Antimutagenicity of the grass, Cynodan dactylon

By

Chandra Prabha D.

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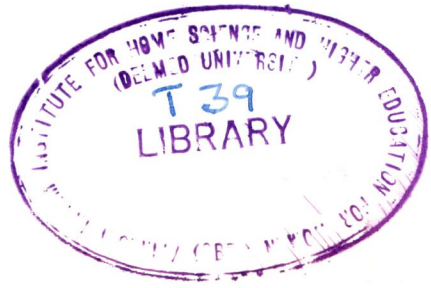

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INTRODUCTION

Cancer is a complex biological phenomena resulting from damage inflicted on the genetic material of different types of cells.

Man is exposed to a multitude of environmental poisons, the global effects of which are difficult to pinpoint in individual cases which leads to different types of cancers [Renner, 1990]. The main human cancers are associated with complex life style related causative, enhancing and inhibiting factors [Weisburger,1991]. It is also becoming increasingly clear that dietary traditions and habits play an important role in the causation and development of a number of major human cancers [Doll et al 1981, National Academy of Sciences, 1982, Wynder et al. 1983].

Many chemicals that can reduce or eliminate the activity of man-made and naturally occuring mutagens in our environment have been identified. Some of these antimutagens occur naturally in food or elsewhere in nature, and others are synthetic. The inhibitory agents present in food are predominantly of plant origin [Watterberg 1983, 1985]. At present there is substantial evidence to show that chemical mutagenesis and carcinogenesis can be inhibited by a large number of naturally occuring compounds of plant origin [Ramel et al 1986].

Interestingly, many of these inhibitors are minor constituents of some of the commonly consumed vegetables, fruits, spices and beverages [Ames, 1983; Wattenberg,1985; Abraham et al 1986; Ramel et al 1986; Ito et al 1989]. In view of these findings, there is growing awareness of the fact that it may be possible to protect humans against the genotoxicity and carcinogenicity of environmental chemicals by manipulating the intake of dietary agents. In order to explore this possibility, there is immediate need to identify the commonly consumed food stuffs which exert a protective effect against a wide spectrum of environmental mutagens/carcinogens.

Consumption of plant foods result in the uptake of the green vegetable containing chlorophyll whose antimutagenic effects against a variety of environmental and dietary mutagenic substances have become apparent in the last few years [Lai et al, 1980, Ong et al, 1986]. Chlorophyll has been shown to be responsible for a high degree of the antimutagenic activity [Kimm et al, 1982]. Similarily, chlorophyllin, a man-made derivative of chlorophyll is also known to inhibit the mutagenic activity of various chemical agents [Hayatsu et al, 1988].

Edible plants and a variety of substances in them such as plant phenolic antioxidants have been reported to inhibit

or to enhance the carcinogenesis and mutagenesis in experimental animals [Newmark, 1987]. Carotenoids and retinol are reported to have pronounced antimutagenic actions [Busk and Ahlberg, 1983, Renner, 1985] as well as preventive effects on the development of cancer in different organs.

Plant foods including fruits and vegetables are good sources of dietary fibre and many are found to possess significant hypolipidaemic effects when included in diet. Composition of the dietary fibre in these food varies to a great extent. Hypolipidaemic effect of dietary fibre is known to be dependent upon the level of its different fibre constituents. Dietary fibre from different sources while asserting the hypolipidaemic action also affects the biological utilisation of diet [Agarwal and Chauhan, 1991].

Wattenberg [1983] reported that foods contain a large number of anticarcinogens and antimutagens which are known to inhibit cancer formation. Epidemiological studies showed that vegetarians have a lower risk of cancer than the non-vegetarians as green coloured raw vegetables had most of the polyphenols, aromatic isothiocyanates, methylated flavonoids, coumarin etc. that are known to inhibit the cancer formation.

The development of research in the area of antimutagens and anticarcinogens has been primarily devoted to the reduction of risk factor in humans. This has been done using

a variety of in vitro and in vivo test systems. This extensive work has led to the identification of a broad range of inhibitors of plant origin, belonging to over 20 different chemical classes [Gichner and Veleminsky, 1988; Hayatsu, 1982; Stich and Rosin, 1986; Wattenberg, 1985].

Nowadays, research is being carried out to test the antimutagenic effects of the various medicinal plants. One such medicinal plant is the grass Cynodan dactylon.

The grass, Cynodan dactylon is reported to reduce the body acidity, tones up the nervous system, purifies the blood, controls tuberculosis and leprosy. The extract of grass also controls the diabetes, asthma and blood pressure [Subramaniam, 1978]

Voluminous literature is available on the distribution of mutagens, comutagens and antimutagens in our environment and lifestyle-related processes. Since the present dissertation deals only with the grass Cynodan dactylon, a brief review of the literature available on the mutagens/co-mutagens/antimutagens present in plants is presented in the following chapter.

Review of Literature

II REVIEW OF LITERATURE

Many factors in our environment are potential causative agents of cancer. These include a broad spectrum of chemicals both naturally occuring and synthetic of both simple and complex structure. They are widespread in the air we breathe. the water we drink, the food we eat and regions in which we work and live. Also chemical exposure due to occupation or to drugs has been known to cause human cancers [Hiatt et al, 1977; Higginson,1979, 1980; Vainio et al., 1980; Weisburger et al, 1980; Wynder,1980; Doll and Peto, 1981; Higgenson and Muir, 1981; IARC, 1982].

Chemical carcinogens and mutagens represent a spectrum of agents that vary in their activity. Of all the chemicals that we are exposed to, a relatively small proportion have been tested to determine their cancer causing potential. Nearly 20% of them have been found to be tumorigenic in test animals [Maugh, 1978,a,b]. Data on more and more such chemicals are being made available day-after-day following in depth research worldwide.

Many epidemiological studies clearly indicate that 80% of human cancers are caused by environmental factors associated with food, water and air [WHO, 1963]. A large number of researh work show that 40% of cancer incidences

are directly or indirectly connected with malnutrition, dietary habits and life style. These factors may promote or prevent the cancer depending on the relative intakes and combination of nutrients and carcinogenic components of the diet [Wynder, 1984, Palmer,1985, Reddy and Cohen, 1987].

To protect our health from adverse effects of environmental mutagens, it is more important to evaluate the effects of modifying factors. It has been increasingly recognized that identification and elimination of carcinogens or co-carcinogens from the environment will play an important role in the prevention of cancer in humans. A variety of short-term test systems have now been made available through extensive research.

A brief review of the literature available pertaining to the present study is discussed under the following headings.

1. Introduction
2. Dietary factors that correlate with cancer
3. Plants as the source of food
   - 3.1 Plants as source of carcinogens/mutagens
   - 3.2 Plants as a source of procarcinogens/promutagens
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4. Mechanism of anticarcinogenesis.
5. Conclusion.

1. INTRODUCTION

A wide range of food stuffs exist in our environment which might contain both the mutagenic/carcinogenic and antimutagenic/ anticarcinogenic agents. Nowadays, much of the studies focus on the components of the environment that have been suspected to induce or enhance human cancers.

Recent investigations on the detection of natural and synthetic mutagenic factors by means of microbial assays revealed a number of environmental chemicals, especially those found in foods, may be adversely affecting human health [Nagao et al., 1977]. Less than deserving attention has been given to substances in the environment that may render protection against chemical mutagens or carcinogens acting as initiators in the carcinogenic process.

2 .DIETARY FACTORS THAT CORRELATE WITH CANCER

Important causative factors that have elicited keen interest among cancer investigators in recent years are diet and nutritional factors [Srinivas, 1990].

Diet, apart from being an important causative factor for cancer, also modulates cancer by several other mechanisms. For example, dietary fibre, protease inhibitors, certain vegetable constituents and antioxidants, modulate cancer either by themselves or synergistically.

According to Doll and Peto(1981) there are five possible ways or means by which diet may affect the incidence of cancer.

1) Ingesting powerful, direct-acting carcinogens or their precursors
2) Affecting the formation of carcinogens in the body
3) Affecting transport, activation or deactivation of carcinogens
4) Affecting promotion of cells that are already initiated, and
5) Overnutrition.

Nutritional deficiencies act as predisposing factors to cancer at particular sites in the body, by enhancing the tissue susceptibility for the carcinogens. Deficiency of vitamin A in the diet enhances the risk of lung, colon and urinary bladder cancers. Deficiencies of vitamin C and β-carotene enhance the risk of stomach cancer. Iron deficiency is associated with oesophageal cancer. A low selenium concentration in the diet is associated with incidence of colon, rectum and breast cancer. Magnesium deficiency enhances the risk of thymic lymphosarcoma in rats. Thus, nutrient deficiencies modulate carcinogenesis [Srinivas,1990].

Several studies have been carried out to investigate the effects of the nutrients on cancer. But since scope of the present dissertation is concerned with plants, literature pertaining only to plants are discussed below.

3. PLANTS AS A SOURCE OF FOOD

Studies carried out on populations from different parts of the world indicate that certain vegetables in the diet may have anti-carcinogenic properties [Sharma,1990; Weisburger,1991]. Low incidence of various types of cancer have been associated with increased consumption of different vegetables. For example, cancers of breast, colon and prostate are less frequent in people consuming legumes and cereals[Kroes et al.,1986] and those of stomach and oesophagus in populations eating a high amount of fruits and vitaminous vegetables [Joossens et al., 1985; Hoursan et al.,1986; Mirvish,1986; Munoz et al.,1988;Tsuda et al.,1988].

Low incidence of cancers of gastrointestinal and respiratory tract have been shown to be associated with increased consumption of cruciferous vegetables and those of intestine with vegetables containing a high amount of fibre, and those of larynx, oesophagus and lung with vegetables rich in carotene, like carrot, tomato and spinach [Koo, 1986; Gao,1987; Shibato et al.,1989]. Such dietary intervention is however, not always, effective since human diet is a mixture

of different compounds. Most components of the diet are of plant origin either directly or through animal products.

3.1 Plants as a source of carcinogens/mutagens

The plant food contains a great veriety of natural mutagens and carcinogens as well as many natural antimutagens and anticarcinogens. Many of these mutagens and carcinogens may act through the generation of oxygen radicals. Recent widespread use of short-term tests for detecting mutagens and the increased number of animal studies on plant substances [Kapadia,1982] have contributed to the identification of many natural mutagens, teratogens and carcinogens in the human diet [Miller et al., 1981].

Some plants have been found to induce cancer directly . For example, extracts of the plant Krameria ixina, and Annona muricata, when injected to the experimental animals resulted in the formation of fibrosarcoma [Sharma,1990]. Some aromatic oils [Croton oil, camphor oil and sassafras oil] extracted for medicinal, edible and cosmetic purposes have also been shown to be carcinogenic in mouse liver [Sharma,1990].

Safrole [1-allyl;3,4-methylene dioxybenzene] and estragole [1-allyl-4-methoxy benzene] are weak hepatocarcinogens which occur in certain spices and essential oils [Miller et al., 1983]. Studies have implicated that 1'-

hydroxy estragole and 1 - hydroxy safrole, the proximate carcinogenic metabolites of estragole and safrole were found to be mutagenic for S.typhimurium TA 100 in the absence of fortified liver microsomes while no mutagenicity was detected for safrole or eugenol with or without added NADPH-fortified liver preparations [Miller et al., 1979].

Psoralens, which have been detected in different parts of family Umbelliferae, were found to be potent photosensitizers and are highly mutagenic in the presence of activating long wavelength ultraviolet light. They readily intercalate into DNA strands where they form light-induced mono or di adducts with pyrimidine bases [Scott et al., 1976].

The mutagencity of a number of flavonoids and pyrrolizidine alkaloids occuring in edible plants has been reported [Shelby and Matsushima,1981]. Although the mechanism of carcinogenicity of quercetin [a flavonol] are not known, quercetin has demonstrated significant effects on DNA synthesis in neoplastic cells [Podhajcer et al., 1980].

Studies with four pyrrolizidine alkaloids [clivoxine, symphytine, senkirkine and petasitenine] yielded results when tested in an S9 mediated mutagenicity assay in TA 100 using the pour-plate technique and preincubation and were found to

be carcinogenic when fed to rats [Shelby and Matsushima,1981].

3.2. Plants as a source of promutagens / procarcinogens.

Many chemicals are not mutagenic or carcinogenic by themselves but are known to be biologically transformed into mutagens /carcinogens within the system. A very large number of chemicals are released into the environment which may be taken up by the plants. Within the plant the chemical may cause mutagenic damage to the plant itself leading to the formation of mutagenic /carcinogenic metabolites.

For eg., colchicine derived from the plant Colchicum autumnale, may be used for hampering cell division, but under certain conditions it can exert a completely different effect on cell division. Other plants which have shown such variable effects are 3 species of Brassica family, some members of the families Asteraceae and Fabaceae (Sharma,1990).

Mutagenicity of the aromatic amines 2 amino fluorence and 4 aminobiphenyl in vitro was inhibited by the plants flavonoid catechin, apparently by direct action with the proximate carcinogen (Steele et al,1985).

Lesca (1983) has claimed a potent protection effect of ellagic acid and lesser activity of ferulic and chlorogenic acids against B(a)P induced lung tumour formation in mice.

Several of the plant phenolics eg., caffeic, and ferulic acid are potent reactants with nitrite ion (Newmark and Mergens, 1981) and therefore have been demonstrated to be potent blockers of nitrosamine formation in vitro and in vivo (Kuenzig et al,1984). This property may be particularly important in preventing in vivo nitrosation in the stomach in areas of high nitrate intake.

Inhibition of carcinogenesis induced by non aromatic carcinogens may also be inhibited by plant phenolics. Mori et al, (1986) have shown an inhibiting effect of chlorogenic acid on methylazoxymethanol (MAM) acetate induced carcinogenesis in the colon and liver of hamsters. Mandal et al ,(1986) reported a preliminary study indicating that ellagic acid inhibited methylbenzylamine induced esophageal tumors in rats.

Several reports have indicated that ellagic acid inhibits binding of carcinogens to DNA. Del Tito et al,(1983) demonstrated potent hydroxylase in vitro, resulting in decreased DNA binding by benzo(a)pyrene.

Gold and Dixit (1986) demonstrated the efficiency of ellagic acid in inhibiting the mutagenic activity of the direct - acting mutagen N-methyl-N-nitrosourea (MNU). They suggested that ellagic acid has a definite affinity for DNA and acts only to inhibit 0-6 substitution and not N-7 by MNU.

3.3 Plants as a source of comutagen/cocarcinogen

One of the earliest known chemical cocarcinogen is croton oil from the seeds of the plant croton tiglium, (Co burn et al., 1980). Some naturally occuring cocarcinogens in food include citrus oils particularly D-limonene and (Sterculic and malvolic acids)cyclopropenoid fatty acids from cotton seed (Hendricks et al., 1980). Various phenols and their derivatives are also reported to be cocarcinogens (Kikuzaki and Nakatani, N. 1989).

Chilli and its pure alkaloid capsaicin and ginger and its phenolics gingeril and shogaol have been found to be mutagenic (Nagabhusan et al., 1987).

Phorbol esters present in herbal teas are reported to be the promoters of carcinogenesis (Hirayama and Ito, 1981).

Phorbol -12-myristrate-13-acetate a well known promotor is found to induce cancer in mouse (Kozumbo and Cerutti,1986).

3.4 Plant as a source of anticarcinogens/antimutagens.

Extracts of a number of plants including certain vegetables have been shown to have anticancer properties. These observations evoked a keen interest among scientists and resulted in large scale screening of higher plants for the isolation of anti cancer drugs in different centres all over the world. More than 3000 species of higher plants have been reported to have yielded one of more compounds, effective against various types of cancer (Sharma,1990).

Wood et al (1982a) showed in vitro that caffeic, chlorogenic ferulic amd ellagic acids, particularly the latter, are potent inhibitors of mutagenesis against benzo(a)pyrene diol epoxide, in strain TA 100 of S.typhimurium assay and also in a mammalian cell assay.

Huang et al (1983a) reported the comparative inhibition of the mutagenicity of polycyclic aromatic hydrocarbons by 27 plant flavonoids Tannic acid and several hydroxylated anthraquinone and cinnamic acid derivatives, all repesentative of plant phenols present in human foods, were found to be inhibitors of the mutagenicity of polycyclic aromatic hydrocarbous (Huang et al,1985)

Teel (1986), suggested that ellagic acid binds covalently to DNA and ellagic acid could inhibit

benzo(a)pyrene binding to DNA in some rat tissues, positively by lowering benzo(a)pyrene metabolism to its active metabolites.

Su and Coworkers(1986) screened 230 traditional medicinal plants for their antioxidant activity in relation to their medicinal effects and found 22 plants with stronger activity. The antimutagenic chemicals present in food products belong to several chemical groups such as vitamins (Guttenpaln,1977; Lo and Stich,1978; Baird and Birnbaum, 1979; Rosin and stich, 1979; Shamberger et al.,,1979, Busk and Ahlborg, 1980, Osawa et al.,,1980, Thorgeirsson et al., 1980, Busk and Ahlburg, 1982, Calle and Sullivan, 1982; Wood et al., 1982(b), tracemetals (Jacobs et al.,, 1977, Rosin and stich, 1979; Shamberger et al 1979, Calle and Sullivan, 1982, Teel and Kain, 1984), aminoacids and their derivatives (Moriya et al, 1978; Rosin and Stich, 1978,; De Flora et al., 1984; Wilpart et al., 1985) fatty acids,(Hayatsu et al, 1981), pyrrole pigments (Arimato et al 1980,a,b) and plant phenolics (Wood et al., ,1982(b), Huang et al., 1983 b, Steele et al, 1985).

Plant foods are excellent sources of vitamins and minerals . Also they provide adequate amounts of flavonoids, ellagic acid, indole and other agents that contribute to increased levels of enzymes that can detoxify carcinogens

including hydroxy radicals and reactive oxygen. Plants also serve as good sources of compound that lower risk of cancer by inhibiting cell duplication processes and favour differentication.

VITAMIN A

Vitamin A levels have been determined in prospective and retrospective investigations and the risk of cancer of the lung, kidney, urinary bladder and breast was lower in individuals with higher plasma levels (De luca et al, 1981, Kvale et al, 1983, Koo,1986, Moon,1988, Rogers et al 1983, Bendich, 1989, Moon,1989, Ziegler, 1989).

Several in vivo and in vitro studies suggest that retinoids can prevent or limit carcinogenesis at different sites.(Sporn, et al,1976). In experimentally induced tumours in animals Vitamin A and its derivatives have been shown to have a protective action, in tumours of the salivary gland of rats (Alam et al, 1984) in the skin of hairless mice (Bollag, 1974; Mathews-Roth, 1982), in the bladder and mammary gland (Sporn and Newton, 1981, Sporn, 1983) gastro intestinal tract and in oral mucosa of hamsters (Shklar et al, 1980).

β- CAROTENE.

β- Carotene, the essential precursor to vitamin A is an excellant antioxidants and radical trapping agents,

especially for peroxyl and hydroxy radicals. β-carotene is considered to be one of the most important anticarcinogens in the diet(Burton, and Ingold, 1984). It protects animals against the carcinogenic effects of ultraviolet light alone and in combination with psoralen (Korhhauser et al,1986), at both the initiation and the promotion stage (Suda et al,1986) β-carotene amd similarpolypyrenes are present in carrots and in all food that contains chlorlphyll. They appear to be the plants main defense against singlet oxygen generated as a by product from the interaction of light and chlorophyll.

VITAMIN C

Vitamin C, their derivatives and other blocking agents have been tested in a variety of foods with ultraviolet radiation, benzo(a) pyrene and nitrite (forming nitroso carcinogens). It may be inversely associated with human uterine cervical dysplasia. Vitamin C is thought to account in part for the reduced risk of cancer due to endogenous production of carcinogens from nitrites (Correa, 1982, Tannenbaum et al, 1991) Okshima and Bartsch (1982) have demonstrated that nitrosamines could also be formed within the digestive tract Vitamin C and their derivatives have been tested in a variety of foods and found to be effective in inhibiting nitrosamine formation.

National Research council (1982) has recommended adequate amounts of Vitamin C to reduce the risk of cancer. Also studies have shown that adequate levels of Vitamin C in the diet serve to react with endogenously produced nitrite in individuals consuming appreciable amounts of nitrate, either from food sources or where the drinking water contains appreciable levels of nitrate (Kosaka et al, 1989).

VITAMIN E.

Being a major free radical trap in biomembranes, vitaminE has a protective effect against carcinogenesis induced by radiations. Vitamin E destroys nitrite, an essential component in the food chain associated with the production of cancer (Bright-See,1983; Mirvish, 1986, Chen et al, 1988) Vitamin E especially together with vitamin C is an excellant nitrite trapping agent.

SELENIUM

Selenium is considered to be one of the most powerful antimutagenic and anticarcinogenic agents so far known. (Griffin, 1982; Vernie, 1984; Shamberger, 1985; Hocman, 1988). Dietary selenium requirements at controlled levels above minimum requirements has been correlated with anticarcinogenic properties in both laboratory animal studies and human epidemiologic investigations (Chortyk and Schlotzhaver, 1984).

However, the anticarcinogenic effect of selenium has been questioned by many contradicting reports. Low concentrations of selenium may be a risk factor in human cancer. Selenium influences some DNA repair mechanisms by decreasing their effectiveness, thereby potentiating the mutagenic activity of some chemical compounds. Thus, under certain conditions, the chemopreventive agent may also act a comutagen (Balansky, 1991).

GLUTATHIONE

Glutathione is present in food and is one of the major antioxidants and antimutagens in the soluble fraction of cells. The glutathione transferases are major defenses against oxidative and alkylating carcinogens (Warholm, et al, 1981). Glutathione with its reactive free thiol group is a highly potent blocking agent (Chasseaud, 1976), Dietary glutathione has been shown to be an effective anticarcinogen against aflatoxin (Novi, 1981).

Glutathione functions in many cellular processes, including catalysis, transport and reductive phenomena. It functions to protect cells against toxic compounds of endogenous and exogenous origin. Together with glutathione-s-transferase it catalyses the conjugation of a whole range of xenobiotics and hence plays a critical function on

detoxification and cellular protection.(Shallom and Chitinis,1990).

The glutathione is abundant in all organs. The glutathione transferases are most active in the liver, (Grover,1977). Many electrophilic species generated by phase I metabolism of carcinogens have been shown to be conjugated with glutathione in both in vivo and in vitro experiments (Jernstrom et al., 1982).

DIETARY FIBRE

Dietary fibre consists of plant cell walls and components of these walls vary widely in structure and composition depending on cell type, plant species and stage of development (Bacic et al.,,1988). Epidemiological studies indicate that certain types of dietary fibre may protect against the development of colon cancer (Burkitt, 1978; Willett, 1989). The dietary fibre may adsorb certain mutagens or cancer promoters in the digestive tract and these are carried out of the body adsorbed onto undigested dietary fibre. Thus the effective concentrations of these substances available to initiate or promote cancerous changes in the gut mucosal cells are lowered. Certain mutagens and cancer promoters have been shown to adsorb onto a range of dietary fibres in vitro (Smith - Barbaro et al, 1981; Barnes et al,1983; Kada et al,1984; Takeuchi et al, 1988; Roberton et

al, 1990). Recent studies have shown that dietary fibre protects the bowel by adsorbing cancer initiating compounds. (Ferguson et al, 1990).

PLANT PHENOLOCS

Plant phenolics are usually widely distributed in the food supply if the plant foods are utilised shortly after harvesting.

Wargovich (1985) using a short term assay in mice of aberrations of colon epithelial cell nuclei to detect DNA damaging effects of initiating carcinogens found that oral feeding of several plant phenolics (Ferulic acid , Ellagic acid, Curucumin and Quercetin) failed to inhibit 1,2-dimethylhydrazine genotoxicity (Wargovich and Newmark,1983). However, when B(a)P was administered intrarectally, the oral administration of the plant phenols Caffeic, ferulic and ellagic acid and quercetin produced significant inhibition of nuclear aberrations (Wargovich et al., 1985).

Tannic acid and several hydroxylated anthraquinone and cinnamic acid derivatives, were found to be potent inhibitors of carcinigenesis (Huang et al, 1985.)

Several of the plant phenolics eg., Caffeic and feuulic acids are potent reactants with nitrite ion (Newmark and Mergens, 1981) and therefore have been demonstrated to be

effective blockers of nitrosamine formation in vitro and in vivo (Kuenzig et al, 1984).

PYRROLE PIGMENTS

Pyrrole pigments were found to inhibit mutagenesis induced by certain classes of carcinogens. Arimato and Co workers [1980 a,b] showed that hemin, biliverdin, chlorophyllin and protoporphyrin were effective in inhibiting the activity of a series of mutagens in expriments using the Ames Salmonella/ microsome assay.

Chlorophyll and copper chlorophyllin have also been shown to be effective in inhibiting mutagenesis induced by the carcinogens(Lai,1979;Lai et al, 1980]. Chlorophyll ingested in human food is essentially not absorbed. The chemical changes of chlorophyll induced by the monogastric mammaliam digestive system produce a molecule more closely resembling chlorophyllin. This suggest that digestive processes may play a part in activating ingested chlorophyll to an effective blocker of mutagenesis within the lumen of the lower gastrointestinal tract.

4. MECHANISM OF ANTICARCINOGENSIS IN PLANTS

Anticarcinogenic properties of plant extracts have been suggested to be due to antimutagenic properties of the active principles occuring in these plants. The extracts act at

different levels of cell division and may lead to the formation of cancer at the stages of initiation, promotion and progression. Plant extracts with antimutagenic activities are often anticarcinigenic as well.

In understanding the inhibition of cancers, both the modes of action and of detoxification of the known carcinogens and mutagens have to be taken into account. Xenobiotics [both carcinogens and mutagens]are metabolised in two stages

1) Biotransformation of phase I metabolism

2) Conjugation of phase II metabolism

During detoxification of toxic agents the oxidative reaction of phase I result in the formation of metabolites which subsequently undergo conjugation at phase 2 by enzymes like epoxide hydrolase or gluthathione transferase to form conjugates. The later are then eliminated from the body.

In contrast, activation of the chemical leads to the formation of proximate carcinogens or reactive intermediates. The later are poor substrates for the conjugating enzyme. Therefore the reactive intermediates undergo a non enzymatic interaction with intracellular constituents like proteins RNA and DNA. Such covalent binding leads to the formation of neo antigens , mutation, cancer induction and subsequent cell death. The two possible alternative routes of oxidative bio

transformation resulting in detoxification or activation indicate two different modes of oxygenation.

Two families of enzymes are responsible for the alternate pathways. The cytochromes P_{450} result mainly in detoxication while the cytochromes P_{448} flavoprotein mono oxygenases and non enzymic free radical hydroxylations result in oxidative activation.

In protecting against cancer, the plant products may act at different stages of carcinogenesis and through various interactions.

Extracellular protection can be given by the inhibitor by acting on the carcinogen during the first stages of carcinogensis. The penetration of the carcinogen into the cell may be hindered as done by putresin, fatty acitds and aromatic amino acids. Toxicants or carcinogens already present may be removed by fibrous plant products through

i) binding by fibres of initiators and promotors

ii) modification of their mechanism and

iii) increase in faecal bulk.

The endogenous formation of carcinogens can also be hampered. The reaction between the nitrite and nitrosatable amines or amides in the acidic gastric environment gives rise to N-nitroso compounds a majority of which has been found to

be carcinogenic. This reaction has been inhibited by complex mixtures including tea and coffee. Vegetables and fruits may be attributed to the presence of vitamin C and E and natural phenolics in these plant products.

Activation of mutagens in the alimentary tract is mediated by enzymes with peroxidase and NADPH- oxidase activities present in vegetables like cabbage and broccoli and also by plant extracts containing thiols and various anti-oxidants.

In stage II of cancer formation, anti-cancer activity has been shown by several plant products acting inside the cell. These agents may modulate the mechanism by (i) inhibiting cell replication (ii) sequestering mutagens in non-target cells so as to render them inactive as well as by (iii) inhibiting activation of promutagens and (iv) inducing detoxication.

The principle reactive groups are retinoids, thiols and phenolic compounds. The reactive molecules of carcinogens can be blocked from acting with nucleophilic sites of DNA by reacting with the active electrophiles (as by sulphur compounds) or by scavenging reactive oxygen (as antioxidants) or through directly binding with those sites (eg. Ellagic acid and retinoids).

Some plant products may increase the repair of DNA damage thus reducing the chances of carcinogenesis (cinnamaldehyde, coumarins, Umbelliferons, vanillin and thiols) while others (beans) may inhibit error prone DNA repair as shown by protease inhibitors.

After the formation of neoplastic cells, promotion of tumour can be prevented in addition by scavenging free radicals (antioxidants) inhibiting cell proliferation and inducing cell differentiation (retiniods, glucocortcoids and vitamin D_3). Progression of tumours may further be hampered by plant harmones and protease inhibitors acting on growth factors and immunoregulators like retinoids, acting on the immune system.

5.CONCLUSION

The aforemention brief review strongly supports the protective role that plants and /or individual components isolated from them could play in rendering protection against grave diseases including caner. More attention should be focussed on the potential anticarcinogenic properties which could be residing in plants which are known to have medicinal properties. One such plant, most widely distributed, is the grass, Cynodan, dactylon, commonly referred to as Arugampul.

Arugampul is recognised to have medicinal properties and is used asa folk remedy against various disorders. It is reported to reduce the body's acidity, tone up the nervous system, purify the blood, control diabetes, asthma and blood pressure (Subramaniam, 1978). In the present dissertation it was undertaken to study the antimutagenic properties, if any, of this grass, using a battery of short term test systems. Biochemical evaluation to support the antimutagenic studies were also conducted alongside.

The various methods employed for these are discussed in detail in the following chapter.

Experimental Procedure

Preparation of the extracts.

Since the mutagenicity/ antimutagenicity of the grass, Cynodan dactylon, is fairly unexplored, it was decided that different extracts of grass can be prepared and tested. The various extracts prepared for the present study are as follows:-

Water extract:-

10 g of the washed and blotted dry grass was cut into small pieces and homogenized in sterile water. The homogenate was stored in the dark at 4° C for 72 hours with occasional shaking to facilitate complete extraction. After 72 hours, the contents were filtered through cotton guaze into a pre-weighed container. The resultant filterate was freeze-dried in a lyophiliser. The residue was again weighed with the container and the difference in weight calculated.

Saline extract:-

10 g of the grass which was washed and wiped dry was cut into small pieces and homogenized in physiological saline solution (pH 7.0). The homogenate was stored in the dark at 4° C for 72 hours with occasional shaking to ensure complete extraction. After 72 hours, the contents were filtered through cotton guaze into a pre-weighed container. The resultant filterate was freeze-dried in a lyophiliser. The

residue was again weighed with the container and the difference in weight calculated.

Organic extract:-

In order to check whether the components of the grass which are lipid soluble showed a mutagenic / antimutagenic effect, an organic extract was prepared.

10 g of dried grass which was blotted dry, cut into small pieces and homogenized using petroleum ether (40° - 60° boiling range). The whole of the homogenate was carefully transferred into a flask and stored in the dark at 4°C for 72 hours with occasional shaking of the contents. After 72 hours, the contents were filtered through cotton guaze into a pre-weighed container. The resultant filterate was allowed to evaporate at room temperature. After the evaporation of the ether, the residue was again with the container and the difference in weight was calculated.

Acid extract:-

It is well known that some mutagens are converted to their active mutagenic form in the stomach, under the acidic conditions. In the present study, we also explored such a possibility by preparing an acid extract of the grass.

10 g of dried grass which was wiped dry, was cut into small pieces and then homogenized using 0.1N Hcl(pH 1.8).

The whole of the homogenate was carefully transferred into a flask and stored in dark at 4°C for 72 hours with occasional shaking of the contents. After 72 hours the contents were filtered through cotton guaze into a pre-weighed container. The resultant filterate was neutralised with 0.1N sterile sodium bicarbonate and the extract was freeze-dried in a lyophilizer. The residue was again weighed with the container and the difference in weights were calculated.

The water, saline and acid extracts were dissolved in sterile distilled water, while the organic extract was dissolved in sterile dimethyl sulphoxide (DMSO) to give the required concentration.

2. Bacterial mutagenicity assay.

Bacterial strains of Salmonella typhimurium TA 98 and TA 100 were obtained from Prof. B.N. Ames, Biochemistry department, University of California, Berkeley, California, U.S.A.

The standard mutagens 4- Nitrophenylene diamine (4-NPDA) and Benzo(a)pyrene (B(a)P) were a king gift from Dr.(Mrs.) S.V. Bhide, Head, Carcinogenesis Division, Cancer Research Insititute, Bombay, India.

Principle:-

A set of histidine - requiring strains is used for mutagencity testing. These strains are incapable of growth in the absence of histidine in the growth medium. When a mutagen is added to the culture, the strain is mutated back, thereby losing the histidine - dependence for its growth. The number of revertant colonies resulting after the action of mutagen depends on the potency of the compound.

The type of mutation in the histidine operon in the strains are different, thereby enabling the identification of frameshift mutagens (those which are mutagenic towards TA 98) and base-pair substituting mutagens (those which are mutagenic towards TA 100).

The properties of the tester strains and the genotype confirmation tests are listed in Appendix - I.

Spontaneous Reversion.

Spontaneous reversion of the tester strains to histidine independence is measured routinely in mutagenicity experiments and is expressed as the number of spontaneous revertants/ plate. Each tester strains reverts spontaneously at a frequency that is characteristic of the strain.

Each mutagenicity assay conducted included control plates without the test compounds to assess the Spontaneous

Revertant (SR) frequency. The SR frequency was approximately close to the number specified by Ames (Ames et al.,1975; Maron and Ames, 1983).

Metabolic activation system:-

The S-9 fraction of the liver homogenate from mouse induced with phenobarbital, along with cofactors was uses as the metabolic activation system (Appendix -II).

Testing the mutagenicity of the extracts.

For the mutagenesis assay, the minimal glucose agar, Vogel Bonner (VB) salt solution (25x), Top agar and Histidine -Biotin solution were prepared and stored as described in Appendix -III.

Assay procedure.

To 2 ml of the molten top agar (45°C) 0.1 ml of overnight culture and 0.1 ml of test compound (containing various concentrations of the compound) were added in succession. The contents were mixed gently and thoroughly and poured over the basal agar and spread evenly. After the top agar had solidified, the plates were incubated inverted at 37°C for 48 hours. At the end of 48 hours, the number of histidine - revertant colonies were counted. Whereas in the presence of S-9 mix, all the above procedures were same except that 0.05 ml of S-9 mix was added along with the test compound and the assay was carried out as above.

Each assay included 5 sets of plates as follows:-

a) The overnight grown cultures were serially distrubuted and known amounts of these cultures were plated on nutrient agar plates to determine the number of viable bacteria in the inoculum. The plates were incubated at 37°C overnight and the number of colonies were counted.

b) The spontaneous revertants in the inoculum were determined as above, with the exception that 0.1 ml of the culture was added to the top agar before pouring onto the plate.

c) The number of revertants induced by the test substance was determined by plating 0.1 ml of the culture and 0.1 ml of appropriate concentration of the test compound on minimal agar plates supplemented with trace amounts of histidine.

d) The number of spontaneous revertants in the presence of S-9 mix were determined as in the above step with 0.05 ml of S-9 mix.

e) The number of spontaneous revertants due to the addition of S-9 mix were determined by adding 0.1ml culture and S-9 mix.

After counting the number of viable bacteria in the inoculum, the number of induced revertants were finally converted per 2 x 10^8 bacterial cells and this was referred to as revertants per plate in subsequent results (Maron and Ames, 1983).

Each concentration was tested in 4 plates and the results are expressed as Mean $\pm$ SD of 4 plates. The results of the Ames test are expressed as revertants/ plate from 2 x 10^8 bacterial cells.

Testing the co-mutagenicity / antimutagenicity of the extracts.

The co-mutagenicity /antimutagenicity of the extracts were done both in the presence or absence of the metabolic activation mixture. The testing can be done as follows:-

The standard mutagens - that is, sodium azide for TA 100 and 4-NPDA (4-Nitrophenylene diamine) for TA 98 in the absence of S-9 mixture and benzo(a)pyrene for both strains in the presence of S-9 mixture were plated along with the grass extract and the test was carried out as described in the mutagenicity assay (Appendix-III).

The concentrations of standard mutagens used were as follows:-

4-NPDA - 10 µg/plate.

Sodium azide - 1 µg/plate

Benzo(a)pyrene - 1 µg/plate

The results of the Ames test are expressed as revertants /plate from 2×10^8 bacterial cells.

3. Mammalian clastogenic assay - Micronucleus test.

Following the bacterial assay, the grass extracts were tested for their clastogenic property in an in vivo mammalian system. This effect was tested by studying the micronucleus inducing capacity of the extracts in the bone marrow of Swiss Laca mice. This study also comprised of two phases.

a) Testing the clastogenic effect of the grass extracts.

b) Testing the coclastogenic/anticlastogenic effect of the grass extracts.

Animals:-

The experiment was conducted on the Swiss Laca male mice. purchased from Pasteur Institute of India, Coonoor. The animals were acclimatized for two weeks in our laboratory.

8-10 weeks old mice, weighing about 23 - 25 g were selected for the assay.

Principle

Micronucleus test is a test for screening chemicals causing chromosomal damage. The test is based on the principle that chromatin fragments which may be produced by the clastogenic agents or spindle poisons, lag behind during anaphase due to chromosomal breakage and spindle malfunction and are included into the nucleus of the daughter cells. These small fragments subsequently give rise to micronuclei which are present in the cytoplasm of the daughter cells.

The acid and water extracts, were dissolved in sterile water and the organic extract in the sterile DMSO, to give the required concentration and are used for the micronucleus assay.

Micronucleus assay.

Testing the clastogenic effect of the grass extract

The method described by Schmid, (1975) was followed to conduct the test. The extracts were injected twice intraperitoneally, at an interval of 24 hours. The animals were sacrificed 30 hours after the first injection by cervical dislocation.

Preparation of bone-marrow smears:-

From the freshly killed animals, both femora are removed in toto, by cutting through pelvis and tibia. The bones were then freed from adhering muscle by the use of cotton guaze.

By gentle traction, the distal epiphyseal portion was torn off together with the rest of the tibia and the surrounding muscle. The proximal end of the femur was carefully shortened with a scissors until a small opening of the marrow canal became visible. The femur was submerged into 5 ml of physiological saline solution held in a centrifuge tube and the bone marrow was aspirated into the physiological saline. After several gentle aspirations and flushings, the process was repeated from the distal end of the femur.

The centrifuge tubes containing the marrow cells suspended in physiological saline were centrifuged at 1000 rpm for 5 minutes. The supernatant was drained out without disturbing the pellet. The pellet was then mixed thoroughly with the help of the pasteur pipette in its capillary part. A drop of this was placed on a clean slide and a smear was made by pulling the material behind a polished cover glass held at an angle of 45°. Atleast 3-4 slides were prepared from each animal. The smears were allowed to air dry.

The slides were strained in May-Grunnwald solution (Appendix-IV) for 3 minutes followed by diluted May-Grunnwald solution(1:1 in distilled water) for 2 minutes. They were then rinsed twice with distilled water. The smears were then stained with Giemsa solution (Appendix -IV) for 10 minutes.

After Giemsa staining, the slides were rinsed well with distilled water and blotted dry by pressing gently between two layers of filter paper. The backside of the slide was then cleaned with methanol. The slides were cleared in xylene for at least 5 minutes and mounted with coverslips using DPX mountant.

Scoring:-

The areas with good staining and proper morphology of the erythrocytes were selected by screening at 10 X magnification. These areas were then scored under oil immersion for the presence of polychromatic erythrocytes (PCE) which were stained blue, and normochromatic erythrocytes (NCE) which were stained pink, with or without micronuclei. The NCE were scored for the presence of micronuclei to evaluate whether artifacts of staining has occured. The ratio of number of PCE to the number of NCE (P/N ratio) was also calculated as a measure of toxicity . (The P/N ratio is close to unity for non-toxic doses).

The results were expressed as Mean $\pm$ SD of percentage number of PCE with micronuclei of 4 animals.

4 Effect of grass extract on the activity of the xenobiotic conjugating enzyme (Glutathione-s-transferase) in mice liver.

The activity of the xenobiotic conjugating enzyme (GST) was assayed in Swiss Laca mice of 8 weeks old.

Treatment of animals.

The extracts (75 μg/dose) were injected intraperitoneally 72,48 and 24 hours prior to the enzyme assay. Control animals were injected with 0.1 ml of distilled water. All the animals were fasted overnight and killed by cervical dislocation. The assays of the components of the drug metabolising system were done in the cytosolic fractions of the liver.

Estimation of glutathione -s -transferase activity

Glutathione-s-transferase activity was assayed in the cytosolic fractionm by the method of Habig et al., (1974), (Appendix - VI).

GST activity was calculated using the extinction co-efficient of the product formed (9.6 mM-1 cm-1) and values have been expressed as Mean ± SD of nmoles of CDNB conjugated/min/mg protein.

5. Biochemical evaluation of grass, for the presence of known antimutagenic compounds.

The grass was analysed for the following components:-

a. Vitamin A:-

Vitamin A content was analysed by the method described by Neeld and Pearson (1963) ,(Appendix -VII).

b. Vitamin C:-

Vitamin C was estimated by the method of Roe and Kuether (1943), (Appendix VIII).

c. β - Carotene.

β-Carotene was estimated by the method described in Appendix IX.

d. Crude fibre.

The crude fibre was estimated according to the method of AOAC, 1975 (Appendix X)

e. Chlorophyll.

The total Chlorophyll and cholorophyll a and b content were estimated as per the method described in Appendix XI.

f. Selenium

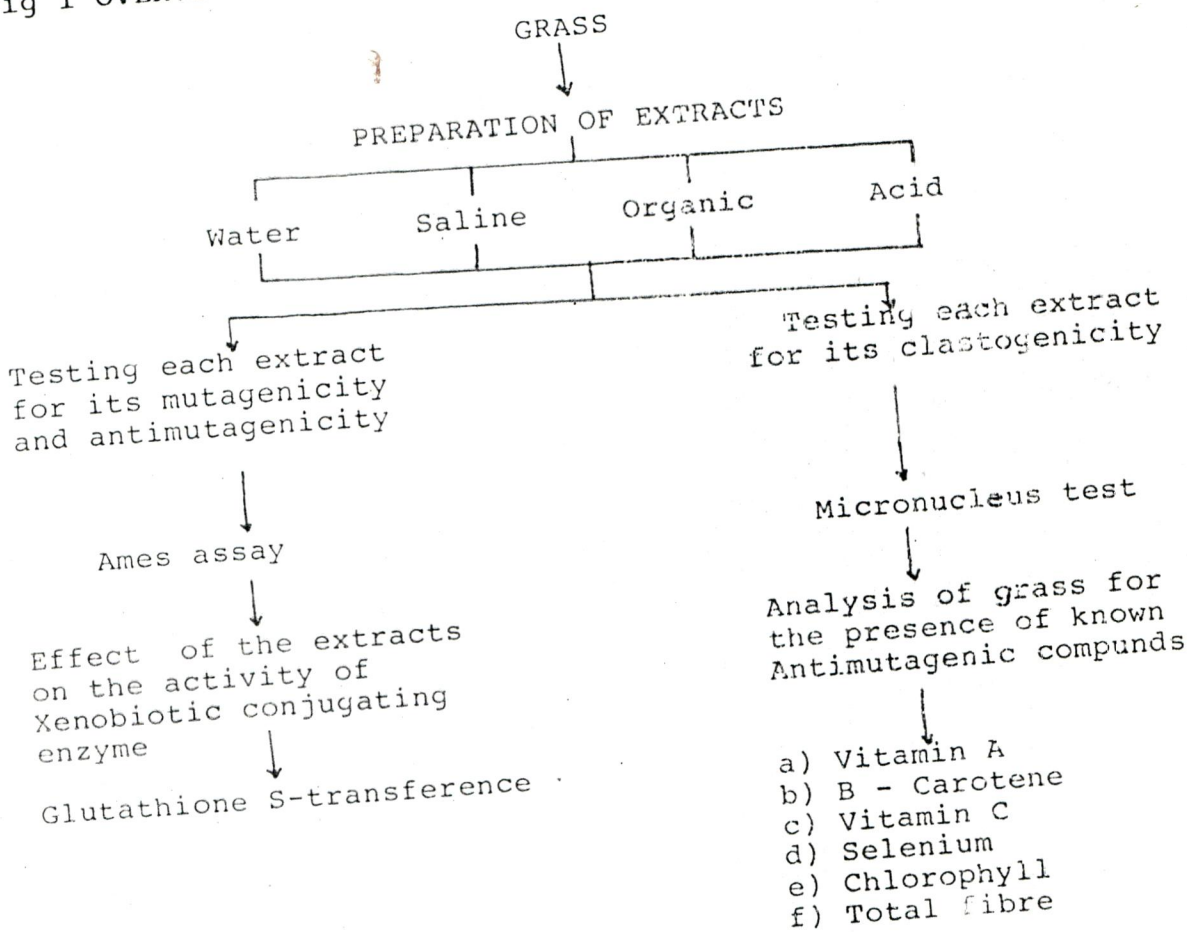
The Selenium level in the grass was estimated by the method of Duncan and Parker (1974) and Brodie, 1979 (Appendix -XII).

Results and Discussion

IV. RESULTS AND DISCUSSION

The following scheme gives an overview of the experimental design of the present study.

Fig I OVERVIEW OF THE PRESENT STUDY



MUTAGENICITY STUDIES USING AMES TEST

The Ames Salmonella/microsome assay (Maron and Ames,1983) was used to study the mutagenicity of grass extracted with water, saline,acid and organic solvent. This study comprised of two important phases as follows:-

1) Testing the mutagenicity of the extracts:

a) To test the direct-acting mutagenic effect of the sample in TA 98 (frame shift mutagens) and TA 100 (base pair substituting mutagens)

b) To test the formation of mutagens upon the metabolic activation in TA 98 and TA100

2. Testing the Co-Mutagenic/Antimutagenic effects:-

a) To test the influence of the sample extracts on direct-acting mutagens in TA 98 and TA 100

The results obtained in these are discussed below:-

The doses of the extracts to be tested were arbitarily selected as 25,50,75 and 100µg per plate, to study the mutagenicity as well as the dose-dependence of such effect. The colony counts obtained with the various extracts in both TA98 and TA100 with and without metabolic activation are depicted in Table I

It was observed that all the four extracts of grass did not exhibit mutagenicity in both the strains, both with or without S 9 mixture at all the dose levels tested.

Green leaves are known to contain several antioxidant compounds (Gopalan,etal,1987). which are nonmutagenic in nature. Green leafy vegetables have also been shown to be nonmutagenic both with and without metabolic activation

Table I. Mutagenicity of the grass extracts in S.typhimurium.

Extracts	Dose µg/plate	Number of His + Revertants in TA 98		TA 100	
		-S9	+S9	-S9	+S9
SR	-	30±5	34±1	105±4	116±2
Water	25	28±4	35±6	103±2	112±7
	50	30±2	36±4	104±2	113±3
	75	30±7	36±1	104±9	113±8
	100	31±1	37±8	105±7	115±2
Saline	25	27±2	33±7	103±5	113±4
	50	28±5	34±3	103±9	114±2
	75	29±4	34±5	104±3	115±7
	100	31±4	35±5	105±1	116±3
Organic	25	31±7	33±2	107±1	115±3
	50	33±2	34±8	108±3	116±1
	75	33±8	36±2	108±9	116±3
	100	35±2	36±7	110±3	117±4
Acid	25	26±2	33±4	104±1	113±2
	50	27±6	33±9	104±8	114±1
	75	28±1	34±8	105±3	114±8
	100	28±4	35±3	107±1	115±5
4-NPDA	10	542±11	-	-	-
Sodiumazide	1	-	-	392±14	-
B(a)P	1	-	447±7	-	462±10

Values are Mean ± SD of four plates.

SR = Spontaneous Revertants.

(Amudhavalli, 1991). Our results of non-mutagenicity of grass extracts agree with these reports.

In the second phase of the Ames test, the effects of the grass extracts was tested on the effect of standard mutagens. For this, the extracts were added along with a standard mutagen characteristic of the strain to the top agar before pouring on to the basal agar plates as explained in the experimental procedure. The results obtained are depicted in Table II

All the four extracts of grass showed a strong antimutagenic effect against the standard mutagens. This effect was more pronounced in the presence of S 9 mixture. The antimutagenicity also showed dose dependence.

Green leaves have been shown to contain various compounds like vitamins and chlorophyll which are established antimutagens (Shenoy, and Choughvely, 1989). The antimutagenicity observed with grass extracts may be attributed to the presence of such compounds in the grass. In the present study,

we have also analysed grass for the presence of these compounds, the results of which are presented later in this chapter.

Table II - Comutagenicity/antimutagenicity of the grass extracts in S-typhimurium.

Extracts	Dose µg/plate	Number of His + Revertants in			
		TA 98		TA 100	
		-S9	+S9	-S9	+S9
		542±11	447±7	392±14	462±10
Water	25	492±4*	393±2*	351±17*	432±8*
	50	483±7*	379±11*	340±5*	417±7*
	75	465±3*	353±5*	321±7*	392±11*
	100	432±13*	332±7*	301±3*	387±7*
Saline	25	489±3*	398±7*	343±1*	440±7**
	50	479±8*	353±8*	324±8*	423±9*
	75	453±7*	314±3*	301±6*	397±15*
	100	419±12*	302±8*	297±7*	373±4*
Organic	25	485±7*	383±11*	357±12*	438±6*
	50	463±2*	370±6*	332±8*	412±4*
	75	451±11*	343±14*	314±7*	402±12*
	100	439±17*	321±8*	292±11*	390±8*
Acid	25	495±5*	397±11*	343±17*	442±7**
	50	473±7*	373±17*	330±12*	437±14**
	75	460±8*	357±15*	312±7*	412±11*
	100	442±12*	313±2*	298±3	398±7*

Values are Mean ± SD of 4 plates.

Spontaneous Revertants for the strains were:-

TA 98 = 30±5 (-S 9) and 34±1 (+S 9)

TA 100 = 105±4 (-S 9) and 116±2(+S 9)

* - Statistically significant $p<0.01$
** - Statistically significant $p<0.05$

MAMMALIAN SYSTEMS

After testing the antimutagenicity of the extracts in the bacterial systems, the potency of the extract must also be evaluated in whole mammalian system. In the present study, the clastogenic and anticlastogenic effects were investigated in the micronucleus test using the Swiss Laca Male Mice.

The micronucleus test conducted using grass extract can be broadly divided into 2 phases:-

1) Testing the grass extracts for clastogenicity
2) Testing the effect of grass extracts on the clastogenicity of standard mutagen Benzo(a)Pyrene.

Two doses of grass extracts were chosen for the present study viz., 100 μg/g body weight and 200 μg/g body weight. Since the saline and water extracts had a similar effect, We carried out the micronucleus test with water, organic and acid extracts.

Table IIIdepicts the results obtained with the extracts. It was observed that all the extracts did not induce significantly higher number of micronuclei as compared to the untreated controls.

Table III -Clastogenicity of the grass extract in micronucleus test.

Treatment	Dose	Number of MNPCE's %	P/N ratio
Untreated control	-	0.02 ± 0.04	1.3
Water	50µg/gx2	0.14 ± 0.08	0.99
	100µg/gx2	0.08 ± 0.04	0.995
Organic	50µg/gx2	0.14 ± 0.10	0.99
	100µg/gx2	0.06 ± 0.09	0.99
Acid	50µg/gx2	0.22 ± 0.07	0.995
	100µg/gx2	0.10 ± 0.06	0.987

Values are expressed in Mean ± SD of 4 animals.

MNPCE - Micronucleated polychromatic erythrocytes.

P - Polychromatic erythrocytes

N - Normochromatic erythrocytes

In the second phase of the study, only 200 μ g/g body weight was tested along with B(a)P.

One striking observation made was the reduction in the toxicity of B(a)P by the grass extracts. Animals injected with B(a)P alone showed toxicity symptoms like inability to move, paralysis like effect on the hind legs and difficulty in breathing. These effects persisted for about 1 to 2 hours after which the animals recovered.

However, those animals injected with grass extracts following B(a)P administration did not show any toxic symptoms. (Fig.II)

Table IV lists the extent of micronucleated cells induced by B(a)P in the presence and absence of grass extracts. B(a)P induced a highly significant number of micronuclei in the bone marrow cells of mice. (Fig III).

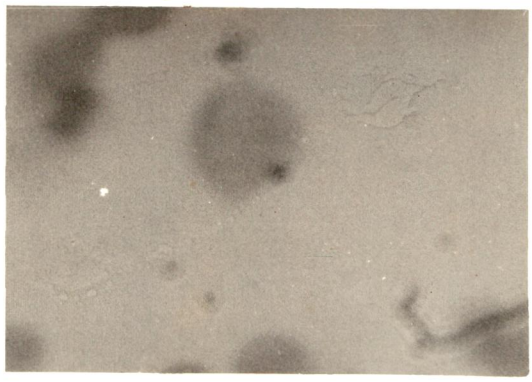
The P/N ratio which reflects the toxicity also showed values over unity indicating toxicity by this agent.

When grass extracts were administered along with B(a)P, the number of micronucleated cells were significantly decreased (Table IV). The values were comparable to those of untreated controls .In other words administration of grass extracts completely inhibited the clastogenicity of B(a)P.

I. Reduction of the toxicity of B(a)P by grass in Swiss Laca Mice.

I. Injected with B(a)P alone

II. Injected with B(a)P and grass extract



II. An erythrocyte in the bone-marrow of a mouse treated with B(a) P showing micronucleus (May-Grunnwald - Giemsa x 1000)

Table IV - Anticlastogenicity of 200µg of grass extract /g body weight against Benzo(a)pyrene in micronucleus test.

Treatment	Number of MNPCE's %			
	Without grass extract	P/N ratio	With grass extract	P/N ratio
Water extract	0.66±0.05	1.45	0.05±0.05*	0.81
Organic extract	0.66±0.05	1.45	0.06±0.08*	0.836
Acid extract	0.66±0.05	1.45	0.05±0.05*	0.779

Values are expressed as Mean ± SD of 4 animals.

MNPCE's - Micronucleated polychromatic erythrocytes.

P - Polychromatic erythrocytes

N - Normochromatic erythrocytes

* - Statistically significant $p<0.01$

The P/N ratios were also below one, indicating that toxic effects were suppressed by grass extracts.

BIO- CHEMICAL EVALUATION FOR THE PRESENCE OF KNOWN ANTIOXIDANTS IN THE GRASS.

Under in vivo conditions, several dietary agents have been reported to have a protective effect against the mutagenicity of chemicals Hayyatsu et al 1988).Some such agents are the vitamins.

The strong antimutagenic and anti clastogenic action of grass extracts may be due to the presence of the antioxidants in green leaves. To confirm the presence of such compounds in grass, the levels of the following antioxidants were estimated in 100 g of the grass.

Vitamin A, β- carotene, vitamin C, chlorophyll a, chlorophyll b, total chlorophyll, selenium and crude fibre. The values obtained are shown in Table V.

The carotenoids and retinol are reported to have pronounced antimutagenic actions. Retinol was found to be antimutagenic towards benzo(a) pyrene (Calle and Sullivan, 1982; Busk et al 1982) in Ames test with metabolic activation. However it was reported to be ineffective in the mouse micronucleus test. This could be due to the possible

Table -V - Levels of antioxidants in 100 g of grass.

Antioxidants	Levels
Vitamin A	93.75 ± 0.25 µg
β-Carotene	78±2 mg
Vitamin C	187 ±2 mg
Chlorophyll -total	18.3 ±0.3 mg
a	14.4 ±0.9 mg
b	4.0 ± 0.2 mg
Selenium	1.52 ±0.02 µg
Crudefibre	28.0 ±0.2 g

Values are Mean ± SD of duplicates.

poor availability of vitamin A in the in vivo system following oral administration (Hayatsu et al, 1988) .

The anticlastogenic activity of β-carotene may be due to its antioxidative property that eliminates the free radicals by formation of complexes with one of its double bonds or by affecting the processes of enzymatic activation of the mutagens (Renner, 1990).

The most thoroughly studied vitamin which has been found to inhibit mutagenicity of a variety of carcinogens is is ascorbic acid. It was found to inhibit mutagenicity of dimethyl nitrosamine (Gutten plan,1977; Lo and Stich,1978). The effect of ascorbic acid was attributed to be due to its antioxidant action.

Chlorophyllin, a man-made derivative of chlorophyll, has been shown to be an antioxidant (Shibata et al., 1989) .

Studies by Arimoto et al.(1980) and Negishi et al.(1989) indicate that chlorophyllin may interact with mutagens, either in the metabolically active form , to form a complex and it is this complex formation that inactivates and/or decreases the availability of mutagens to attack DNA.

A study reported by Newmark and Mergens et al.(1981) indicates chlorophyllin to be a potent antimutagen, more effective than retinol, β - Carotene, Vitamin C or Vitamin E.

Dietary selenium, at control levels above minimum requirements has been correlated with anticarcinogenic properties in both laboratory animal studies and human epidemilogic investigations (Chortyk and Schlotzhaver, 1984). Minimum requirement for selenium in man is estimated to range from 0.05 to 0.1 ppm in the diet.

The anticarcinogenic property of selenium may be due to two of its known functions including its role in the active site of the enzyme glutathione peroxidase and its interaction with heavy toxic metals. Glutathione peroxidase inactivates hydroperoxides and lipoperoxides thus protecting cell constituents against free radical damage. Such heavy metal toxins, such as cadmium , a known carcinogen and mercury lower glutathione peroxidase activity by interacting with selenium.

Balansky, (1991) reported that selenium influences some DNA repair mechanisms by decreasing their effectiveness thereby potentiating the mutagenic activity of some chemical compounds.

Hence, in any case, when evaluating the anticarcinogenic and antimutagenic properties of selenium and the prospect of its being used as a chemo preventive agent against cancer, it should be taken into account that under certain conditions this trace element can act as a comutagen.

Dietary fibre adsorb certain mutagens or cancer promoters in the digestive tract. Roberton et al. (1991) observed that Italian rye grass (Lolium multiflorum) which is mainly composed of unlignified walls strongly adsorbed DNP (1,8 - dinitro pyrene) a hydrophobic mutagen.

Extrapolation of these data to the in vivo situation would indicate that increased consumption of grasses and plant material containing cork cells, for eg., potato skins should be effective in removing hydrophobic mutagens from potential contact with colonic mucosal cells.

EFFECT OF THE EXTRACTS ON THE ACTIVITY OF XENOBIOTIC CONJUGATING ENZYME

The detoxification by the glutathione-s-transferase (GST) system forms one of the most important defence mechanisms (Smith et al 1977) . In the present dissertation, the effect of the various grass extracts on the activity of GST were tested . The result obtained are listed in Table VI

Table VI - Levels of GST activity in the Swiss Laca Mice.

Treatment	Dose x 3	GST activity n moles of CDNB conjugated/min/mg protein
Distilled water(vehicle for acid and water extracts)	0.1 ml	631.6 ± 35.7
DMSO (Vehicle for organic extract)	0.1 ml	460.8 ± 65.1
Water extract	75 µg/g b wt	912.0 ± 67.9*
Acid extract	75 µg/g b wt	936.0 ± 33.9*
Organic extract	75 µg/g b wt	641.9 ± 23.3*

Values are Mean ± SD of 4 animals.

* - Statistically significant as compared to corresponding vehicle control.

esophagus, high enough to be considered as protective agents against carcinogenesis. Glutathione levels were also significantly elevated in the 3 tissues by the plant products. (Aruna et al; 1990).

In the present disertation, it was observed that the grass extract possessed both antimatagenic and anticlastogenic effects. Also the extracts were found to induce the levels of GST to a highly significant level, when compared to the vehicle control. The observations of the present study are summarised in the next chapter.

Summary and Conclusion

V. SUMMARY AND CONCLUSION

The great and unique importance of plant foods to the mankind is well known to all of us. The whole existence of the human race is dependent on these plants. Humans have gained much knowledge about plant foods that are considered to protect the health.

Foodstuffs are the main sources of naturally occuring anti carcinogenic agents that hinder the formation of carcinogens from precursors or that act protectively to lessen or eliminate the effects of carcinogenesis. Dietary intake of natural antioxidants could be an important aspect of the body's defence mechanism against these agents. Many antioxidants are being identified as anticarcinogens. Characterizing and optimising such defense systems may be an important part of a strategy of minimizing cancer and other age-related diseases.

Cancer occupies second place among the killer diseases in the world. Hence special attention is being made to identify the naturally occuring anticarcinogens and in elucidating their mechanism of action as a practical means of inhibiting cancer. This fact forms the basis of the present study.

In the present study , entitled, "Antimutagenicity of the grass Cynodan dactylon", the grass was studied for its antimutagenic effect and anticlastogenic effect. Supporting studies were also conducted. The results can be summarized as follows:-

Mutagenicity	-	Negative
Antimutagenicity	-	Strongly positive
Clastogenicity	-	Negative
Anticlastogenicity	-	Strongly positive
Presence of antioxidants	-	To a high extent
Effect on GST	-	Inducing to a high extent.

The grass was extracted with various agents like water, organic solvent, saline and acid. To find the effect of the grass extract in the physiological conditions, saline extract was prepared. An organic extract was prepared to find out the effects due to the lipid soluble components of the grass. In order to find the effect of the grass in the stomach conditions, an acid extract was also prepared.

By using the Ames test, all the four extracts were tested for the mutagenicity and found to be non-mutagenic, in both the strains TA 98 and TA 100, in the presence and absence of the metabolic activation mixture. As the next phase of the study, the extracts were tested for the co-mutagenicity /antimutagenicity, in both the strains, in the presence and absence of the metabolic activation (S 9)mixture against specific standard mutagens. The extracts were found

to suppress the effects of the mutagens to a significant extent. This effect also exhibited dose-dependence.

Following the assay in the bacterial systems, the effects were studied in the mammalian systems. The extracts were tested for their clastogenicity on the bone-marrow cells of mice. It was observed that all the extracts did not induce significantly higher number of micronuclei as compared to the untreated controls, showing that they are non-clastogenic.

To study the anticlastogenicity, the grass extracts were administered along with the standard carcinogen B(a)P. The number of the micronuclei induced by B(a)P, was found to be significantly lower in combination with grass extracts as compared to that of B(a)P alone. In fact, the grass extracts completely inhibited the clastogenicity of the standard clastogen that was administered.

The antimutagenic and anticlastogenic effect of the grass extracts may be due to the presence of the various antioxidants like the vitamins, trace minerals and other phenolic compounds. Green leafy vegetables and fruits are excellent sources of vitamins and chlorophyll. They also provide minerals, flavonoides, ellagic acid, indoles and other agents that contribute to increased levels of enzymes

that can detoxify carcinogens including hydroxy radicals and reactive oxygen and others that lower risk by inhibiting cell duplication processes and favour differentiation.

In the present study, the grass was analysed for its vitamin A, β - Carotene, Vitamin C, Selenium, Chlorophyll and crude fibre content. It was found that grass contained considerable amount of all the above compounds. The antimutagenicity and anticlastogenicity of the grass may be well due to the presence of such antioxidants and other as yet unidentified agents.

The effects of grass extracts on GST activity were also tested in the liver of the Swiss Laca Mice. The extracts were found to induce the enzyme activity to a significant extent when compared to that of the vehicle control. The enhanced action of GST prevents the carcinogens from reaching or reacting with critical target sites, thus reducing the impact of the carcinogenic assualt.

From the present study, it is clear that the grass, Cynodan dactylon, has a good potential in protecting against the mutagens and /or clastogens that we are exposed to. From these results, we can also deduce the anticarcinogenic action of the grass. However, this needs to be confirmed by long term animal studies.

Our results also indicate that the protective effect of grass may be due to the presence of antioxidants and known antimutagenic compounds in it.

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Appendix

APPENDIX-I

PROPERTIES AND GENOTYPE OF THE TESTER STRAINS

In addition to the histidine mutation, the standard tester strains contain other mutations that greatly increase the sensitivity in detecting the mutagens. The rfa mutation causes partial loss of the lipopolysaccharide barrier that coats the surface of the bacteria, thus increasing the permeability of large molecules such as benzo(a)pyrene [B(a)P], that do not penetrate the normal cell wall (Ames et al.1973). The other mutation (uvrB mutation) involves the deletion of a gene coding for the DNA excision repair system ,resulting in greatly increased sensitivity in detecting many mutagens (Ames,1971;1973).

TA 100 and TA 98 have been developed by transferring a resistance transfer factor (R factor) to the standard tester strains and TA 1535 and TA1538 respectively.These two new strains (TA 100 and TA 98) have been extremely sensitive in detecting a number of mutagens, and are recommended for use in general mutagenesis testing (Maron and Ames,1983).

Maintenance of tester strains

Upon receiving the strains, they were inoculated into nutrient broth (8 g nutrient broth, 5 g NaCl, 1000ml distilled water) and allowed to grow at 37°C. The genotypes of the tester strains were confirmed as described below and

the cultures streaked on nutrient agar plates(Master Plates) and also stored in sterile vials (which were cryopreserved in liquid nitrogen).

Confirming genotypes of tester strains

(a) Histidine requirements : The His^- character of the tester strains was confirmed by demonstrating the histidine requirement for growth on selective agar plates.(Biotin is also required by all of the standard tester strains because of the uvrB deletion which extends through the bio gene).

Each plate (with 0.1 ml of 0.5 mM biotin and with or without 0.1ml of 0.1M histidine) were streaked with the strains,incubated overnight at 37°C and examined for growth. The histidine requirement was shown by growth observed only in the his/bio plate,but not in the control plate with only biotin.

(b) rfa mutation: The presence of rfa mutation was checked by testing the permeability of large molecules.Crystal violet is used for this purpose. Sterile filter paper disc, onto which 10#l of crystal violet solution (1mg/ml) has been delivered, was carefully placed on the solidified top agar to which the bacterial culture had been added. After overnight incubation at 37°C, a clear zone of inhibition was observed around the disc, indicating the presence of rfa mutation, permitting large molecules like crystal violet to enter and

kill the bacteria.

(c) uvrB mutation : The uvrB mutation is quite stable and can be confirmed by demonstrating uv sensitivity in strains that contain this mutation. For this, the cultures were streaked on the nutrient agar plates. The plates were partially covered (so that half of each streak was covered) with a piece of cardboard. The plates were then irradiated with a germicidal uv lamp at a distance of 33 cm,for 6 seconds (for non-R-factor, viz., TA 1535 and TA 1538) to 8 seconds (for R-factor strains, viz ., TA 98 and TA100). The plates were then incubated overnight at 37°C after which time it was observed that the bacteria grow only on the un-irradiated side of the plate.

d) R-factor: The presence of R-factor should be tested routinely by the presence of ampicillin resistance, because the plasmid is somewhat unstable and can be lost from the bacteria (McCann et al.,1975). For this, plates containing ampicillin (25#g/ml) in the basal agar were prepared and the cultures were streaked on this. After 12-24 hours incubation at 37°C, growth was observed only along the streaks made with the R-factor strains (TA 98 and TA100) and not along the streaks of other strains.

APPENDIX II

Induction of Drug-Metabolising Enzyme in mouse

Most carcinogens are inactive when present in the environment. Upon entering the system they are converted to active metabolites by the carcinogen metabolising enzymes. The enzymes are of 2 major types (1) the activating enzymes and (2) the detoxifying enzymes. The components of the drug metabolising system include aryl hydrocarbon (benzo (a)pyrene) hydroxylase(AHH), Cytochrome b_5 and CytochromeP_{450}, amino pyrene N-demethylase (APND), ethoxy resorufin-o-deethylase (ERRD), epoxide hydroxylase (EH), glutathione -s-transferase(GST) and reduced glutathione. Many chemicals are used to induce the drug metabolising enzymes. The most commonly used inducers include poly chloro bi phenyl mixture (Aroclor 1254) and PhenoBarbital (PB). In the present dissertation, PB was used according to the methods reported earlier (Kunz, et al.,1987).

Swiss Laca Male mouse 8-10 weeks old weighing about 25g was injected with PB at a dose of 1mg/g body weight intraperitoneally for three consecutive days.

Preparation of S-9 fraction

After PB administration for 3 consecutive days, on the 4th day the animal was killed by cervical dislocation,

following overnight fasting. The liver was quickly excised using sterile surgical tools, washed with cold isotonic Kcl, blotted dry between sterile filter paper folds and weighed. At 20% homogenate was prepared in cold isotonic Kcl. The homogenate was spun at 9000 x g in a refrigerated centrifuge at 4C for 15 mins. The supernatant (S9 fraction) was distributed into sterile 2ml vials after adjusting the protein concentration to 40 mg/ml.

The protein estimation was carried out according to the method described by Lowry et al (1951).

Protein estimation by Lowry's method

Protein was estimated according to the method of Lowry et al(1951).

Principle

This method is based on the principle that different proteins contain different amounts of aromatic amino acid residues which react with Folin Ciocalteu reagent giving a blue colour which is read in a colorimeter.

Procedure

The protein sample was suspended in 1 ml of 1N NaOH at 100°C for 4-5 mins. 5 ml of alkaline copper reagent was added to it and the mixture was allowed to stand at room temperature for 10 mins. 0.5 ml of Folin -Ciocalteu reagent

was added rapidly and the contents in the tube mixed throughly.

The amount of protein in the sample was calculated with a standard curve prepared using bovine serum albumin.

Preparation of the co-factors and the S-9 mixture

The cofactors used are $Mgcl_2$, Kcl, Glucose-6-phosphate, NADP and phosphate buffer.

The S9 mixture was prepared fresh just prior to each assay as follows :

S-9	- 0.1ml
$Mgcl_2$	- 8.0 μmoles
Kcl	- 33.0 μmoles
Glu-6-Po_4	- 5.0 μmoles
0.1 M phosphate buffer	- 100 μmoles

The S-9 fraction was stored in deep freeze (-80°C).

APPENDIX III

AMES TEST

Preparation of solutions for the Mutagenesis assay

Minimal Glucose (Basal agar) Plates

For the mutagenesis assay, the minimal glucose agar medium was prepared as follows.

Agar	-	21 g
Distilled water	-	910 ml
25 x VB salts	-	40 ml
40 % glucose	-	50 ml

The VB salt solution and glucose solution were autoclaved separately and mixed to the agar solution (also autoclaved) just before pouring onto the petriplates.

Vogel Bonner (VB) Salt Solution (25 x)

Warm distilled water	-	67 ml
Magnesium sulphate ($MgSO_4$ $7H_2O$)	-	1 g
Citric acid monohydrate	-	10 g
Dipotassium hydrogen phosphate	-	50 g
Sodium ammonium phosphate ($NaHNH_4$ $4H_2O$)	-	17.5 g

The salts were added in the order indicated to warm water (45°C). Each salt was allowed to dissolve completely before adding the next. The volume was adjusted to 200 ml and the solution was autoclaved.

Top Agar

Agar	-	0.6 g
Sodium chloride	-	0.5 g
Distilled water	-	100 ml

Histidine Biotin Solution

This solution contain 0.5 mM biotin and histidine.

L.Histidine	-	0.7758 mg
D.Biotin	-	1.2215 mg
Distilled water	-	10 ml

The agar was dissolved by autoclaving. The solution was cooled to 45°C and histidine - biotin solution was added to it (10 ml to 90 ml of top agar solution) and distributed into 2.0 ml aliquots in sterile tubes for the assay.

APPENDIX IV

Preparation of Staining Solutions

May - Grunnwald stain

May Grunnwald powder	-	250 mg
Methanol	-	100 ml

The stain was dissolved in methanol by mixing thoroughly for 5 minutes. The solution was then filtered through whatman filter paper No.I and stored at 0-4°C.

Giemsa Solution

Giemsa powder	-	800 mg
Glycerol	-	50 ml
Methanol	-	50 ml

Stain was dissolved in 50 ml of glycerol at 60°C with regular shaking for 45-60 min. The solution was cooled to room temperature and 50 ml of methanol was added and mixed thoroughly for 5-10 minutes. The resultant solution was allowed to stand overnight and filtered through whatman filter paper and stored in dark at 0-4°C.

APPENDIX V

Preparation of Microsomal and cytosolic fractions

The liver was quickly excised, washed throughly, with ice-cold, isotonic KCl (1.15 %) blotted dry, weighed and homogenized to obtain a 20 % homogenate in KCl and the homogenate was centrifuged at 9.500 g for 15 minutes at $4^{o}C$ in a refrigerated centrifuge. The supernatant was further spun at 105,000 g for 60 minutes in a ultra centrifuge at $4^{o}C$. The supernatant (cytosolic fraction) was used for the assay of glutathione-S-transferase.

APPENDIX VI

Estimation of GST activity

GST activity was assayed in the cytosolic fraction by the method of Habig *et al.* (1974).

Principle

The enzyme was assayed by its ability to conjugate GSH with 1-chloro, 2,4-dinitrobenzene (CDNB), the extent of conjugation causing a change in the absorption at 340 nm.

Procedure

The assay was done at 25°C, under conditions giving activities linear with respect to incubation times and protein concentrations for atleast 3 mins.

The enzyme activity was determined by monitoring the change in absorbance at 340 nm. 0.1 ml of both substrates (1 mM GSH and 1 mM CDNB in ethanol) were taken in 0.1 m phosphate buffer (pH 6.5) to make a volume of 2.9 ml. The reaction was started by adding 0.1 ml of cytosol to this mixture. The readings were read against distilled water blank. Complete assay mixture without enzyme, served as control to monitor non-specific binding of the substrates (final concentration of ethanol in the assay system was always less than 4 %).

GST activity was calculated using the extinction coefficient of the product formed (9.6 mM^{-1} cm^{-1}) and values have been expressed as mean $\pm$ SD of n moles of CDNB conjugated/min/mg protein.

APPENDIX VII

Estimation of Vitamin A

The Vitamin levels were analysed by the Trifluoroacetic acid (TFA) method described by Neeld and Pearson (1963).

Principle

This method is based on the measurement of the unstable blue color formed by the interaction of vitamin A with TFA, the intensity of which is a function of the concentration of vitamin A.

Procedure

To 20 % homogenate of the grass, equal volume of saponification mixture (2N KOH in 90 % alcohol) was added to the homogenate and heated under gentle reflux for 20 minutes at 60°C. 25 ml of water was added to the mixture after cooling to room temperature and the solution was transferred to a separating funnel. It was then extracted thrice using 25, 15 and 15 ml of petroleum ether (boiling range 40°C - 60°C). The ether extracts were pooled and washed with 50 - 100 ml of distilled water repeatedly until the wash water was free of alkali. The petroleum ether extract was then dried by adding anhydrous sodium sulphate. The volume of the extract was noted and aliquots were taken in triplicates.

The vitamin A levels in the aliquots were analysed by the TFA method described by Neeld and Pearson (1963). The aliquot was evaporated to dryness in a 60°C water bath. The residue was taken immediately 0.1 ml of chloroform and 0.1 ml of acetic anhydride and 1.0 ml of TFA reagent (TFA : chloroform, 1:2, prepared fresh) were added to it. The mixture was rapidly transferred to a cuvette and the absorbance was measured at 620 nm, against a reagent blank, exactly 15 seconds after the addition of the TFA reagent.

A standard curve was made using retinyl acetate solution in chloroform containing concentrations ranging from 0.5 - 2.0 ug.

The results are expressed as Mean $\pm$ SD of ug vitamin A/g of the grass.

APPENDIX VIII

Estimation of Vitamin C

Vitamin C was estimated by the method of Roe and Kuether (1943).

Principle

The method is based on the oxidation of ascorbic acid to dehydroascorbic acid (achieved by shaking with norit) and the subsequent conversion of dehydroascorbic acid to diketogulonic acid, which couples with DNPH is the presence of thiourea (a mild reducing agent) to give red coloured osazones which are estimated colorimetrically.

Procedure

2 g of the grass was homogenized with 50 ml of 4 % oxalic acid and the homogenate was treated with 100 mg of acid washed norit.

(Acid washed norit : 1 litre of 10 % HCl was added to 200 g of norit in a large flask, heated to boiling and filtered under suction. The cake of norit was transfered to a beaker, 1 litre of distilled water was added to it and stirred thoroughly and filtered. This was repeated until the washings was free of ferric ions. Then the norit was dried overnight at 110° - 120°C).

It was shaken well, kept for 10 minutes and then filtered. The volume of the filterate was noted down and aliquots taken in duplicates. Added 0.5 ml of the DNPH reagent and a few drops of 10 % thiourea solution. The tubes were incubated at 37°C for 3 hours. After incubation, 2.5 ml of 85 % sulphuric acid was added in cold, drop by drop, with no appreciable rise in temperature. DNPH reagent and thiourea were added to the blank only after the addition of sulphuric acid. The red colour developed was read at 540 nm after 30 minutes incubation at room temperature.

The standard ascorbic acid was prepared in 4 % oxalic acid to contain 10 - 100 ug and treated in the same procedure.

The results are expressed as mean $\pm$ SD of mg vitamin C/g of the grass.

APPENDIX IX

ESTIMATION OF CAROTENOIDS

The β - carotene content was estimated according to the method adopted by Mahadevan and Sridhar (1984).

Chopped 2 g of the fresh grass and homogenised with 20ml of distilled methanol,and filtered using the Whatman NO. 42 filter paper. Repeated the extraction twice, until the tissue is free from the pigments. Pool the filtrates and partition with equal quantity of Peroxide free ether thrice using a separatory funnel. Added water if necessary to produce two layers during the initial ether extraction. The ether phase contains the carotenoids. Evaporate the combined ether extract under reduced pressure at 35 C in a rotary evaporator or on a hot water bath. Dissolve the residue in minimum quantity of ethanol. Add 60 % of aqueous KOH at the rate of 1ml for every 10 ml of the ethanol extract to saponify it. This will remove chlorophylls and interfering lipids and also clears the esterified carotenoids. Keep this mixture in the dark in pressure of N_2 or boil it for 5-10 min or leave it overnight at room temperature. Added equal amount of water and partition twice with peroxide- free ether . Evaporated the ether under reduced pressure and dissolve the residue in minimum volume of ethanol. Measure the absorbance of this solution at 450nm in a spectrophotometer.

APPENDIX X

ESTIMATION OF CRUDE FIBRE

Method of AOAC-1975

According to the method of AOAC -1975, the crude fibre was estimated.

About 2.0g of moisture and fat free sample are weighed in a 500ml beaker and 200ml of boiling 0.255N[1.25% W/V]H_2SO_4 was added. The mixture was boiled for 30 mins, keeping the volume constant by the addition of water at frequent intervals. A glass rod inserted in the beaker helps smooth boiling. At the end of this period, the solution is filtered through a muslin cloth and the residue washed with water till free from acid. The material is then transferred to the same beaker and 200ml of boiling 0.373N[1.25%]NaOH added. After boiling for 30 mins [keeping the volume constant as before], the mixture is filtered through muslin cloth . The residue is washed with hot water till free from alkali, followed by washing with some alcohol and ether. It is then transferred to a crucible, dried overnight at 80-100C and weighed (We). The crucible is heated in a muffle furnace at 600C for 2-3hours cooled and weighed again (Wa). The

difference in weights [We-Wa] represents the weight of crude fibre.

$$\text{Crude fibre} = \frac{[We-Wa] \times 100}{\text{Weight of sample taken}}$$

APPENDIX XI

ESTIMATION OF CHLOROPHYLL

The Chlorophyll content was estimated according to the method adopted by Mahadevan and Sridhar (1984).

Extraction and Estimation

Chlrophylls are present as green pigments in all green plant tissues. Chlorophyll a and Chlorophyll b occur in higher plants. Disease development affects not only the total Chlorophyll content but also alters the ratio between Chlorophyll a and b. Measurement of green pigments may indirectly denote the severity of disease especially in systemic virus diseases. Chlorophyll estimates may also be required to relate other biochemical changes in the plant tissues.

Method

Cut 1 g fresh leaves into small pieces and homogenized in a mortar with excess acetone, with a pestle. Decant and filter the supernatant on a Buchner funnel through Whatman No. 42 filter paper. Add sufficient quantity of 80% acetone and repeat the extraction. Transfer the content from the mortar to the Buchner funnel and wash the brei with acetone until colourless. Pool the filterates and make up the volume to 100 ml in a volumetric flask. Transfer 5ml of the extract

into a 50ml volumetric flask and dilute by making up the volume with 80% acetone. Measure the absorbance at 645 and 663 nm for the determination of Chlorophyll a and b and total Chlorophyll. For routine measurements of total Chlorophyll content only, measure the absorbance of the extract at 652nm using a light path of 1 cm.

Using the specific absorbtion coefficients for Chlorophyll a and b at 663 and 645nm in 80% acetone. the following simulteneous equations can be set up for measuring Chlorophyll concentrations.

$$\text{i)} \quad A_{663} = 82.04\ C_a + 9.27\ C_b$$

$$\text{ii)} \quad A_{645} = 16.75\ C_a + 45.6\ C_b$$

$$\text{Total Chlorophyll (Mg/g)} = 20.2\ A_{645} + 8.02 A_{663}$$

$$\text{Chlorophyll a(Mg/g)} = 12.7\ A_{663} - 2.69\ A_{645}$$

$$\text{Chlorophyll b(Mg/g)} = 22.9\ A_{645} - 4.68\ A_{663}$$

The Chlorophyll content can be calculated on a fresh weight basis employing the following formula.

$$\text{Total Chlorophyll (Mg/g)} = \frac{20.2\ A_{645} + 8.02\ A_{663}}{a \times 1000 \times w} \times V$$

$$\text{Chlorophyll a (Mg/g)} = \frac{12.7\ A_{663} - 2.69\ A_{645}}{a \times 1000 \times w} \times V$$

$$\text{Chlorophyll b (Mg/g)} = \frac{22.9\ A_{645} - 4.68\ A_{663}}{a \times 1000 \times W} \times V$$

where, a = length of path light in the cell (usually 1 cm)

V = volume of the extract in ml

W = fresh weight of the sample in g

APPENDIX .XII

Analysis of Selenium by atomic absorption spectrophotometry.

Method of Duncan and Parker, 1974 and Brodie, 1979.

Selenium level in the grass was estimated by the method of Duncan and Parker, (1974) and Brodie, 1979.

Principle:

The sample in solution on suspension was heated to a high temperature by burning it in a flame. The flame broke up the chemical bonds between the molecules and enabled the individual atoms to float freely in the sample area. In this condition, the atoms (unexcited) absorb ultraviolet or visible radiation. The warelength bands in which each element is absorbed is narrow. Hence at a particular warelength the absorption is measured. The amount of light absorbed gives a direct indication of the amount of metal that is present.

Procedure:

Dissolve 1 g of pure selenium powder in 80 ml of 1:1 nitric acid, heated gently to initiate the reaction, cool if the reaction proceeds vigorously. Dilute to 1 litre to give 1000 ug/lit selenium.

From the above stocks a series of standards, (5,10,15,20 and 25 ppm) were prepared. A set of lower standards cointaining 1,2,3,4 and 5 ppm were also prepared.

The lamp current selected for the estimation of selenium was 7mA. The fuel used was acetylene along with air as its support. The selected warelength for selenium was 196 nm.

Interference:

Chemical. interference from most elements is within an allowable 5%. Only barium, lead, lithium, sodium and strontium depress the selenium absorbance by 25, 55, 12, 16 and 15% respectively. The air acetylene flame reduces all of these interference.