use the leaves, flowers, stems, berries, and roots of plants to prevent, relieve, and treat illness. Marshmallow root, hyacinth, and yarrow have been found carefully tucked around the bones of a Stone Age man in Iraq. These three medicinal herbs are continued to be used today for their anti-inflammatory and diuretic properties.

Shen Nong recommended the use of Ma Huang (also known Ephedra in the Western world), against respiratory distress. Ephedrine, extracted from Ephedra, is widely used as a decongestant. The records of king Hammurabi of Babylon include instructions for using medicinal plants. Hammurabi prescribed the use of mint for digestive disorders. Modem research has confirmed hat peppermint does indeed relieve nausea and vomiting by mildly anesthetizing the lining of the stomach Pharmacognosy is the study of medicinal and toxic products from natural plant sources. The plant-derived drugs is mainly due to the current widespread belief that "Green Medicine" is safe and more dependable than the costly synthetic drugs, many of which have adverse side effects.

Industries are now interested in exploring parts of the world where plant medicine remains the predominant form of dealing with illness. The World health Organization (WHO) estimates that 4 billion people, 80% Of the world population, presently use herbal medicine for some aspect of primary health care. WHO notes that of 119 plant-derived pharmaceutical medicines, about 74% are used in modem medicine (WHO, 2002) in ways that correlated directly with their traditional uses as plant medicines by native cultures? Major pharmaceutical companies are currently conducting extensive research on plant materials gathered from the rain forests and other places for their potential medicine value. Scientists have also realized that the study of the native regions can provide enormously valuable clues in the search for improved health.

There are over 350,000 plants on the earth and only a few of the medicinal plants have been studied scientifically. The efficacy of many medicinal plants has been validated by scientists abroad, from Europe to the Orient. Thanks to modem technology, science can now identify some of the specific properties and interactions of botanical constituents. With this scientific documentation, we now know why certain herbs are effective against certain conditions. Several such researches helped viewing various medicinal plants with potential use as drugs. Initial steps include the study of plants for their antimicrobial properties against various pathogenic strains and later their potential use as drugs is evaluated. Considering the importance of plants as a source of medicine even today we have selected catharanthusroseus, family- Apocyanaceae, which is in use for centuries in the treatment of many ailments. However it is less explored plant for its varying activities, hence an effort has been made here to investigate the potential uses of this plant.

MATERIALS AND METHODS

Collection of Source

The specimens of Vincarosea (C. roseus) Linn were collected from Gudiyattam. The Herbarium of the specimen is deposited in the museum. The Vincarosea (C. roseus) Linn leaf and flower were collected and crushed into powder and the extract was collected by using different solvents.

Phytochemical Screening

The different chemical tests were performed for establishing profile of the extract for its chemical composition, the following chemical tests for various phyto constituents in the acetone, Ethanol and aqueous extracts were carried out as described below (Harborne, 1974), the following tests were performed (anonymous
1996).

Preparation of Extract's

The dried leaf of Vincarosea Linn were subjected to extraction using the solvents (acetone, Ethanol and aqueous) in increasing order of polarity. The prepared extracts were then subjected to preliminary phytochemical analysis. It was found plant possesses carbohydrates, protein, alkaloids, flavonoids and Tannin. The leaves of plants were dried in shade, separated and made to dry powder. A weighed quantity (5gm) of the powder was subjected to continuous hot extraction in separate apparatus with solvents such as acetone, Ethanol and aqueous respectively. The extract was

evaporated under reduced heat using water bath until all the solvent has been removed to give an extract sample..

Microsopic and Macroscopic Structures of Vincarosea Linn.

Macroscopic: Leaves are about 4 to 6 mm, sub globose, black, shiny with a whitish scar of aril, nutty flavor, noodour. Microscopic: T.S.shows an outermost thick yellowish layer of cuticle; testa shows a single layer of radially elongated, brown and thick walled palisade like cells showing linealucida and with stellately lobed lumen as seen in surface view; a wide zone of sclereids with thick walled highly sinuous, light yellow to yellowish-brown lignified cells showing radiating canals on their walls in surface view; tegmen consists of parenchymatous cells; ground tissue of the embryo consists of angular to hexagonal parenchyma cells with oil globules; starch grains absent.

Phytochemical Analysis

Carbohydrates, proteins, fats and oils are utilized as food by man and animals. Other chemical compounds in plants apart from these listed above are phytochemical. Such compounds usually exert peculiar, unique and specific active physiological effects responsible for their therapeutic and pharmacological functions. Activities of such naturally occurring compounds are generally responsible for changes, which are utilized to satisfy man's desires. Phytochemical studies afford revelation and understanding of phytoconstituents, as much as possible conserving their bioactivities, and are on how to standardize them; compared with the crude herbal methods that are most easily standardized. These complex substances of diverse nature occur mostly in plant based foods; they are in very small amounts in grams or mg or pg./Kg of samples. They do not add to body calorie and are numerous in types. This Phytochemical are applied mostly for preventive and healing purposes. About 25% of prescribed drugs are obtained from Phytochemical in higher plants. Plants are safe means of obtaining drugs. About 25,000 higher plants have promising Phytochemical, half of which are located in tropical forests; 60% of these have their biological activities established, while about 15% of them have their phyto-compounds isolated and reported (Hamburger and Hostettman, 1991).

Phytochemical Screening (Qualitative): Detection of Alkalolds: Mayer's Test

TO a few mL of filtrate, a drop or two of Mayer's regent was added by the sides of the test tube. A white creamy precipitate indicated the test as positive.

TEST FOR PHYTOSTEROL: Salkowski's test

To 1ml of the extract add few drops of chloroform and filter the mixture using Whatmann filter. To the filterate added few drops of conc. Sulfuric acid and shaken well. A golden yellow color confirms the presence of triterphenoids or triterphens.

Detection of Carbohydrates and Glycosides: Benedict's Test

To 0.5 ml of filtrate, 0.5ml of Benedict's reagent was added. The mixture was heated on a boiling water bath for 2 minutes. A characteristic brick red colored precipitate indicated the presence of sugar.

Detection of Glycosides: Bontrager's Test

To 2 mL of extract, 3 mL of chloroform was added and shaken. Chloroform layer was separated and 10% ammonia solution was added to it. Pink color indicated the presence of glycosides.

Detection of Saponins: Foam Test

The extract (50mg) was diluted with distilled water and made up to 20 ml. The suspension was shaken in a graduated cylinder for 15 minutes. A two cm layer of foam indicated the presence of saponins.

Detection of Proteins and Amino Acids

The extract (100mg) was dissolved in 10ml of distilled water and filtered through Whatmann No. l filter paper and the filtrate was subjected to tests of proteins and amino acids.

Biuret Test

An aliquot of 2mI of filtrate was treated with one drop of 2% copper sulphate solution. To this, I ml of ethanol (95%) was added, followed by excess of potassium hydroxide pellets. Pink color in the ethanolic layer indicated the presence of proteins.

Detection of Phenol Compund: Ferric chloride Test:

The extract (50mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. A dark green color indicated the presence of phenols.

Detection of Tannins: Ferric chloride Test;

To extracts a few drops of 1% netural ferric chloride solution was added formation of blackish blue color indicatyes the presence of tannins.

Detection of Steroids: Liebermann Bur Chard Test:

To 0.5mI of the extract add 2ml of acetic anhydride and 2mI of concentrate H2S04 along the sides of the tube. The formation of green color indicates the presence of Steroids.

Detection of Flavonoids: Shinoda Test:

To the alcoholic solution of extract a few fragments of magnesium ribbon and concentrated hydrochloric acid was added. Appearance of red to pink colour after few minutes indicates the presence of flavonoids.

Ferric Chloride Test:

Few drops of neutral ferric chloride solution were added to little quantity of alcoholic extract. Formation of blackish green color indicates the presence of phenolic nucleus.

Lead Acetate Test:

To the extract, a few drops of aqueous basic lead acetate solution were added. Formation Of yellow precipitate indicates presence of flavonoids.

Zinc-hydrochloric acid reduction Test:

The alcoholic solution was treated with a pinch of zinc dust and few drops of concentrated hydrochloric acid. Formation of magenta color after few minutes indicates the presence of flavonoids.

Alkaline Reagent Test / NaOH Test:

To alcoholic solution added few drops of sodium hydroxide solution. Intense yellow colour which disappeared after adding dilute HCL indicates the presence of flavonoids.

Delection of Volatile Oil

To 2mI of the extract, add 0.1ml of dilute Sodium hydroxide followed by dilute HCL which forms a white colored precipitate.

Quantitative Estimation: Estimation of Tannin Contents

Tannin contents of flour were measured by Folin-Denis method (Schanderi, 1970). Sodium tungstate (100g) and phosphomolybdic acid (20g) were dissolved in 750mI distilled water and later 50ml phosphoric acid was added into the solution. Mixture was refluxed for 2 hr and volume was made to one liter with distilled water. Preparation of carbonate solution Sodium carbonate (350g) was dissolved in one liter water at 70^0C. Solution was allowed to stand overnight and then it was filtered through glass — wool. Preparation of standard tannic acid solution Tannic acid (10 g) was dissolved in 10 ml distilled water. 2.5»1 stock solutions was diluted to 100 ml with distilled water. Each ml contained 25 µg of tannic acid.

Ground sample (0.5 g) was taken in a 250 ml conical flask and 75 ml distilled water was added to it. It was heated and boiled for 30 min and then centrifuged at 2000 rpm for 20 min. The supematant was collected in 100 ml volumetric flask and volume was made up to the mark. In a 100 ml flask containing 75mI water. I ml sample extract, 5ml Folin-Denis reagent and 10 ml sodium carbonate solution were added and volume was made up. Contents of the flask were shaken well and then absorbance was measured at 700mm after staying for 30 min. A blank was prepared with water instead of sample and standard graph was produced by using 0-100 gg tannic acid.

Estimation of Total Phenolic Content

Total phenolic content was estimated using the Folin-Ciocalteu Method (Lachmanet al.,200).Samples (100µg) were mixed thoroughly with 2 ml of 2% Na2C03.After 2 min.100pl offlin-Ciocalteu reagent was added to the mixture. The resulting mixture was allowed to stand at room temperature for 30 min and the absorbance was measured at 743 nm against a blank. Total phenolic content was expressed as gram of Gallic equivalents per 100 gram of dry weight of the plant samples.

RESULTS AND DISCUSSIONS

Figure 1: Fresh leaves with flower of Vincarosea Linn. (NithyaKaIyani)

l' igure 2: Dried leaves of Vincarosea [.inn. (NithyaKaIyani)

Figure 3. Vincarosea Linn. (Nithya Kalyani) after dried and grinded.

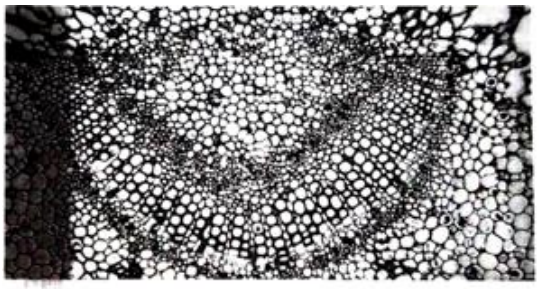

Fig-5:Vincarosea L.inn. (NithyaKalyani) Internal structure Microscopical view

Preliminary Screening of Vinca Rosea Linn

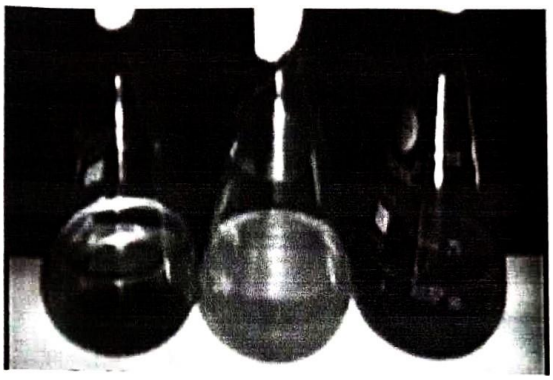

Figure-6: Extracts of Vincarosea Linn. (NithyaKalyani) in different solvents

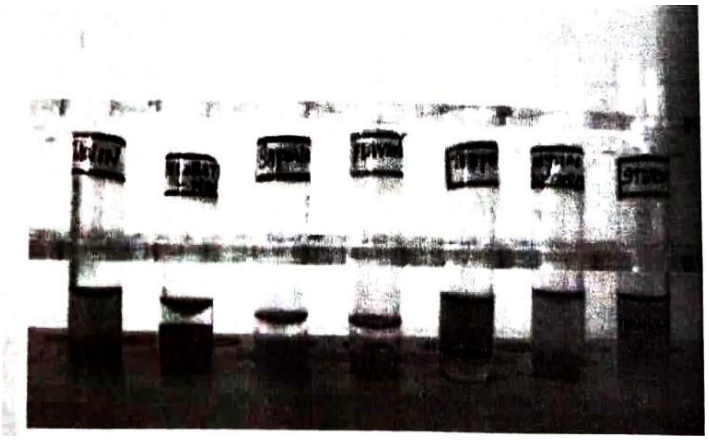

Figure-7: Preliminary Phytochemical screening of acetone extract

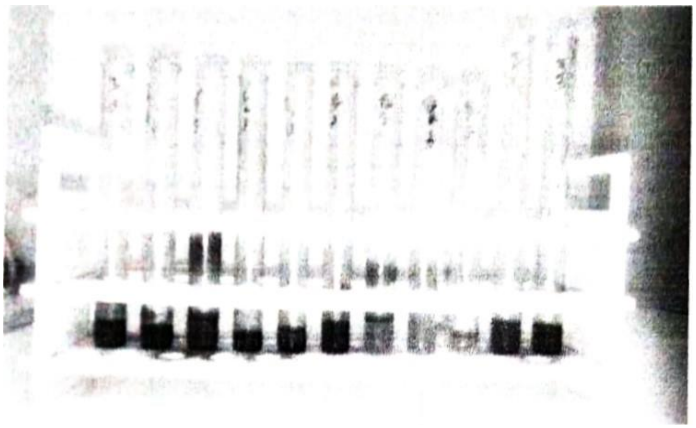

Figure-8: Preliminary Phytochemical screening of Ethanol extract

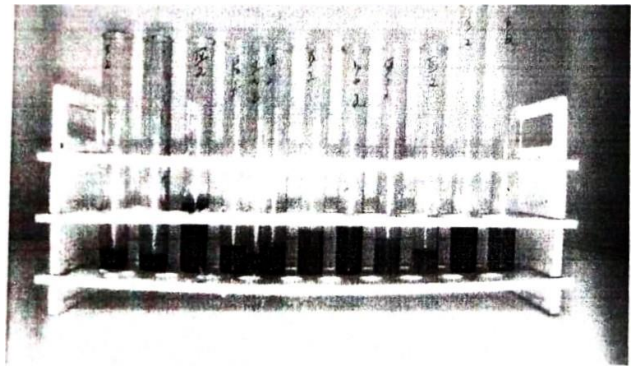

Figure-9: Preliminary Phytochemical screening of Water extract

Table-I: Preliminary phytochemical screening of Vincarosea Linn.

Compound	Acetone	Ethanol	Aqueous
Carbohydrates	+	+	+
Alkaloid	+	+	+
Protem	+	+	+
Tannin and	+	+	+
Flavonoid	++	+	+
Triterpenoids and Terpenoids	+	+	+
Steriod	+	-	-
Saponin	+	+	+
Volatile Oil	+	+	+
Glycosides	+	++	+

Quantitative Estimation of Phenol

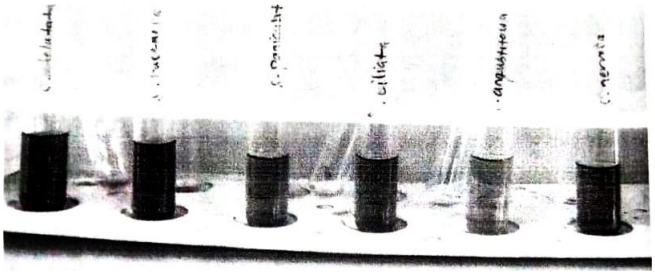

Figure-10: Quantitative Estimation of Phenol

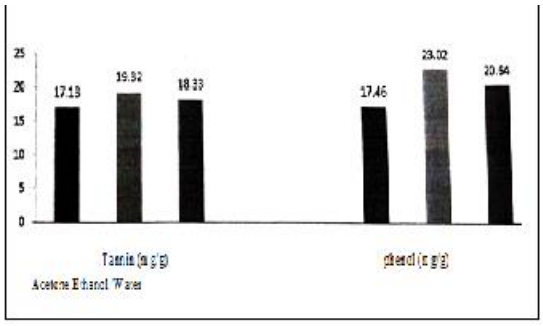

Figure-11: Graphical Representation of Tannin and Phenol

Table-2: Tannin and phenol content of Vincarosea Linn.

S.NO.	Extracted Solvent	Phenol (mg/g)	Tannin(mg/g)
1.	Acetone	17.46	17.46
2.	Ethanol	23.02	23.02
3.	Water	20.64	20.64

Discussion

The preliminary phytochemical and antioxidant activity of Vincarosea Linn. Leaf grinded powder was done the phytochemical constituents were extracted by successive solvent extraction and identified by chemical tests. These tests showed the presence of various phytochemical constituents like Carbohydrate, Alkaloid, Protein and Amino acid, Tannin & Phenol, Flavanoids, Triterpenoids, Steriods, Saponin, fixed oil, Volatile oil, glycosides. Resin, Anthroquinones and balsams Ethanolic and aqueous extracts shows the presence of majority of phyto-constituents.

The plant extractive studied could be an answer to the people seeking for better therapeutic agents from natural sources which is believed to be more efficient with little or no side effects when compared to the commonly used synthetic chemotherapeutic agents. The present study verified the traditional use of Vincarosea Linn. For human ailments and partly explained its use in herbal medicine as rich source of phytochemicals with the presence of tannins, phenols, saponins, steroid, flavanoid and terpenoids. Thus this plant can be utilized as an alternative source of useful drugs. Further studies are needed with this plant to isolate, characterize and elucidate the structure of the bioactive compounds of this plant for industrial drug formulation.

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