ANTIBACTERIAL, ANTIOXIDANT AND PHYTOCHEMICAL SCREENING OF *Hibiscus rosa sinensis, Acorus calamus, Cucurbita maxima and Moringa oliefera*

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CERTIFICATE

This is to certify that the thesis entitled "ANTIBACTERIAL, ANTIOXIDANT AND PHYTOCHEMICAL SCREENING OF *Hibiscus rosa sinensis, Acorus calamus, Cucurbita maxima and Moringa oliefera*" submitted to National Institute of Technology; Rourkela for the partial fulfilment of the Master degree in Life science is a faithful record of bonafide and original research work carried out by Debasis Nayak **under my supervision and guidance.**

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DECLARATION

I hereby declare the thesis entitled **"ANTIBACTERIAL, ANTIOXIDANT AND PHYTOCHEMICAL SCREENING OF *Hibiscus rosa sinensis, Acorus calamus, Cucurbita maxima and Moringa oliefera* "**, submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of Dr. Bismita Nayak, Assistant Professor, Department of Life Science , National Institute of Technology, Rourkela. No part of this thesis has been submitted by any other research persons or any students.

Date:
Place: Debasis Nayak

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ABSTRACT

A large number of the plants are claimed to possess the antibiotic properties in the traditional system and are also used extensively by the tribal people throughout the world. It is now mostly thought that nature has given the cure of every disease in one form or another. Plants have been known to cure people from various diseases in Ayurveda. Effects of crude extracts of the petals of *Hibiscus rosa sinensis, cucurbita maxima*, leaves of *Moringa oliefera* and rhizome of *Acorus calamus* were studied for the antibacterial and antioxidant activity. The research showed that the methanolic extract were more effective than the aqueous extract. The antioxidant activity of the four samples were carried out using DPPH which showed that out of the four plants *Moringa oliefera and Acorus Calamus* has better antioxidant properties which could be very useful against reactive oxygen species that are formed during oxidative stress.

INTRODUCTION

The term "natural products" ranges from an extremely large and diverse range of chemical compounds derived and isolated from plants, animals and microorganism. The interest for natural products can be traced back up to many years of their usefulness to human beings, and till present time it is still very helpful. Compounds and extracts derived from the Mother Nature's diversity have found uses in medicine i.e allopathic, homeopathy and Ayurveda, agriculture, beauty products, and health products in ancient and modern societies around the world. Therefore, the visionary to access natural products, understanding their usefulness and derivation applications has been a major driving force in the field of natural product research. Biodiversity is a term which is commonly used to denote the variety of species and the multiplicity of various forms of life. But this is like a small piece of sand in a desert so it is totally unimaginable how deeper we can succeed in the path of finding the natural products. In addition, there is an enormous sea of secondary metabolites usually confined to a particular group of organisms, or a particular species, or it can be a single strain growing under certain environmental conditions. In most cases we don't know really what specific biological role these products play, except that they represent a treasure of chemical compounds that can be interesting and beneficial to us. Tens of thousands of natural products have been described, but we are not even an inch close to document all the species, there are almost certainly many more thousands of compounds waiting to be discovered.

Natural products generally belong to any of the following category:

1. An entire organism which could be a plant, an animal, or a microorganism that has not been subjected to any kind of processing or treatment other than a simple process of preservation (e.g., drying)
2. Part of an organism which could be leaves, roots, steam, bark, flowers of a plant, an isolated organ from an animal
3. The wholesome compound which could be an alkaloid, sugars, coumarin, glycosides, lignin, steroids, flavonoids, terpenoids, is isolated from plants, animals or microorganisms [Samuelsson, G. 1999].

However, in most cases the term 'natural products' refers to secondary metabolites, small molecules (mol wt. <2000 amu) produced by an organism that are not strictly necessary for the survival of the organism. Perceptions of secondary metabolism include products of overflow metabolism as a result of nutrient limitation, shunt metabolism produced during the idiophase, defence mechanism regulator molecules, etc. [Cannell, R. J. P. 1998]. Natural products can be obtain from any terrestrial or marine source for example from plants source paclitaxel from *Taxus brevifolia*, from animal source animals vitamins A and D from cod liver oil, or from microorganisms such as doxorubicin from *Streptomyces peucetius.*

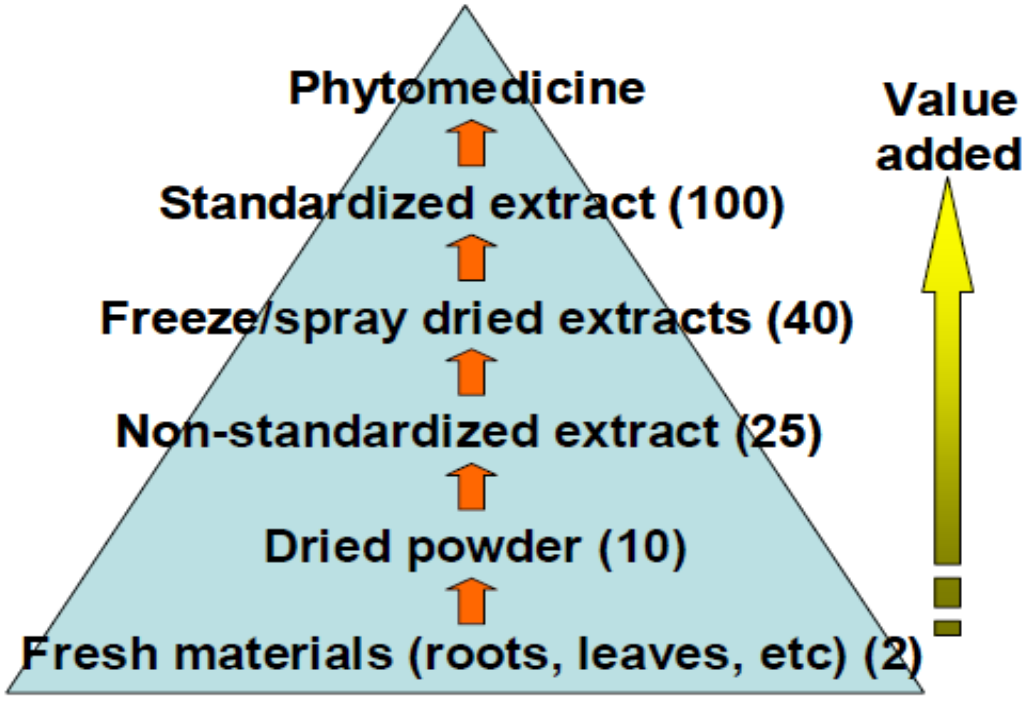


Fig: 1- Increasing value of herbal products with processing and standardisation (Ismail 2003)

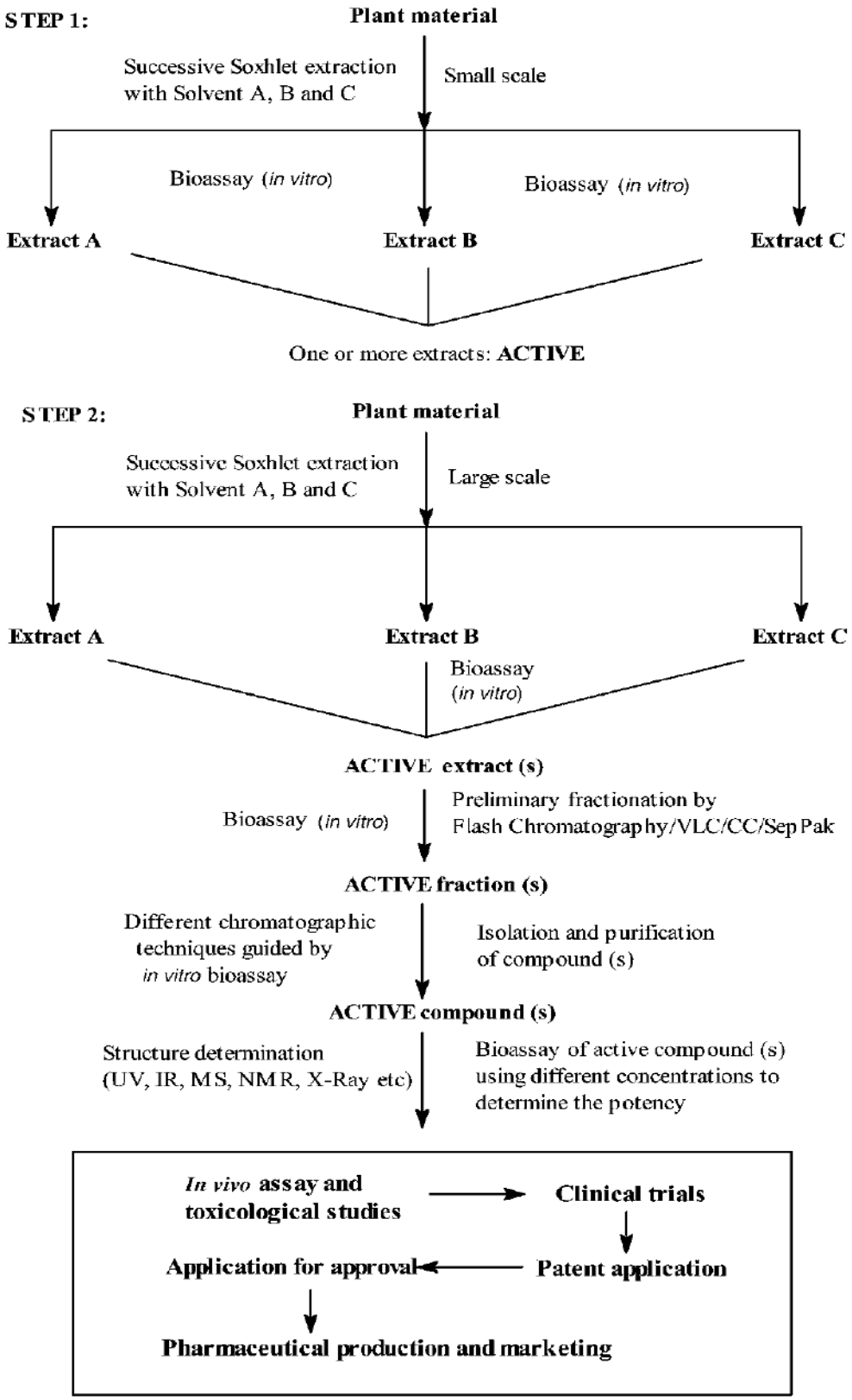


Fig: 2- An example of natural product drug discovery process (Adapted from- *Natural products isolation*- by S.D Sarker)

Nature has been a home of therapeutic agents for thousands of years, and an extraordinary number of recent drugs have been derived from natural sources, many based on their practice in traditional medicine. Over the last century, a number of bestselling medicines have been developed from natural products such as vincristine from *Vinca rosea*, morphine from *Papaver somniferum*. In recent years, a significant renewal of interest in natural products as a possible source for new medicines has been witnessed among academic circles as well as pharmacological corporations. More specifically, according to Cragg *et. al*., . 39% of the 520 new permitted drugs between 1983 and 1994 were natural products or their by-products, and 60–80% of antibacterial and anticancer drugs were from natural origins. In 2000, nearly 60% of all drugs in scientific trials for the range of cancers had natural origins. In 2001, eight (azithromycin, pravastatin, cyclosporine, amoxicillin, clavulanic acid, simvastatin, ceftriaxone, and paclitaxel) of the 30 maximum-marketing medicines were natural products or their by-products. Apart from natural product-derivative new medicine, natural products are also used straight in the ‘‘natural’’ therapeutic production, which is rising quickly in Europe and North America, as well as in traditional treatment programs being assimilated into the primary health care organizations of Mexico, the People's Republic of China, Nigeria, and other emerging countries. The use of herbal drugs is once again becoming attractive in the form of food enhancements, nutraceuticals, and complementary and supplementary medicine. Natural products can fund to the quest for new drugs in three different ways:

1. By substituting as new drugs that can be used in an original state e.g., vincristine from *Catharanthus roseus.*
2. By providing natural ‘‘building blocks’’ used to fuse more composite particles e.g., diosgenin from *Dioscorea floribunda* for the production of oral contraceptives.
3. By demonstrating new modes of pharmacological action that allow broad production of unique analogues e.g., synthetic analogues of penicillin from *Penicillium notatum.*

Natural products will positively continue to be considered as one of the key sources of new drugs in the ages to come because

1. They offer unmatched structural diversity.
2. Numerous of them are relatively small (<2000 Da).
3. They ensure ‘‘drug-like’’ properties (i.e., they can be absorbed and metabolized).

Advent, introduction, and progress of numerous new and extremely specific in vitro bioassay procedures, chromatographic approaches, and spectroscopic approaches, particularly nuclear magnetic resonance (NMR), have made it greatly easier to screen, isolate, and identify prospective drug like compounds quickly and precisely. Computerisation of these approaches now makes natural products feasible for high-throughput screening (HTS).

Bacterial infections contribute basically to general health problems of man and have been reported to be responsible for over 50% of deaths recorded in developing countries. This challenging threat posed by bacterial species appear not to have an explanation in view as some conventional antibacterial drugs have been unsuccessful in their activity against the pathogens due to the development of drug resistance [Lamikanra, 1981; Cimanga *et. al*., 1991]. Multiple drug resistance in pathogenic microorganisms have been frequently reported in current years throughout the world, mainly in developing countries, due to undiscriminating use of commercially available antibiotics in the treatment of infectious diseases [Service, 1995]. Though, the resistance development by microbes cannot be clogged, suitable action will reduce the death and health care costs by using antibiotic resistant inhibitors of plant origin [Ahmad and Beg, 2001]. Moreover, traditional remedies utilizing plants still occupy a central place among rural communities of developing countries for curing various diseases in the absence of an efficient primary health care system [Ali *et. al*., 2001; Pandey 2003]. The search for antimicrobials of plant origin has been mainly stimulated by the fact that some of the major antibacterial agents have considerable drawbacks in terms of limited antimicrobial spectrum. Till today resistance in bacteria is most prevalent. This increasing resistance to antibiotics has therefore resulted in the search for leads for new organic molecules from plants with antimicrobial properties [Cimanga *et. al.,* 1991].

Resistance towards prevailing antibiotics have become wide spread among bacteria and fungi so new class of antimicrobial substances are urgently required. Since plants have coevolved with pathogens they have understandably been developed the chemical protection pathways against the parasitic organisms. Therefore, it is reasonable to expect a variety of plants compounds with specific as well as general antimicrobial activity and antibiotic potential [Wade. D, Science 1997]. There are in fact many studies which reveal the presence of such compounds with antimicrobial properties in various plants part [Alamaghout et. al., 1985]. The bioactive substances in plants are produced as secondary metabolites [Williams

et. al., 1989] which may not be developmental stage specific but also organ and/or tissue specific while plant leaf, stem, and root extracts have been widely evaluated for bioactive compounds, screening of plant flowers has not been extensive.

The flower petals which transmit physical protection to the corresponding components including parenthesis, androecium, and gynoecium and developing embryos in the pollinated gynoecium can be expected to produce potent bioactive compounds along with it may display anti-cancerous and antioxidant activity.

Phytochemicals are in the firmest sense of the word, are the chemicals produced by plants. Frequently, though, the word refers to only those chemicals which might have an impact on health, or on flavour, texture, smell, or colour of the plants, but are not essential by humans as vital nutrients.

Antioxidant generally means "against oxidation." An antioxidant is any substance that delays or prevents weakening, loss or destruction by oxidation [Hatano, T *et. al.*, 1988]. A free radical is a compound by means of one or more unpaired electrons in its outer orbit [Jesberger J.A, 1991]. Such unpaired electrons make these species very unstable therefore relatively reactive with other molecules due to the existence of unpaired electrons [Karlsson, J., 1997] and they try to pair their electrons and generate a more stable compound. Free radicals are formed uninterruptedly as regular by-products of oxygen metabolism during mitochondrial oxidative phosphorylation. Thus the mitochondrion is the main basis of free radicals [Przedborski, S.*et. al.*, 1998; Fahn, S. *et. al*., 1992]. The most hazardous free radicals are the atomic and molecular varieties of oxygen which is known as Reactive Oxygen Species (ROS). While ROS are not strictly free radicals, they are highly reactive with the molecules around them [Sharma, H.1998]. ROS is a combined term, which includes not only the oxygen radicals (O and OH) but also some non-radical derivatives of oxygen, but also hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and ozone (O) [Sjodin, T., 1990].

However, almost all organisms are protected from free radical attack by resistance mechanisms such as a defending antioxidant system that declines the rate of free radical formation, and a another system to produce chain-breaking antioxidants that scavenge and stabilize free radicals. When free radical manufacturing rate exceeds the capability of the antioxidant defence mechanisms considerable tissue injury occurs. [Rahman *et. al*., 2007].

Therefore, antioxidants having free radical scavenging activities may have some great significance in the prevention and therapeutics of free radical aided diseases.

Before doing any purification and isolation work, natural products have to be extracted from the biomass. The main aim could to isolate a known metabolite or to isolate and illustrate as many compounds as conceivable for an organised phytochemical analysis. An initial extraction is executed characteristically on a small quantity of material to attain a primary extract. Once definite metabolites have been recognized in the initial extract, then it may be desirable to isolate them in larger magnitudes. As natural products are so diverse and show distinct physicochemical properties, the question arise is how can these metabolites being extracted proficiently from the material under exploration. Solvent-extraction methods can be used for small initial scale laboratory research on in bulk for industrial purpose.

REVIEW OF LITERATURE

2.1: TERPINOID

Isoprenoids, also known as terpenoids, are the major family of natural compounds, comprising of >40 000 different molecules. The isoprenoid biosynthetic pathway produces both primary and secondary metabolites that are of great significance to plant growth and persistence. Terpenoids are well-defined as secondary metabolites using molecular structures comprising carbon backbones are made up of isoprene (2-methylbuta- 1, 3-diene) units. Isoprene comprises five carbon atoms and as a consequence, the number of carbon atoms in any terpenoids is a multiple of five. The terpenoids comprises of two isoprene units, containing ten carbon atoms. Among the primary metabolites produced by this pathway are: the phytohormones- abscisic acid (ABA); gibberellic acid (GAs) and cytokinins; the carotenoids; plastoquinones and chlorophylls involved in photosynthesis; the ubiquinones required for respiration; and the sterols that impact membrane structure. Many of the terpenoids are commercially interesting because of their use as flavours and fragrances in foods and cosmetics examples menthol, nootkatone and sclareol or because they are important for the quality of agricultural products, such as the flavour of fruits and the fragrance of flowers like linalool [Aharoni, A. *et. al.,* . 2004; Pichersky, E. *et. al.,* . 1994]. In addition, terpenoids can have medicinal properties such as anti-carcinogenic (e.g. Taxol and perilla alcohol), antimalarial (e.g. artemisinin), anti-ulcer, hepaticidal, antimicrobial or diuretic (e.g. glycyrrhizin) activity [Bertea, C.M. *et. al*., 2005; Haudenschild, C, 1998; Lin, Z.J. *et. al.*, 2005; McCaskill, D.1998; Rodriguez-Concepcion, M. 2004]. The terpenoids have also been shown to be of great ecological significance [Degenhardt, J. *et. al.*, 2003; Pichersky, E *et. al.*, 2002].

The steroids and sterols in animals are biologically produced from precursors of terpenoid. Sometimes terpenoids are added to proteins to increase their attachment to the cell membrane the process known as isoprenylation [Sacchettini JC *et. al*, 1997]. These compounds and their derivatives also belong to other drugs such as validol, menovasin,

turpentine, bromkamfora etc. Turpentine is extensively used as external drugs, and it is the main raw material for other products on the base of terpenoids.

2.2: FLAVONOID

Flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized, according to their chemical structure into flavones, anthocyanidins, isoflavones, catechins, flavonols, chalcones and flavanones. More than 4,000 flavonoids have been recognised, many of which occur in vegetables, fruits and beverages like tea, coffee and fruit drinks. The flavonoids have provoked considerable interest recently because of their potential valuable effects on human health-they have been testified to have been shown to have several biological properties including anti-inflammatory, hepatoprotective anti-thrambotic and antiviral activities many of which may be associated, partially at least, to their antioxidant and free-radical-scavenging ability.[Robak, J *et. al.,* 1988]. The antiradical property of flavonoids is directed mostly toward HO and 0_2 as well as peroxyl and alkoxyl radicals [Husain, S. R *et. al.,* 1987]. Furthermore, as these compounds present a strong affinity for iron ions their antiperoxidative activity could also be ascribed to a concomitant capability of chelating iron. [Morel, I *et. al.,* 1993; Afanasav, I.B. *et al*, 1989].

One of the undeniable functions of flavonoids and related polyphenols is their role in defending plants against microbial attack. This not only comprises their presence in plants as constitutive mediators but also their accumulation as phytoalexins in response to microbial attack [Grayer *et. al*., 1994; Harborne, 1999]. Because of their extensive ability to prevent spore germination of plant pathogens, they have been suggested also for use against fungal pathogens. There is an ever growing interest in plant flavonoids for treating human diseases and particularly for monitoring the immunodeficiency virus which is the contributing agent of AIDS. The majority of flavonoids documented as constitutive antifungal agents in plants are flavanones, isofavonoids or flavans. The recognition that a flavone glycoside, namely luteolin 7-(200-sulphatoglucoside), is an antifungal component of the marine angiosperm *Thalassia testudinum* is remarkable [Jensen *et. al*., 1998].

Skaltsa *et. al.,* (1994) claim that acylated flavone glycosides existing in the leaf hairs of *Quercus ilex* affords the plant useful protection against the damage of UV radiation. The main experiment here was to calculate the photosynthetic proficiency of de-haired leaves.

Indeed, there is a substantial reduction in photosystem II photochemical efficacy in treated leaves.

Numerous recent papers report the consistent presence of antibacterial activity among favonoids. Thus, the retrochalcone licochalcone C (4, 40-dihydroxy-20-methoxy-30-prenyl) is active against *Staphylococcus aureus* with a MIC of 6.25 µg/ml [Haraguchi *et. al*., 1998]. Also, the compound 5, 7-dihydroxy-3, 8-dimethoxy favone has an MIC of 50 µg/ ml towards *Staphylococcus epidermis* [Iniesta *et. al*., 1990]. Again, the substance 5, 7, 20, 60-tetrahydroxy-6-prenyl-8-lavandulyl-40-methoxyfavanone completely inhibits the progress of *S. aureus* at concentrations among 1.56 and 6.25 µg/ml [Iinuma *et al*., 1994]. The above favanone is chiefly active against antibiotic resistant strains of *S. aureus* and could have some activity towards treating patients, who unintentionally pick up this infection while in hospital.

Das and Pereira, 1990 have shown that a carbonyl group at C-4 and a double bond between C-2 and C-3 are also important features for high antioxidant activity in flavonoids. Butein and other 3, 4-dihydroxychalcones are more active than analogous flavones because of their ability to achieve greater electron delocalisation [Dziedzicet *et. al.*, 1983]. Likewise, isoflavones are frequently more active than favones because of the stabilising effects of the 4-carbonyl and 5-hydroxyl in the former [Dziedzic and Hudson, 1983]. In the antioxidant action of o-dihydroxyfavonoids metal chelation is an important factor [Shahidi *et. al*., 1991].

Flavonoids have been stated to possess many useful properties, containing anti-inflammatory activity, enzyme inhibition, antimicrobial activity, oestrogenic activity [Havsteen B et al, 1983; Harborne JB *et. al.*, 1999], anti-allergic activity, antioxidant activity [Middleton Jr E et al, 1986], vascular activity and cytotoxic antitumor activity [Harborne JB *et. al*., 1992]. For a group of compounds of relatively homogeneous structure, the flavonoids inhibit a mystifying number and variety of eukaryotic enzymes and have an extremely wide range of activities. In the event of enzyme inhibition, this has been assumed to be due to the interaction of enzymes with different parts of the flavonoid molecule such as carbohydrate, phenyl ring, phenol and benzopyrone ring [Havsteen B *et. al.*, 1983].

A recent area of research that is of particular interest is the deceptive inhibitory activity of some flavonoids against human immunodeficiency virus (HIV). In vitro studies have shown that baicalin inhibits HIV-1 infection and replication. Inhibition of HIV-1 entry into the cells expressing CD4 and chemokine co-receptors [Li BQ *et. al.*, 2000], and antagonism of HIV-1 reverse transcriptase [Li BQ *et. al*., 1993] by the flavone O-glycoside

have been demonstrated by Li and colleagues. Baicalein [Ono K *et. al*., 1989], robustaflavone and hinokiflavone [Lin YM *et. al*., 1997] have also been shown to inhibit HIV-1 reverse transcriptase, as have several catechins, but catechins inhibit other DNA polymerases and their interaction with the HIV-1 enzyme is therefore thought to be of non-specific nature [Moore PS *et. al*., 1992]. In addition, it has been demonstrated that several flavonoids, including demethylated gardenin A and 3, 2''-dihydroxyflavone, inhibit HIV-1 proteinase [Brinkworth RI *et. al*., 1992]. Robinetin, myricetin, baicalein, quercetagetin [Fesen MR. *et. al*., 1994] and quercetin 3-O-(2''-galloyl)'-l-arabinopyranoside [Kim HJ *et. al.*, 1998] inhibit HIV-1integrase, although there are concerns that HIV enzyme inhibition by quercetagetin and myricetin is non-specific [Ono K *et. al*., 1990]. It has also been reported that the flavonoids chrysin, apigenin and acacetin prevent HIV-1 activation via an unusual mechanism that possibly involves inhibition of viral transcription [Critchfield JW *et. al*., 1996]. Interestingly, in a study by Hu and his co-group, chrysin was reported to have the highest therapeutic index of 21 natural and 13 synthetic flavonoids against HIV-1 [Hu CQ, *et. al*., 1994]. Several research groups have investigated the relationship between flavonoid structure and inhibitory activity against HIV-1 and its enzymes [Lin YM *et. al.*, 1997; Brinkworth RI *et. al*., 1992; Fesen MR *et. al*., 1994; Critchfield JW *et. al*., 1996; Hu CQ, *et. al.*, 1994]. Furthermore, at least two groups have proposed mechanisms of action for HIV-1 enzyme inhibition [Brinkworth RI *et. al*., 1992; Fesen MR *et. al*., 1994].

Flavonoids show interactions with cytochrome P_{450} [Ng T B *et. al*., 1996], anti-leukemic properties [Hodek, P *et. al*., 2002] and mild vasodilators properties useful for the treatment of heart disease [Hodek, P *et. al*., 2002].

2.3: SAPONIN

Saponins are a group of secondary metabolites found widely distributed in the plant kingdom as plant glycosides, characterized by a skeleton resulting of the 30-carbon precursor oxidosqualene to which glycosyl residues are attached along with it they have sturdy foaming property. Conventionally, they are subdivided into triterpenoid and steroid glycosides, or into triterpenoid which are found primarily in dicotyledonous plants but also in some monocots, spirostanol, and furostanol saponins. [Hostettmann, K. *et. al*., 1995]. Steroid saponins occur chiefly in monocotyledons family such as the Lilliaceae, Agavaceae, Droscoraceae and in certain dicotyledons, such as foxglove [Hostettmann *et. al*., 1995]. Oats are unusual because they contain both triterpenoid and steroid saponins [Price *et. al*., 1987]. Steroidal

glycoalkaloids are found principally in members of the family belonging to Solanaceae, which includes potato and tomato. The saponins formed by oats and tomato have been studied in detail in relation to their potential role in the defences of plants against phyto-pathogenic fungi [Osbourn, 1996].

They are stored in plant cells as inactive precursors but are readily converted into biologically active antibiotics by plant enzymes in reply to pathogenic attack. These compounds can also be regarded as Ò preformed Ó, since the plant enzymes that activate them are already present in healthy plant tissues [Osbourn, 1996]. The natural role of saponins in plants is thought to be protection against attack by pathogens and pets [Price *et. al*., 1987; Morrissey *et. al*., 1999]. These molecules also have substantial marketable value and are processed as drugs and medicines, foaming agents, sweeteners, taste converters and cosmetics [Hostettmann *et. al*., 1995].

Saponin containing plants are used as traditional medicines, especially in Asia, and are intensively used in food, veterinary and medical industries [Hostettmann K *et. al*., 1995]. The pesticidal activity of saponins has long been reported [Irvine FR, 1961] Saponin-glycosides are very lethal to cold-blooded organisms, but deceptively not to mammals (Hostettmann K *et. al*., 1995; Hall JB *et. al*., 1991). Plant extracts containing a high percentage of saponins are commonly used in Africa to treat water supplies and wells contaminated with disease vectors; after treatment, the water is safe for human drinking [Hall JB *et. al*., 1991].

Various studies have shown the effect of saponins on the immune system. Saponins induce a strong adjuvant effect to T-dependent as well as T-independent antigens & it also induces strong cytotoxic CD8+ lymphocyte responses and potentiate the response to mucosal antigens [Kensil C.R.1996] Saponin based adjuvants have the ability to modulate the cell mediated immune system as well as to enhance antibody production and have the advantage that only a low dose is needed for adjuvant activity [Oda K., *et. al.*, 2000].

Saponins have been extensively used as adjuvants for several years and have been incorporated in several veterinary vaccinations. The adjuvant act of saponins was, however, not so noticeable in some of the non-mammalian species tested [Cossarini M, 1985; Grayson T.H *et. al*., 1987]. Saponin has both stimulatory effects on the components of specific immunity and non-specific immune reactions such as inflammation [de Oliveira C.A.C., *et. al*., 2001] and monocyte proliferation [Delms F *et. al.*, 2001] The mechanisms of immune-stimulating action of saponins have not been evidently understood, Saponins apparently

induce production of cytokines such as interleukins and interferons that might mediate their immune-stimulant effects [Kensil C.R., 1996]. Saponins have been shown to interpolate into cell membranes, from side to side by interaction with cholesterol, forming 'holes' or pores, their specific capability to form pores in membranes has backed to their common use in physiological research (Choi *et. al*., 2001; Menin., 2001; El Izzi *et. al*., 1992; Plock *et. al.,* 2001; Authi *et. al*., 1988). It is currently unfamiliar if the adjuvant effect of saponins is related to pore formation, which may permit antigens to gain access to the endogenous pathway of antigens presentation, promoting cytotoxic T-lymphocyte (CTL) response [Sjölander A.*et. al*., 2001]. It was believed that the adjuvant activity of saponins could be associated to branched sugar chains or aldehyde groups or to an acyl residue having the aglycone [Kensil C.R., 1996]. Latter lablab sides and soya saponins were found to display strong adjuvant activity in spite of lacking acyl residues and owning only un-branched sugar chains Oda *et. al,*. concluded that the overall conformation of functional groups exaggerated adjuvant action of saponins. Saponins have been known long to possess a lytic action on erythrocyte cell membranes and this property has been used for their detection. The haemolytic action of saponins is alleged due to be their affinity for the aglycone moiety of membrane sterols, mainly cholesterol [Glauert *et. al*., 1962], with which they form undissolvable complexes [Bangham & Horne, 1962]. The quantity of glycosides prerequisite for permeabilisation is much lower for cholesterol-rich lipid layers than cholesterol-free membranes [Gogelein *et. al*., 1984].

Saponins isolated from plants such as fenugreek [Petit *et. al.,* 1993], Phellodendron and Aralia cortex [Kim *et. al*., 1998], *Pueraria thunbergiana* [Lee *et. al.,* 2000], and *Calendula officinalis* [Yoshikawa *et. al*., 2001] have revealed to have hypoglycaemic effects. Petit *et. al*., (1993) established chronically higher plasma insulin levels, possibly initiated by stimulation of the b-cells in male Wister rats were given 10 and 100 mg fenugreek extract/300 g body weight mixed with food while Matsuda *et. al*., (1999) did not find insulin-like or insulin-releasing activity in rats were given oleanolic acid glycosides orally. The hypoglycaemic act here was due to suppression of transportation of glucose from the stomach to the small intestine and the inhibition of glucose transport across the brush border of the small intestine. The saponin momordin was also found to be significantly dose-dependent to inhibit gastric discharging [Matsuda *et. al*., 1999]. The inhibitory activity here was dependent on the level of serum glucose and facilitated at least in part by the capsaicin-sensitive sensory nerves and the central nervous system. Yoshikawa *et.*

al., (2001) revealed that the oleanolic acid 3-monodesmosides with hypoglycaemic activity also inhibited gastric emptying showing a correlation between the two properties, while other saponins also did not affect gastric emptying.

2.4: TANNIN

Tannins are naturally occurring plant polyphenols that have a characteristic of binding and precipitating proteins. They can have a large influence on the nutritive value of many foods eaten by humans and feedstuff eaten by animals. Tannins are found commonly in fruits such as grapes, persimmon, blueberry, tea, chocolate, legume forages, legume trees like *Acacia* spp., *Sesbania* spp., in grasses i.e; sorghum, corn, etc.

The characteristics of tannins are that they are oligomeric compounds with numerous structure units with free phenolic groups, molecular weight fluctuating from 500 - 20,000, soluble in water, with exception of some high molecular weight structures they are able to bind proteins and form insoluble or soluble tannin-protein complexes. Presently there is a cumulative interest in tannins as bioactive component of foods as well as biological antioxidants. Tannins are an exceptional group of water soluble phenolic metabolites of relatively high molecular weight and having the ability to complex strongly with carbohydrates and proteins [Chavan *et. al*., 2001]. In the past, tannins have been viewed as one of the anti-nutrients of plant origin because of their capability to precipitate proteins, inhibit the digestive enzymes and decline the absorption of vitamins and minerals [Khattab *et. al.*, 2010]. However, lately several health benefits have been recognized for the intake of tannins and some epidemiological associations with the decreased frequency of chronic diseases have been established [Serrano *et. al*., 2009]. Abundant studies have demonstrated supposedly significant biological effects of tannins such as antioxidant or radical scavenging activity as well as inhibition of lipid peroxidation and lipoxygenases in vitro [Amarowicz *et. al*., 2000; Gyamfi *et. al*., 2002], antimicrobial and antiviral [Dolara *et. al.,* 2005; De Bruyne *et. al*., 1999], antimutagenic [Dolara *et. al.,* 2005; Carlsen *et. al*., 2010], and antidiabetic properties [Matsui *et. al*., 2001; Anderson *et. al*., 2002]. The antioxidant activity of tannins results from their free radical and reactive oxygen species-scavenging properties, as well as the chelation of transition metal ions that modify the oxidation process [Serrano *et. al*., 2009]. Antioxidants have also been reported to provide synergistic benefits for the treatment of diabetes because of their insulin enhancing potential [Madhujith *et. al*., 2004].

2.5: PHENOLS

Phenolic compounds are some of the most widespread molecules among plant secondary metabolites, are known to act as natural antioxidants and antinitrosating agents which are of great significance in plant development. They are involved in various processes comprising rhizogenesis [Curir *et. al.,* .1990], vitrification [Kevers *et. al*., 1984], resistance to biotic and abiotic stress [Delalonde *et. al*., 1996], and redox reactions in soils [Takalama *et, al*., 1992]. Additionally, they serve as flower pigments, act as constitutive protection agents against invading organisms, function as signal molecules, act as allelopathic compounds, and affect cell and plant growth [Dakora, 1995; Dakora *et. al*., 1996; Ndakidemi and Dakora, 2003], are important natural animal toxicants [Adams, 1989] and some may function as pesticides [Vidhyasekaran, 1988; Waterman and Mole, 1989; Beier, 1990]. They are also functional components of the rhizosphere and its soil organic matter [Haider *et. al*., 1975; Martin, 1977]. They have long been recognised as allelochemicals for weed control [Rice, 1984; Putnam and Tang, 1986] phytoestrogens in animals [Adams, 1989] and plant defence molecules [Vidhyasekaran, 1988]. In the rhizosphere, they work as important precursors for the production of soil humic substances [Haider *et. al.,* 1975]. A regular intake of phenolic compounds is assumed to decrease the incidence of certain forms of cancer, and for that reason they are normally regarded as chemo-preventive agents. [d′Ischia M *et. al*., 2006; Craig WJ *et. al*., 1999] The antioxidant properties of phenols are determined by their radical scavenging ability and consequent inhibitory action on lipid peroxidation under oxidative stress situations, which link with their substitution pattern. [Rigobello MP *et. al*., 2004].

2.6*: Hibiscus rosa sinensis*

In addition to its traditional use *Hibiscus rosa sinensis* has anti-inflammatory, demulcent, aphrodisiac, emmenagogue, refrigerant, anodyne, laxative, emollient and various researchers had described the use of the flower to treat heart disorders [J. Anjaria, *et. al*., 2002]. Sachdewa and Khemani established the antidiabetic activity of HRS in diabetic rats and the effect was analogous with glibenclamide. It has been also shown to be valuable in fever and bronchial catarrh. It is known to have various activities like antitumor, antidiarrheal, antiestrogenic antispermatogenic, androgenic, antiphologistic [S. D. Kholkute and K. N. Udupa 1976], antiimplantation [D. R. K. Murthy et. al., 1997], wound Healing [N. Vasudeva and S. K. Sharma 2008], anticonvulsant [B. Shivananda Nayak et. al., 2007]. It mainly consists of cyaniding, anthocyanins, quercetin, hydrocitric acid, kaempferol,

hydrocitric acid, flavonoids and so forth [H. Yamasaki *et. al.*, 1976]. These chemical constituents were reported in plant i.e. cyanidin, quercetin, flavonoids, hentriacontane, thiamine, riboflavin, niacin and ascorbic acid [Nair R *et. al*., 2005].

2.7: *Acorus calamus*

Roots and rhizomes of *Acorus calamus* generally known as sweet flag, sweet grass and sweet cane (Family: Acoraceae), have been used in the Indian and Chinese systems of the medicine for hundreds of years for its beneficial role in improved learning performance, and its anti-aging effect. [Nishiyama N *et. al*., 1994; Zhou Y *et. al.*, 1994; Zhang Y *et. al*., 1994] According to Arabic, Roman, and later European folk botany, the plant is also an aphrodisiac .Shukla *et. al*., reported that ethanolic extract of AC, decreased GSH and GST, increased dopamine receptors in the corpus striatum, prevented acrylamide-induced hind limb paralysis [Shukla P. K *et. al*., 2002]. The ethanolic extract of AC has been reported to possess the antioxidant activity in an in vitro study [Acuna U *et. al*., 2002]. *Acorus calamus* effectively prevents the noise stress-induced changes in the rat brain. This anti-stressor effect might be due to an increase in brain antioxidative capacity which in turn could be achieved by protection of decreasing GSH, vitamins C, and E levels and restoring free radical scavenger's enzymatic activity. [Sundaramahalingam M. *et. al.*, 2005]. The rhizome alcoholic extract has sedative and analgesic properties and causes depression in blood pressure and respiration rate. Extracts are used to treat intestinal coli, gastritis and gastric ulcers. [Desai I *et. al*., 1984; Bhattacharya A *et. al*., 2001].

2.8*: Cucurbita maxima*

Pumpkin contains naturally active components that comprise of polysaccharides, fixed oils, para-aminobenzoic acid, peptides, sterol, and proteins [Buchbauer G *et. al.*, 1998; Kuhlmann H *et. al*., 1999; Matsui T *et. al.*, 1999; Appendino G *et. al*., 1999]. The fruits are a noble source of carotenoid and γ -aminobutyric acid [Murkovic M *et. al*., 2002; Gonzalez E *et. al*., 2001; Rodriguez-Amaya DB, 1999; Arima HK *et. al*., 1990; Zhang H, 2003].Yet, the presence of antinutrients in pumpkin seeds which have been revealed to have harmful physiological properties on growing rats and chicks confines its nutritional value and hence bounds the usefulness of fresh pumpkin seed as a protein basis for human food [Akwaowo EU *et. al*., 2000; Achinewhu SC *et. al.*, 1990; Nwokolo E *et. al*., 1987]. Several phytochemicals such as polysaccharides, 13-hydroxy-9Z, phenolic glycosides, and 11E-octadecatrienoic acid from the leaves of Cucurbita, proteins from germinated seeds, have

been isolated [Koike K *et. al*., 2005; Bang MH *et. al.*, 2002; Xiang D *et. al.*, 2004; Li QH, Fu CL 2005; Jun HI *et. al*., 2006]. The hypoglycemic chemicals of pumpkin contain polysaccharides from the fruit pulp [Zhang YJ *et. al*., 2002; Yao HY *et. al.*, 2002; Xiong XM, 2000], oil from un-germinated seeds and protein from germinated seeds. These chemicals are intense in fruits of pumpkin therefore; fruit of pumpkin has shown more noticeable hypoglycemic/antihyperglycemic activity. However, protein possessing hypoglycemic activity was not from un-germinated pumpkin seed [Cai TY *et. al.*, 2002]. Hypoglycemic action of polysaccharide isolated from pumpkin containing 8.48% sugar was lower than that from pumpkin comprising 4.29% sugar [Zhang Y *et. al*., 2002]. Both common and sugar-removed pumpkin powder displayed a significant reduction in blood glucose and an upsurge in plasma insulin and protected the diabetic nephropathy [Ju LY *et. al*., 2001; Zhang XP *et. al.*, 2004; Chen JG 2005]. Drop in blood glucose, serum total cholesterol and triglyceride was witnessed in alloxan induced diabetic rabbits applied with pumpkin powder [Zhang ZJ 1998]. Hypoglycemic activity of water-extracted pumpkin polysaccharides was demonstrated and excelled glibenclamide in alloxan-induced diabetic rats ($P < 0.01$) [Zhang YJ 2004; Zhang YJ, 2001; Zuo YM ,2001; Peng H 2002; Xiong XM ,1998]. Anti-hyperglycemic activity of water-extracted pumpkin polysaccharides was observed in normal rats [Zhang YJ *et. al.*, 2002].Crude polysaccharide from pumpkin fruit was reported to reduce branched chain amino acid and have better effect on normal rats than on alloxan-induced diabetic rats [Kong QS *et. al*., 2002].

2.9: *Moringa oliefera*

Moringa oleifera is an extremely valued plant, dispersed in many countries of the tropics and subtropics. It has an extraordinary range of medicinal uses with high nutritive value. Different parts of this plant contain a sketch of important minerals, and are a good source of protein, various phenolics, vitamins, β – carotene and amino acids [Farooq *et. al.*, 2007]. The Moringa plant offers a rich and exceptional combination of zeatin, kaempferom, quercetin and many other phytochemicals. It is very significant for its medicinal value. Numerous parts of the plant such as the roots, seed, bark, leaves, fruit, and immature pods, flowers act as cardiac and circulatory drugs, possess antitumor [Makonnen *et. al.*, 1997], antipyretic, antiulcer, anti nflammatory, antiepileptic [Pal *et. al.*, 1995]. Other chief medicinal properties of the plant include antispasmodic [Caceres *et. al*., 1992], diuretic [Morton, 1991], antihypertensive [Dahot, 1988], cholesterol lowering [Mehta *et. al.*, 2003], hepatoprotective, antioxidant, antidiabetic, [Ruckmani *et. al*., 1998], antibacterial and

antifungal activities [Nickon *et. al*., 2003]. *M. oleifera* parts are being employed for the treatment of different ailments in the indigenous system of medicine, particularly in South Asia [Farooq *et. al*., 2007]. In addition, *M. oleifera* seeds possess water purifying powers [Muyibi and Evison, 1995b; Kawo, 2007] by flocculating Gram positive and Gram negative bacterial cells [Olsen, 1987; Broin *et. al.*, 2002; Kawo, 2007]. *M. oleifera* seeds can also be used as a less expensive bio absorbent for the removal of heavy metals [Sharma *et. al*., 2006].

Precise components of Moringa preparations that have been stated to have hypo-tensive, anti-cancerous and other including 4-(4'-O-acetyl-α-L-rhamnopyranosyloxy)benzyl isothiocy-anate [Abrams B *et. al.*,1993], 4-(α-L-rhamnopyranosyloxy)benzyl isothiocy-anate.[Abuye C *et. al*., 1999], niazimicin [Akhtar AH *et. al.,* 1995], pterygospermin [PC Bell, *et. al.*, 1986], benzyl isothiocyanate [Anwar F *et. al*., 2003], and 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate [Asres K, 1995]. While these compounds are comparatively unique to the Moringa family, it is also rich in a quantity of vitamins and minerals as well as other more commonly predictable phytochemicals such as the carotenoids (including β-carotene or pro-vitamin A).

BIOLOGY OF THE SAMPLES

3.1*: Hibiscus rosa sinensis*

Classification

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Dilleniidae

Order: Malvales

Family: Malvaceae

Genus: Hibiscus.

Species: *Hibiscus rosa sinensis*

Fig 3: *Hibiscus rosa sinensis*

Characteristics

The plant *hibiscus rosa sinensis* belong to the family malvaceae known in Sanskrit as japa or rudrapushpa the roots are cylindrical 5-1 cm in length and 2 cm in diameter, off-white and with light brown transverse tentacles. The roots tastes sweet are mucilaginous. The leaves are simple ovate or ovallancoate and are entire at the base and coarsely toothed at the apex. The corolla consists of 5 petals red and about 8 cm in diameter. The flowers are demulcent, refrigerant, aphrodisiac, emollient and emmenagogue. Petals are used to stimulate thicker hair growth and to prevent premature greying, hair loss and scalp disorders.

3.2: *Cucurbita maxima*

Classification

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Superdivision:	Spermatophyta
Division:	Magnoliophyta
Class:	Magnoliopsida
Subclass:	Dilleniidae
Order:	Violales
Family:	Cucurbitaceae
Genus	Cucurbita
Species:	maxima

Fig 4: *Cucurbita maxima*

Characteristics

They are large low creeping vine having very large leaves those are palmate with a maple shape having small sharp serrations along the margin; flowers are very large bright yellow with messy edge; fruit is a large. Pumpkin seeds are rich in sterols, vitamin E, fatty acids and non-protein amino acids. Pharmaceutically, they are used in treating rheumatism, bladder disorders, wounds, stomach upsets, burns, intestinal worms, bed-wetting, benign prostatic hyperplasia, and certain female reproductive complaints. Pumpkin seeds also possess vitamin B, and many essential minerals such as iron, zinc, and they are very nutritious and stimulating. Other nutrients found in *Cucurbita* seeds are magnesium, phosphorus, copper, potassium, niacin, folic acid, riboflavin, thiamine, pantothenic acid, and antioxidants. Zinc helps the healing process generally useful in treating the enlarged prostate gland and pantothenic acid helps to be in good health.

3.3: *Acorus calamus*

Classification

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Liliopsida

Subclass: Arecidae

Order: Arales

Family: Acoraceae

Genus: Acorus

Species: calamus

Fig 5: *Acorus Calamus* (rhizome)

Characteristics

Acorus calamus is a perennial plant growing to 1 m (3ft 3in) by 1 m (3ft 3in). It starts flowering from May to July and the seeds mature from July to August. The flowers are bisexual and are pollinated by insects. The plant prefers clay soils with slightly acidic or alkaline nature. It cannot grow in the shade and requires wet soil; it can also grow in water. The root is emmenagogue, aphrodisiac, stimulant, carminative, diaphoretic, hypotensive, expectorant, and febrifuge, aromatic, hallucinogenic, analgesic, sedative, stomachic and vermifuge. They are used in the treatment of digestive disorders, bronchitis, sinusitis etc. They are said to have excellent tonic powers of stimulating and stabilizing the appetite. *Acorus* is used externally to treat skin eruptions, neuralgia and rheumatic pains. Chewing the root is said to kill the taste for tobacco.

3.4: *Moringa oliefera*

Classification

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Superdivision:	Spermatophyta
Division:	Magnoliophyta
Class:	Magnoliopsida
Subclass:	Dilleniidae
Order:	Capparales
Family:	Moringaceae
Genus:	Moringa
Species:	oleifera

Fig 6: *Moringa oliefera*

Characteristics:

Moringa oleifera is a small, fast-growing evergreen or deciduous tree. The bark is thick, soft, corky and deeply fissured, the leaves are usually tripinnate, the leaflets are elliptic, the flowers are generally white and fragrant in large panicles, the pods are pendulous green in colour triangular and ribbed within trigonous winged seeds. In traditional Indian medicine various parts of the tree are used therapeutically for treatment of venomous bites, ascites and rheumatism and helps in lowering blood pressure. The root and bark of young trees are considered rubefacient, stomachic carminative, vescicant and abortifacient. The flowers and roots contain an antibiotic that is highly effective in the treatment of cholera. The leaves, rich in vitamin A and C, are considered useful in respiratory ailments. The juice extracted from the leaves has strong antibacterial and antimalarial properties.

MATERIALS AND METHOD

4.1: Extraction

The plant materials were collected from the locality of Rourkela. The leaves and flowers were initially separated from the main plants body and rinsed with distilled water. After wards the samples were dried under shade paper towel in laboratory and then homogenized into fine powder using a mortar and pestle. Were stored in air tight bottles and were used for all the extraction process.

Aqueous extraction

Cold aqueous extraction

10g of each flower and leaves air dried powder was weighed and soaked separately in 50ml cold water in a conical flask stoppered with rubber cork and left undisturbed for 24 hours and then filtered off using sterile filter paper (What Man No: 1) into a sterile conical flak and were subjected to water bath evaporation so that the aqueous solvent was evaporated at its boiling temperature 100°C. The extract was got with the help of muslin cloth and was subjected to centrifugation at 5000Xg for 5 minutes and the supernatant was obtained and stored at 4°C for further use [Farombi *et. al*., 2003].

Solvent extraction

Methanol extract

10g of each leaf and flower air dried powder was weight and was placed in 100ml of organic solvent (methanol) in a conical flask and then kept in a rotary shaker at 190-220 rpm for 24 hours after 24 hours it was filtered with the help of muslin cloth and centrifuged at 5000 rpm for 15 minutes. The supernatant was then collected in a round bottom flask and the solvent was evaporated to make the final volume of one-fourth of the original volume, providing a concentration of 40 µg/0.1ml. It was stored at 40°C in air tight bottles for further studies [Ikram *et. al*., 1984].

4.2: <u>Phytochemical screening</u>

- **Materials required:**

Ferric chloride

Sodium hydroxide

Glacial acetic acid

Chloroform

Sulphuric acid (concentrated)

Acetic anhydride

Fehling solution (A & B)

Potassium dichromate

Hydrochloric acid (diluted)

Nitric acid (concentrated)

- **Procedure**

Phytochemical analysis was carried out for all the extracts as per the standard methods.

1. Detection of alkaloids: Extracts were dissolved individually in dilute Hydrochloric acid and filtered

   Mayer's Test: Filtrates were treated with Mayer's reagent i.e; Potassium mercuric Iodide. Formation of a yellow coloured precipitate specifies the presence of alkaloids.

2. Detection of carbohydrates: Extracts were dissolved individually in 5 ml distilled water and were filtered and those filtered extracts were used to test for the presence of carbohydrates.

   Fehling's Test: Filtrates were hydrolysed with dil. HCl and were neutralized with alkali and heated with Fehling's solution A & B. Formation of red precipitate indicates the presence of reducing sugars.

3. Detection of glycosides: Extracts were hydrolysed with dil. HCl, and then subjected to test for the change of colour of the samples.

4. Detection of steroids and terpenoids

   In 1 ml of methanol plant extract 1ml of chloroform was added and 2-3 ml of acetic anhydride was mixed then 1-2 drops of concentrated H_2SO_4 was added. The dark green colouration of the solution indicates the presence of steroids and pink or red colouration of the solution indicates the presence of Terpenoid.

5. Detection of Saponin

   Froth Test: Extracts were diluted with distilled water to 20ml and this was shaken in a conical flask for 15 minutes. Formation of foam indicates the presence of Saponin.

   Foam Test: 0.5g of extract was shaken with 2 ml of water. The produced foam continues for ten minutes it indicates the presence of Saponin.

6. Detection of phenols

   Ferric Chloride Test: Extracts were treated with 3 to 4 drops of ferric chloride solution. Formation of bluish black colour specifies the presence of phenols.

7. Detection of proteins and amino acids

   Xanthoproteic Test: The extracts were treated with few drops of concentrated Nitric acid. Occurrence of yellow colour indicates the presence of proteins.

8. Detection of flavonoids

   In Methanol extract 10%NaOH was added and dilute HCl was added to that solution. The change of colour from yellow to colourless provides the positive result.

4.3: Antibacterial Assay

- **Microorganism used for Antibacterial Assay**

The microbial strains are standard which were obtained from IGH, Rourkela. The bacterial strains studied are *Bacillus subtilis, Proteus mirabilis, Klebsiella pneumoniae, Escherichia coli, and Pseudomonas fluorescens*.

- **Material**

Soyabean casein digest medium (Tryptone soya Broth)

Mueller Hinton Broth

Mueller Hinton Agar

Agar- agar

- **Culture preparation for Antibacterial Assay**

The cultures were grown on Tryptone soya broth 37 °C for 24 hours in the test tube in an incubator. The turbidity was measured by adjusting to 0.5 Mac Far land standards (108 CFU/ml).

- **Agar Well Diffusion Method**:

Muller Hinton agar plates were prepared and wells of 6mm were cut and swabbed with different cultures and the cut wells were then filled with 50µl of both aqueous and solvent extracts of flowers and leaves separately and the plates were kept for incubation at 37°C for 24 hrs. [Artizzu et. *al*., 1995].

4.4: Antioxidant activity

DPPH assay: free radical scavenging activity

- **Materials:**

2, 2-Diphenyl-1-Picryl hydrazyl

Ascorbic acid

Methanol

Ethanol

- **Procedure :**

The antioxidant activity of *Acorus calamus*, *Moringa oliefera*, *Cucurbita maxima* and *Hibiscus rosa sinensis* methanolic extract and the standard antioxidant ascorbic acid was assessed on the basis of the radical scavenging effect of the stable 2, 2- diphenyl-1-picrylhydrazyl (DPPH) free radical activity according to the method described by Brand-William et al. (1995). The methanol extract with different concentrations (10, 50, 100, 200, 400, 600, μg/ml) were prepared using methanol. Ascorbic acid was used as the standard in 1-100 μg/ml solution. 0.004 % of DPPH solution was prepared in ethanol and 5 ml of this solution was mixed with 5 ml of extract solution and standard solution distinctly. These solution mixtures were kept in dark for 30 min. The degree of DPPH purple decolourization to DPPH yellow indicated the scavenging effectiveness of the extract. The absorbance of the combination was determined at517 nm using UV-Visible Spectrophotometer and ascorbic acid was served as a positive control. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity.

$$\% \text{ DPPH radical-scavenging} = \frac{(\text{Absorbance of control} - \text{Absorbance of test Sample})}{(\text{Absorbance Of control})} \times 100$$

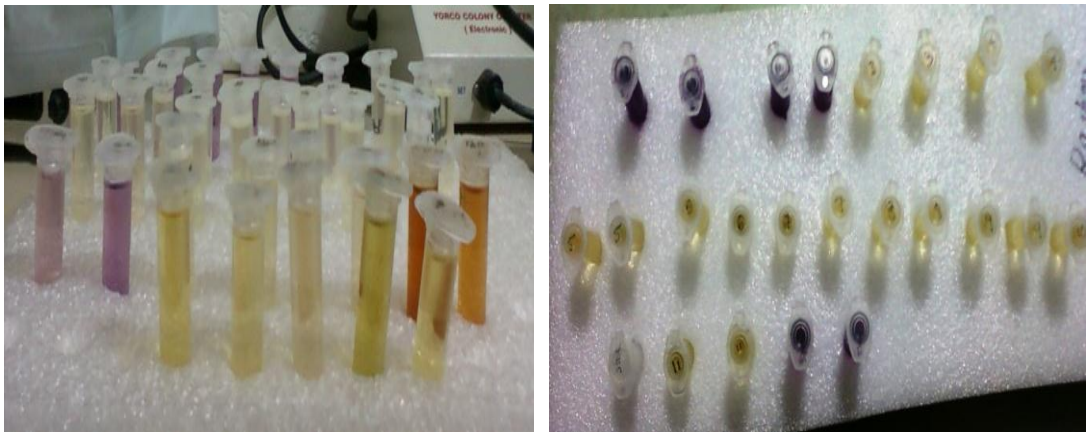


Fig 7: Procedure of antioxidant activity

Reducing antioxidant power assay:

The reducing antioxidant power of plant methanolic extracts was determined by the method of Oyaizu (1986). Different concentrations of plant extracts in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50^oC for 20 minutes. After that 2.5 ml of trichloroacetic acid (10%) was added to mixture. Then the solution was centrifuged for 10 minutes at 3000 rpm. From the upper layer 2.5 ml of the supernatant was mixed with 2.5 ml distilled water and 0.5 ml, 0.1% $FeCl_3$. The absorbance was measured at 700 nm against a blank using UV-Vis spectrophotometer.

Increased absorbance of the reaction mixture indicates the increase in reducing power.

RESULTS AND DISCUSSION

5.1: Phytochemical screening

Table no- 1: Phytochemical assay results

Sl.No	Phytochemicals	*Cucurbita maxima*	*Hibiscus Rosa sinensis*	*Acorus calamus*	*Moringa oliefera*
1	Tannins	+ve	+ve	+ve	+ve
2	Saponin	+ve	+ve	+ve	-ve
3	Flavonoids	+ve	+ve	-ve	-ve
4	Cardiac glycosides	+ve	-ve	+ve	+ve
5	Steroids	+ve	+ve	+ve	+ve
6	Terpenoids	+ve	+ve	+ve	+ve
7	Carbohydrates	-ve	-ve	+ve	-ve
8	Phenols	+ve	-ve	-ve	-ve
9	Proteins	+ve	+ve	+ve	-ve

The phytochemical screening of the methanolic extracts of *Cucurbita maxima*, *Hibiscus rosa sinensis, Moringa oliefera* and *Acorus Calamus* showed that the leaves are rich in, alkaloids flavonoids, tannins, saponnins and glycosides; and also possess antibacterial properties as well as physiological activity [Sofowora, 1993; Ekam and Ebong, 2007]. Flavonoids are most commonly known for their antioxidant activity. They are transformers which modify the body's reactions to carcinogens, viruses, and allergens. They show anticancer, anti-inflammatory, antimicrobial and anti-allergic activity [Balch and Balchi, 2000; Ekam and Ebong, 2007], and may be useful in therapeutic roles [Jisika *et al*, 1992]. Alkaloids are organic compounds that contain nitrogen, and are physiologically active with

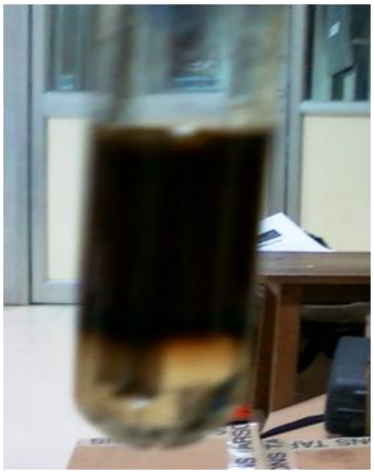
sedative and analgesic properties. They are used in relieving pains, anxiety and depression [Jisika *et al*, 1992].

Saponin

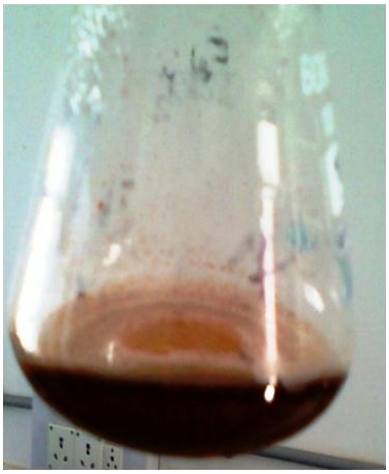
tannins

Terpenoid

protein



Cardiac glycosides



carbohydrate

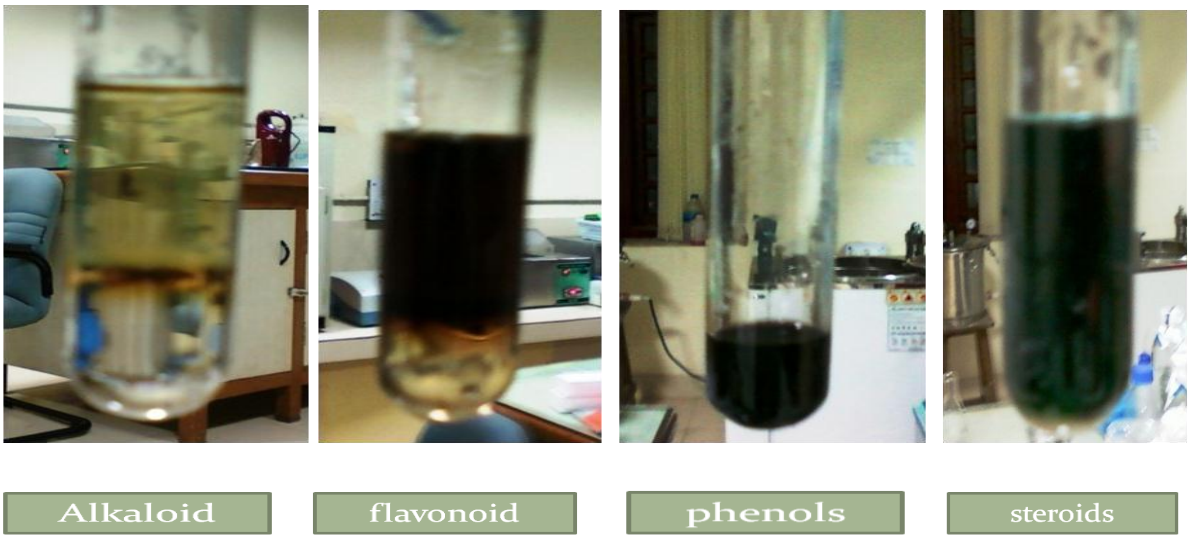


Fig 8: Phytochemical qualitative test

Alkaloids are toxic due to their stimulatory effects, leading to excitation of cells and neurological dysfunction [Obochi, 2006; Ekam and Ebong, 2007]. Glycosides are compounds containing a carbohydrate and non-carbohydrate residue in the same molecule. In these compounds, the carbohydrate moiety is attached by an acetal linkage of carbon-1 to the aglycone. They all contain steroid as aglycone component in combination with sugar molecules. They are important in medicine because of their action on heart and are used in cardiac insufficiency [Balch and Balch, 2000]. Thus, cardiac glycosides are drugs and can be used in the treatment of congestive heart failure and cardiac arrhythmia. They work by inhibiting the Na+/Na+ pump, resulting in an increase in the levels of sodium ions in the myocytes, which then leads to a rise in calcium ions level. This inhibition raises the amount of Ca2+ ions available for concentration of the heart muscle, increases cardiac output and reduces distension of the heart [Bertorello *et al*, 1990; Clausen and Nielson, 1994; Beltowski et al, 1998]. These glycosides are found as secondary metabolites in several plants and animals [Clausen, 1996; Beltowski *et al*, 1998]. However, some glycosides, such as ouabain, are toxic as it inhibits active transport of Na+ in cardiac muscle (sodium pump inhibitor), which results in inhibition of translocases during electron transport chain, and leading to death [Beltowski *et al*, 1998]. Also phloridzin (toxic glycosides) blocks the transport of sugar across the mucosal cells of small intestine and also renal tubular epithelium; it displaces Na+ from the binding sites of carrier protein and prevents the binding of sugar molecule and produces glycosuria [Clausen, 1994; Gloor, 1997; Beltowski *et al*, 1998].

Saponins are used in veterinary vaccines as adjuvant (e.g. foot-and-mouth disease vaccines) helping to enhance immune response. They are also mild detergents and can be used commercially as well as for research [Belch et al, 2000]. They can also be used in intracellular histo-chemistry staining to allow antibody access to intracellular proteins [Belch et al, 2000].

5.2: <u>Antibacterial activity</u>

Table no- 2: Antimicrobial activity of methanolic extracts.

Plant	*Escherichia coli*	*Pseudomonas fluorescens*	*Klebsiella pneumoniae*	*Bacillus subtilis*	*Proteus mirabilis*
Hibiscus rosa sinensis	-ve	-ve	-ve	-ve	-ve
Acorus Calamus	+ve	-ve	+ve	-ve	+ve
Moringa oliefera	+ve	+ve	+ve	-ve	+ve
Cucurbita maxima	-ve	-ve	-ve	-ve	-ve

Table no-3: Antimicrobial activity of Aqueous extracts.

Plant	*Escherichia coli*	*Pseudomonas fluorescens*	*Klebsiella pneumoniae*	*Bacillus subtilis*	*Proteus mirabilis*
Hibiscus rosa sinensis	-ve	-ve	-ve	-ve	-ve
Acorus Calamus	+ve	-ve	-ve	-ve	-ve
Moringa oliefera	+ve	+ve	+ve	-ve	-ve
Cucurbita maxima	-ve	-ve	-ve	-ve	-ve

Antibacterial activity of the aqueous and methanol extracts of *Hibiscus rosa sinensis, Cucurbita maxima, Acorus calamus and Moringa oliefera* were tested against the different test microorganisms are shown in the table no-2 & 3.

The methanolic extract of *Acorus calamus* showed antibacterial property against *Escherichia coli, Pseudomonas fluorescens, Klebsella pneumoniae* and *Proteus mirabilis.* The methanolic extract of *Moringa oliefera* showed its antibacterial activity against *Escherichia coli, Pseudomonas fluorescens, Klebsella pneumoniae* and *Proteus mirabilis.* The aqueous extract of *Moringa oliefera* showed the antibacterial activity against *Escherichia coli, Pseudomonas fluorescens and Klebsella pneumoniae.*

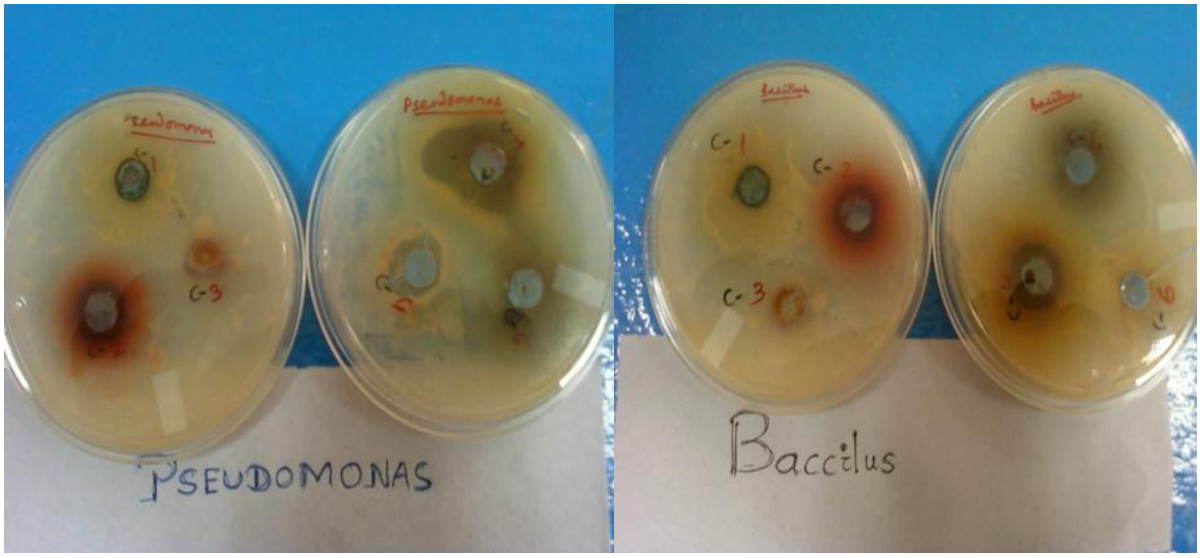


Fig 9: antibacterial activity of the methanolic extracts

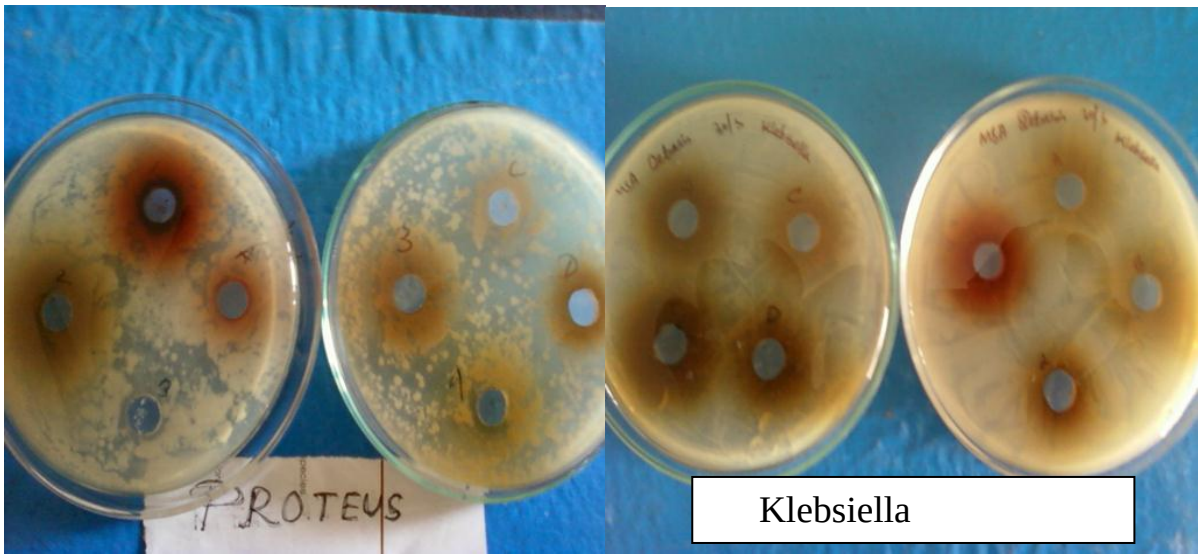


Fig 10: antibacterial activity of the methanolic extract.

From the primary screening, we have been able to identify that methanol extract of, *Moringa oliefera* and *Acorus Calamus* has got phytomedical property it may be due to the nature of biologically active compounds present in hibiscus whose activity are enhanced in the presence on methanol and also methanol has an stronger extraction capacity which could have produced greater number of active constituents responsible for antibacterial activity [Barker *et.al.,* 1995]. The antimicrobial activities can be enhanced if the active components are purified and adequate dosage determined for proper administration.

5.3: DPPH Assay

Table no 4: DPPH assay: Absorbance of the extracts at 517nm

Concentration (µg/ml)	**OD**				
	Ascorbic acid	***Acorus calamus***	***Hibiscus rosa sinensis***	***Cucurbita maxima***	***Moringa oliefera***
0	0	0	0	0	0
1	0.9762	0.9610	0.9891	0.99502	0.9832
10	0.8861	0.8664	0.8982	0.8810	0.9197
20	0.8021	0.7817	0.8207	0.7886	0.8291
30	0.6645	0.6733	0.6388	0.6961	0.6309
40	0.6485	0.6299	0.6626	0.6993	0.6553
50	0.5852	0.5708	0.6070	0.5531	0.5570
60	0.5364	0.5454	0.5291	0.5691	0.5794
60	0.4816	0.4714	0.5027	0.4616	0.5132
80	0.4252	0.4193	0.4415	0.4576	0.4332
90	0.3669	0.3566	0.3724	0.3727	0.3907
100	0.2932	0.2970	0.2771	0.2666	0.2867
200	0.1834	0.1633	0.1719	0.2046	0.2060
300	0.1634	0.1766	0.1649	0.1869	0.1871
400	0.1445	0.1443	0.1302	0.1671	0.1290
500	0.1188	0.1188	0.1409	0.1256	0.1330
600	0.0965	0.1037	0.1290	0.0859	0.1156

Table no- 5: Percentage of scavenging activity of *Acorus calamus*

Concentration (µg/ml)	% of scavenging activity	
	Ascorbic acid	*Acorus calamus*
0	0	0
1	34.26	35.28
10	40.632	41.65
20	45.98	47.356
30	55.2525	54.659
40	56.2963	57.58
50	60.59	61.65
60	63.876	63.27
70	67.565	68.25
80	71.365	71.758
90	75.29	75.98
100	80.25	79.998
200	87.6431	89
300	88.9899	88.102
400	90.2693	90.278
500	92	92
600	93.5	93.012

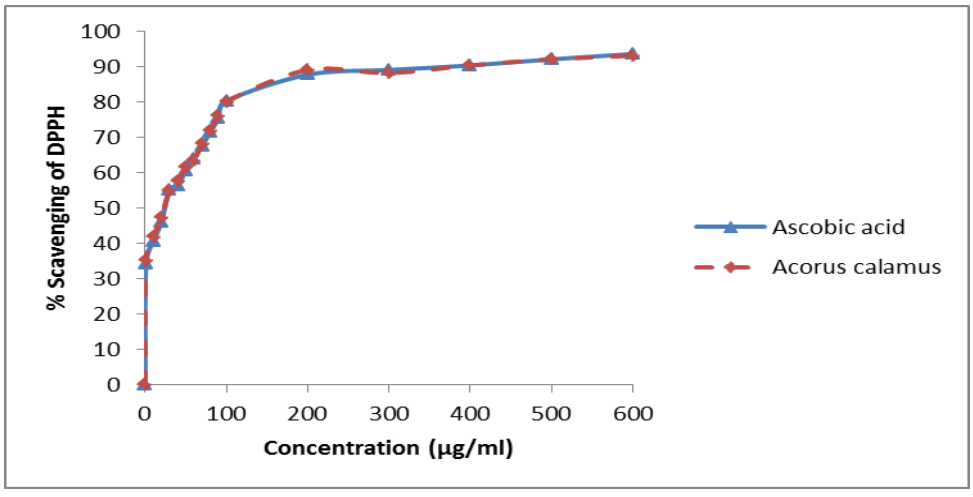


Fig 11: Radical scavenging activity of *Acorus Calamus*

Table no- 7: Percentage of scavenging activity of *Hibiscus rosa sinensis*

Concentration (µg/ml)	% of scavenging activity	
	Ascorbic acid	***Hibiscus rosa sinensis***
0	0	0
1	34.26	33.39
10	40.632	39.51
20	45.98	44.73
30	55.2525	56.982
40	56.2963	55.134
50	60.59	59.12
60	63.876	64.369
70	67.565	66.148
80	71.365	70.265
90	75.29	74.92
100	80.25	81.34
200	87.6431	88.421
300	88.9899	88.89
400	90.2693	91.23
500	92	90.512
600	93.5	91.31

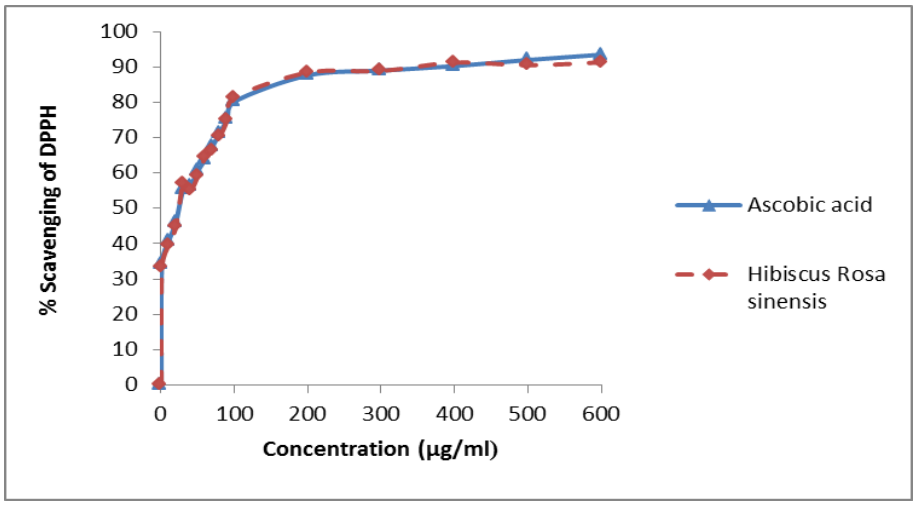


Fig 12: Radical scavenging activity of *Hibiscus rosa sinensis*

Table no-8: Percentage of scavenging activity of *Cucurbita maxima*

Concentration (µg/ml)	% of scavenging activity	
	Ascorbic acid	***Cucurbita maxima***
0	0	0
1	34.26	32.981
10	40.632	40.673
20	45.98	46.892
30	55.2525	53.12
40	56.2963	54.9234
50	60.59	62.754
60	63.876	61.672
70	67.565	68.9132
80	71.365	69.1842
90	75.29	74.899
100	80.25	82.0435
200	87.6431	86.219
300	88.9899	87.409
400	90.2693	88.7421
500	92	91.542
600	93.5	94.21

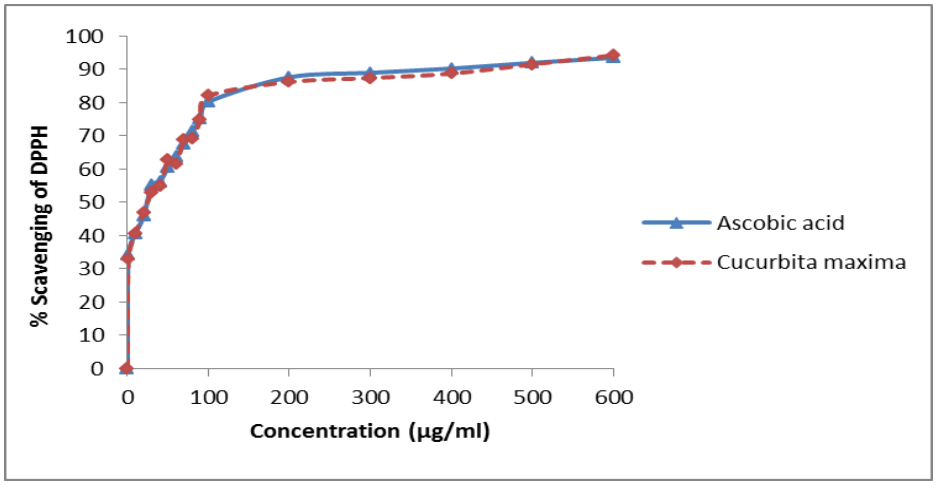


Fig 13: Radical scavenging activity of *Cucurbita maxima*

Table no-9: Percentage of scavenging activity of *Moringa oliefera*

Concentration (µg/ml)	% of scavenging activity	
	Ascorbic acid	***Moringa oliefera***
0	0	0
1	34.26	33.789
10	40.632	38.061
20	45.98	44.162
30	55.2525	57.509
40	56.2963	55.87
50	60.59	62.49
60	63.876	60.981
70	67.565	65.441
80	71.365	70.824
90	75.29	73.687
100	80.25	80.691
200	87.6431	86.127
300	88.9899	87.399
400	90.2693	91.307
500	92	91.04
600	93.5	92.21

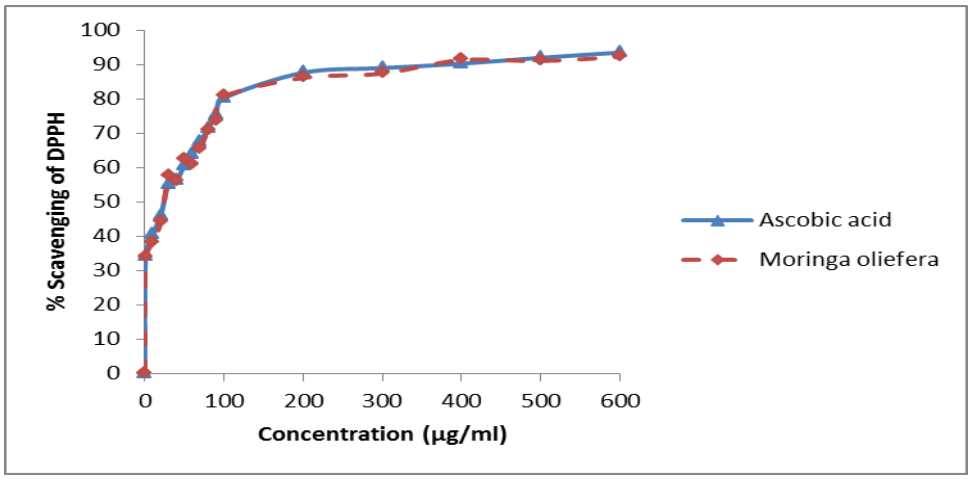


Fig 14: Radical scavenging activity of *Moringa oliefera*

DPPH test is based upon the ability of DPPH, a stable free radical, to decolourize from purple in the presence of antioxidants. It is a direct and dependable method for determining the radical scavenging action. Ascorbic acid was chosen as the standard antioxidant for this test. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for a noticeable deep purple color. When DPPH accepts an electron donated by an antioxidant compound the DPPH becomes colourless, which is quantitatively measured from the changes in absorbance. Highest scavenging was observed with *Acorus calamus* followed by *Moringa olifera.*

Scavenging activity of DPPH radical was found to rise with increasing concentration of the extracts. Additionally, it has been determined that the antioxidant effect of plant products is mainly due to radicalscavenging activity of phenolic compounds such as flavonoids, polyphenols and tannins [Rahman et al, 2007]. The antioxidant activity of phenolic compounds is mainly due to their oxidation reduction properties, which can play an important role in adsorbing and neutralising free radicals, reducing singlet and triplet oxygen, or decomposing peroxides [Hasan *et al.,* 2008]. Oxidative injury now appears as the fundamental mechanism causing a number of human neurologic and other disorders such as autoimmune pathologies, inflammation, viral infections and digestive system disorders including gastrointestinal inflammation and ulcer [Aruoma, 2003]. The present results suggest that all the tested plant extracts have moderate to potent antioxidant activity. Subsequently a variety of constituents are known from the four crude extracts which we studied therefore, it becomes very difficult to ascribe the antioxidant properties selectively to any one group of constituents without further studies it is impossible. Thus further thorough investigations are necessary.

5.4: Reducing antioxidant test

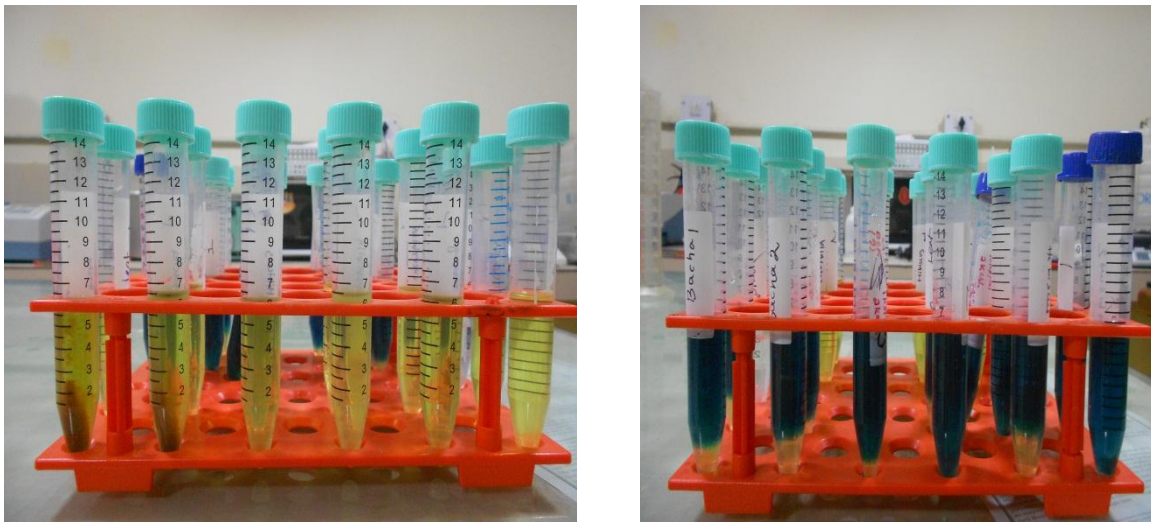


Fig 15- Reducing antioxidant test

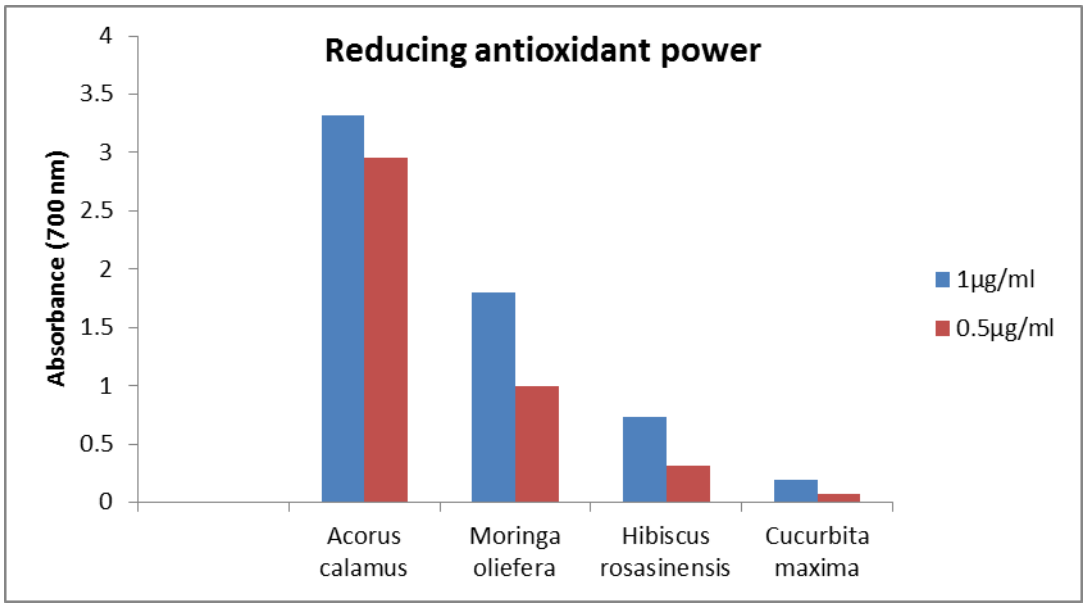


Fig 16: Reducing antioxidant power activity.

With the increase in absorbance there is increase in reducing antioxidant power. So with high amount of extract there is increase in absorbance and with the decrease in extract quantity the absorbance is lowered.

CONCLUSION

Phytochemical analyses as shown in table no 1 revealed that methanolic extracts contain tannins and flavonoids which have been reported to be responsible for the antimicrobial properties in some plant extracts and as a result, it could serve as antimicrobial agents for the treatment of microbial infections.

The antioxidant activity of various medicinal plants can be determined precisely, conveniently, and quickly using DPPH testing. The development in antioxidant activity obtained by using the DPPH method is comparable to trends found using other methods reported in the literature. This method can be used effectively for compact samples without prior extraction procedure and concentration problems, which saves time. The reaction time of four hours and a temperature of 35°C facilitate the extraction and reaction of antioxidant containing compounds with DPPH. Antioxidant activity measured using DPPH accounts partly for the bound and insoluble antioxidants. Relative antioxidant content provides a sign of significance of each of the phytochemical. Antioxidant activity and nutritional labelling data including vitamins, fibres, and minerals will aid in the interpretation of clinical results obtained as various plant products are tested in biological models for chronic disease.

Mostly in many cases the leaves are taken for any research work but here we used the petals of *Hibiscus rosa sinensis* and *Cucurbita maxima* which have very mild antibacterial activity against the pathogens but the leaves of *Moringa oliefera* and the rhizome of *Acorus calamus* showed very potent antibacterial property along with it these two plants have a very good antioxidant and reducing power activity. So we lastly conclude that the leaves of *Moringa oliefera* are medicinally very active against microorganisms and free radicals formed during oxidative stress as seen by their strong phytochemical constituents followed by the rhizome of *Acorus calamus* which has mild antibacterial property but showed good reducing power activity which is dose dependent.

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