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REVIEW ON PLANT DERIVED NATURAL PRODUCTS AND THEIR ANALOGUES WITH CHEMOPROTECTIVE ACTIVITY AGAINST GENOTOXICITY OF CYCLOPHOSPHAMIDE

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ABSTRACT

Cyclophosphamide (CP), a common motherapeutic and immunosuppressive agent, is used in the treatment of wide range of cancers and autoimmune diseases. Besides that it is a well known carcinogen. The genotoxic potential limits its efficacy in the treatment of cancers. The present review was designed to ascertain the chemoprotective potential of natural products and their analogues on cyclophosphamide (CP) using bone marrow micronucleus test in mice, comet assay, chromosomal aberrations (CA) and sister chromatid exchanges (SCE) assay methods. The prevalence of micronuclei, the extent of lipid peroxidation, and the status of the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) in both liver and serum of mice were used as intermediate biomarkers of chemoprotection.

KEYWORDS

Cyclophosphamide; genotoxicity; antioxidants; polyphenols; Chromosome aberration; micronucleus; anticlastogenic.

INTRODUCTION

Cyclophosphamide is a well known anticancer and immunosuppressive agent with a very narrow therapeutic index and it has a complicated process of metabolic activation in the liver to active alkylating metabolites by liver monooxygenases system(cyp450).[1,2] These metabolites interfere with the growth of rapidly multiplying malignant cells by a process of intercalation with the DNA of malignant cells.[3] The effects of genotoxicants can be observed at the level of chromosomes (clastogenesis) through alterations in chromosome structure (chromosomal aberrations) and number (aneuploidy, polyploidy). A broad range of short-term and long-term screening procedures are available to find out the effects of genotoxicants. In general, rodents are used as test systems for monitoring chromosomal aberrations *in vivo*. Various classes of plant derived natural products possess anticlastogenic activity against cyclophosphamide induced genotoxicity.

There are many natural compounds of plant origin which are known to possess protective effects against the genotoxicity and carcinogenicity of drugs and chemicals. Dietary intake of such natural compounds has been suggested as an effective strategy for minimizing the harmful effects of genotoxins and carcinogens. This is possible because of the wide range occurrence of anti-genotoxic compounds in our commonly consumed dietary vegetables, fruits, nuts, cereals, spices and beverages such as tea and coffee.[4] Hence there is need for identifying 'ideal' anti-genotoxic agents. The work should be focused more on to identify the chemopreventive compound with no toxic effect, high efficacy, less expensive and a known mechanism of action.

Plant derived natural products and their analogues Piperine

In various traditional system of medicines Piper nigrum(*Black pepper*) and Piper longum (*long pepper*) are used worldwide and also as household spices.[5] One of the major important constituent of piper species is piperine, which exhibits various pharmacological and biochemical effects including antimicrobial, antifungal and hepatoprotective effects.[6] It also increases the activation of biotransformation enzymes such as glutathione-*S*-transferase (GST) and quinine reductase (QR). Various studies show that piperine possesses antioxidant[7] and anticlastogenic properties. Based on the above properties it is possible to use piperine as genoprotective compound. Various screening procedures are done on rodents and humans and the results conclude that piperine possess anticlastogenic activity.

Saffron

Saffron obtained from dried stigmas of *Crocus sativus* belonging to the family Iridaceae. It is a spice commonly used as flavouring and colouring agent. Over the decades, it has been used in traditional medicine for various diseases.[8] Recently the anticlastogenic and antioxidant effects of saffron have been reported. The constituents of saffron include safranal, crocin and crocetin. Safranal a monoterpene aldehyde which is an essential oil of saffron showed antioxidant activity.[9] Crocin and crocetin which are the carotenoids of saffron possess *inhibitory effect on free radical chain reactions*, because most carotenoids are lipid-soluble and might act as highly-efficienct free radical scavengers. Pretreatment with the aqueous extract of

saffron in experiments with Swiss albino mice significantly inhibited the genotoxicity of cyclophosphamide [10] which is revealed by *decreased comet tail length, tail moment and percent DNA in the tail.* These findings suggest a potential role for saffron as an anti-genotoxic, anti-oxidant and chemopreventive agent.

Garlic

Garlic which is commonly known as allium sativum belongs to the family alliaceae. Garlic contains at least 33 sulfur compounds like aliin, allicin, ajoene, allylpropl, diallyl, trisulfide, sallylcysteine, vinyldithiines, S-allylmercaptocystein, and others.[11, 12] Garlic contains a higher concentration of sulfur compounds than any other Allium species.

Garlic is used as a medicinal herb. It is[Delete] has medicinally important properties like antimutagenic, anticancer, anti-inflammatory, antihypertensive, antimicrobial, antifungal, antidote, hepatoprotective, hyperglycemic, immunomodulation etc. Recent studies have shown that antigenotoxic and antimutagenic effects[13] of garlic are used[add] for various drugs and chemicals. Studies of the anticlastogenic effects of garlic is due to Sulphur rich constituents of garlic such as diallyl sulfide (DAS) and diallyl disulfide (DADS) and are known to induce activities of phase II enzymes,[14] which in turn reduce the genotoxicity of several carcinogens.

Diallyl sulfide and diallyldisulfide had the *highest radical scavenging activity* by inhibiting the interactions of leukocytes which mediate release of superoxide anion. The modulatory effects exerted by the garlic extract against the cyclophoshamide induced genotoxicity was studied in the human lymphocyte cultures, by using chromosomal aberration(CA) and sister chromotid exchange (SCE) assay methods.[15]

Epigallocatechin-3-gallate (EGCG)

Epigallocatechin-3-gallate (EGCG), a compound closely related to Epicatechin gallate (ECG), is a catechin and polyphenolic antioxidant plant metabolite found in abundance in various types of tea, derived from the tea plant *Camellia sinensis*. Cyclophosphamide is also an alkylating agent and after metabolic activation. It gives rise to active mutagenic metabolic phospharamide mustard. EGCG's acts as a anti-oxidant and *protects cells from lipid peroxidation and DNA damage* induced by reactive free radicals. It reacts with electron-rich areas of bio- molecules like proteins and DNA. EGCG was studied for its antimutagenic effect on the chromosomal aberrations[16] and sister chromatid exchanges induced by cyclophosphamide (known mutagen), both in the presence and absence of metabolic activation system in human lymphocytes *in vitro*.

Lipoic acid

Lipoic acid (LA) is an organo-sulfur compound derived from octanoic acid. Lipoicacid is able to scavenge reactive oxygen and reactive nitrogen species *in vitro* .The relatively good scavenging activity of LA is due to the strained conformation of the 5-membered dithiolane ring, which is lost upon reduction to DHLA. In cells, LA is reduced to dihydrolipoic acid, which is generally regarded as the more bioactive form of LA and the form responsible for most of the antioxidant effects.The protective efficacy of DL-α-lipoic acid on the cyclophosphamide (CP)-induced clastogenicity was studied using the *in vivo* micronucleus assay.[17] LA also mediates *upregulation of phase II detoxication enzymes*, i.e. NAD(P)H:quinone oxidoreductase and glutathione-S-transferase (GST). The number of micronucleated polychromatic erythrocytes (MNPCEs) was determined at 24 after CP administration. The chemoprotective effect of lipoic acid at a dose of 200 mg/kg body was found to be stronger than 100 mg/kg body weight dosage, which indicates the dose-dependent protective effect of lipoic acid. However, the protection by lipoic acid was not dependent on the time intervals between lipoic acid and CP administration.

Eicosapentaenoic acid (EPA)

Eicosapentaenoic acid is an omega-3 fatty acid mainly obtained from fish. Eicosapentaenoic acid (EPA) acts against cyclophosphamide (CP)-induced genotoxicity. Eicosapentanoic acid mainly *potentiates the activity of many antioxidant enzymes*, like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) in both liver and serum of mice . There was a dose-dependent reduction in formation CP induced micronuclei by treatment with 100, 200, or 300 mg EPA/kg BW mice.[18] Findings imply that EPA is a potential antigenotoxic, antioxidant and chemopreventive agent

Punica granatum

Punica granatum which belongs to the family Punicaceae, commonly known as pomegranate. In fact, studies on *P. granatum* phytochemistry and pharmacological actions suggest a wide range of potential clinical applications. Antitumour, antibacterial, antidiarrhoeal, antifungal, antiulcer and antioxidant pharmacological properties have been reported for various extracts/constituents of different parts of this plant species.

Recently, the antioxidant activity of *P. granatum* associated with its phytochemicals, such as, polyphenols, flavonoids, and anthocyanidins has gained importance. The *in vitro* antioxidant ability of the pomegranate fruit, rich in polyphenols and anthocyanidins, was higher than that found in green tea, and is considered a powerful antioxidant.[19] Moreover, Guo *et al*. (2007) demonstrated *in vitro,* a powerful DNA damage prevention ability of *P. granatum*. Although extracts from many parts of this plant, including its fruit, seeds, and peel, have been reported to exhibit strong antioxidant activity *in vitro*, *P. granatum* has a preventive effect against chromosome fragmentation and/or damage to the mitotic apparatus, because of *its free radical scavenging.*[20] Mice treated with *P. granatum* showed an absence of mutagenic effects and dose-dependent protective effects against CP induced oxidative DNA damage.

Plumbago zeylanica

Plumbago zeylanica, commonly known as white leadwort, found abundantly in the plains of Bengal and southern India, was tested for its possible *in vivo* protective effect against cyclophosphamide-induced genotoxicity and oxidative stress in Swiss albino mice. Pretreatment with the alcoholic root extract of Plumbago zeylanica (250 and 500 mg/kg body weight orally for 5 days) significantly reduced the frequency of micronucleated polychromatic erythrocytes (MnPCEs), increased the PCE/NCE (normochromatic erythrocyte) ratio in the bone marrow, with changes in the levels of antioxidants there is decrease in the levels of lipid peroxidation products.

Hawthorn or thron apple

Hawthorn (Crataegus microphylla) belongs to Family rosaceae. Active ingredients found in hawthorn are mainly flavonoid (such as vitexin rutin, quercetin and hyperoside, oligomer proanthocyanidins (OPCs, such as epicatechin, procyanidin and mainly procyanidin B-2), flavone-C,triterpene acids (such ursolic acid oleanolic acid and crataegolic acid), and phenolic acid (such as caffeic acid, chlorogenic acid, and related phenolcarboxylic acids). It acts as anticlastogenic against genotoxicity induced by cyclophosphamide. It decreases the ratio of PCE/PCE+NCE (polychromatic erythrocyte/polychromatic erythrocyte + normochromatic erythrocyte). By exhibiting antioxidant activity on 1,1-diphenyl-2-picryl hydrazyl free radical.[21] Mainly flavonoids constituents have antioxidative activity, reduced the oxidative stress and genotoxicity induced by cyclophosphamide .

Zataria multiflora (ZM)

The anticlastogenic activity of Zataria multiflora (ZM) extract was tested in mouse bone marrow cells against genotoxicity induced by cyclophosphamide (CP). Mice were orally pretreated with solutions of ZM extract

prepared at 3 different doses (50, 100, and 200 mg/kg body weight) for 7 consecutive days. CP (50 mg/kg body weight) is administered on the seventh day of treatment and killed after 24 hours for the evaluation of micronucleated polychromatic erythrocytes (MnPCEs) and the ratio of PCE/(PCE + NCE), PCE refers to polychromatic erythrocyte, and NCE refers to normochromatic erythrocyte. Administration of ZM inhibited bone marrow suppression induced by CP. Zataria extract exhibited *concentration-dependent antioxidant activity on 1, 1-diphenyl 2-picryl hydrazyl free radical and lipid peroxidation.* It appeared that ZM because of its antioxidative activity reduced the oxidative stress and genotoxicity induced by CP in mouse bone marrow cells.

Tea

Common use of antimutagens and anticarcinogens in everyday life is an effective measure for preventing human cancer and genetic diseases. Because of its vast antioxidant properties it is used as protective agents against diverse toxic effects. All the three tea extracts (green tea, oolong tea and black tea) [22] have modulates the effects of genotoxicity induced by cyclophosphamide (CP), a commonly used chemotherapeutic drug and a well-known mutagen and clastogen. mice when administered individually. Various tests done on tea extracts using rodent model decreased the micronuclei (MN) induced by CP. Therefore, regular intake of tea may improve the antioxidant status in *in vivo*

Ginseng

Ginseng, the root of *Panax ginseng* is one of the well-known Chinese herbal medicines. *P. ginseng* has a broad range of therapeutic effects including tonic, adaptogenic, immunomodulatory, anti-inflammatory, anti-oxidant, anti-aging, anti-diabetes, anti-mutagenic, anti-cancer and neurovascular modulatory activities. The main active components responsible for the actions of *P. ginseng* and its stem and leaves are the ginsenosides,[23] which have a various biological activities, including anti-inflammatory activity, antioxidant and anti-tumor effects[24] and Clinically, ginseng have been frequently used in combination with chemotherapy to minimise the side effects of anti-cancer drugs. The protective effects of ginseng against the DNA damage and apoptosis induced by CP were evaluated by the alkaline comet assay, in mouse bone marrow cells and peripheral lymphocyte cells.[25,26]

Ginkgo biloba

Ginkgo biloba is a native tree of china belonging to family Ginkgoaceae. Ginkobiloba is composed of 24% flavonoids, glycoside derivates of quercetin, kaempferol, isorhamentin and myrcetin, 6% terpenoids, ginkgolides A, B, C, J, M, and bilobalides, and 0.5 to 1% organic acids acetic, shikimic, p-hydroxybenzoic, vanillic, kynurinic, and ascorbic acids.

The common mechanisms of action of the extract such as antioxidant activity *free radical scavenging and even gene regulation* made possible to use the extract as anticlastogenic compound.[27] The alkylating agent CP is one of a group of anticancer drugs that are administered as inactive prodrugs and that are activated *in vivo* via one or more metabolic steps. It is known that the initial step in the bioactivation of cyclophosphamide in-volves cytochrome P-450-mediated hydroxylation at C-4 (LeBlanc and Waxman, 1990).

The extract was co-administered to mice at doses of 50, 100 and 200 mg/kg (PO) with 24 mg/kg (i.p) CP are effective in reducing the frequency of micronucleated polychromatic erythrocytes. Based on these results, it was suggested that ginkobiloba extract possesses antimutagenic potential. The effectiveness of ginko biloba extract in the modulation of CP mutagenicity and cytotoxicity could be related to the inhibition of some CYP enzymes, which was demonstrated by Qualls et al. (1998). Efficacy of ginkobiloba was evaluated by various screening procedures like comet

assay, micronucleus test and chromosomal aberrations.

Curcumin:

Curcumin, a yellow pigment commonly used as a spice and food colouring agent is obtained from rhizomes of *Curcuma longa* and is a major chemopreventive component of turmeric. The pharmacological functions of curcumin including antioxidant, antidiabetic and hepatoprotective effects have been well described[28,29]. The antimutagenic potential of curcumin has been evaluated using *in vivo* chromosomal aberration assay in Wistar rats. Anticlastogenic activity of Curcumin was evaluated by administration at the dose of 100 and 200 mg/kg bodyweight through gastric intubation for seven consecutive days prior to CP treatment. The animals were sacrificed at the sampling time of 24 h after treatment and their bone marrow tissue was analyzed for chromosomal damage and mitotic index.

In CP treated animals a significant induction of chromosomal aberration was recorded with decrease in mitotic index. However, in curcumin-supplemented animals, no significant induction in chromosomal damage or change in mitotic index was recorded. In different curcumin-supplemented groups, a dose dependent significant decrease in *CP free-radical DNA damage* thereby acting as potent antioxidant[30, 31]. Since mutations induced at the cytogenetic levels are probable causes of cancer, therefore the inhibition of chromosomal aberration by curcumin suggest that the antimutagenic potential of curcumin is related to induced clastogenicity was recorded. Curcumin has two *p*-hydroxy groups and scavenges antioxidant and anticarcinogenic activity. The findings of investigators conclude a dose dependent antimutagenic effect of curcumin on CP induced cytotoxic and clastogenic damage. The molecular mechanisms of antimutagenesis of curcumin are needed to explore further to understand its chemopreventive effects.[32] Curcumin can act as an antigenotoxic agent at low doses ie.. at aconcentreation of 0.5mg/kg, but at high doses ie at about 40mg/kg curcumin itself is genotoxic.[33] The above results revealed that curcumin may play a dual role in genotoxicity according to its doses.

Hesperidin

Hesperidine is a flavonoid mainly obtained from citrus species belonging to family rutaceae.[34] Hesperidine shows anticlastogenic activity by its antioxidant property. Hespiridine has shown to increase the level of catalase and superoxide dismutase. Flavonids like hesperedin, can *selectively inhibit human Cytochrome P450*, 35 reducing the absorption and elimination of toxic compounds. Hesperidine can *stimulate DNA repair pathways*, through transcription regulation or mRNA stabilization and helps in protecting and repairing DNA damage induced by cyclophosphamide. It is obvious that hesperidin, may with antioxidative activity, reduced the oxidative stress[36] and genotoxicity induced by cyclophosphamide in mouse bone marrow cells. Flavonoid constituents with antioxidative activity,[37] may return the GSH level to normal in stress conditions and reduces genotoxicity induced by cyclophosphamide in bone marrow cells. Antioxidant effect of hesperidine was revealed by screening procedures mainly comet assay. Citrus at about a concentration of 400mg/kg is found to be effective as a anticlastogenic compound.[38] The protective effect of citrus extract was investigated by using the micronucleus assay for anticlastogenic activity in mouse bone marrow cells.[39]

Beta-carotene

Beta-Carotene (BC) is a terpenoid mainly obtained from carrot. It is a red-orange natural food colouring agent and an antioxidant,[40] having anticlastogenic property. It acts as anticlastogenic by inhibition of various cytochrome P-450 linked 7-alkoxyresorufin-O-dealkylase enzyme activities,[41] it is demonstrated that in animals supplemented with beta-carotene there is a significant decrease in number of cyclophosphamide induced micronuclei in

polychromatic erythrocytes in bone-marrow of mice .[42]

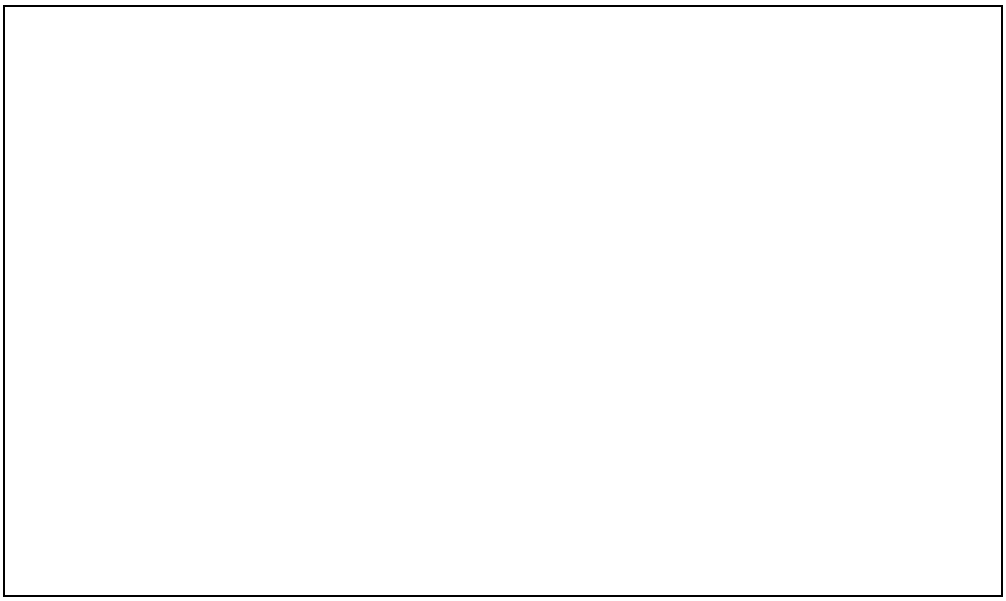
Anthocyanin:

Anthocyanins are the largest group of water-soluble pigments in the plant kingdom. A number of studies have demonstrated that anthocyanins present antioxidant capacity and show inhibitory effects on the growth of some cancer cells. Pretreatment with higher doses of the anthocyanin (10 and 20mg/kg B.W.) there is a significant reduction in the frequency of micronuclei in polychromatic erythrocytes induced by cyclophosphamide. The pattern of reduction ranged from 48% to 57% independent of concentration. No apparent genotoxicity and mutagenicity was found for either the anthocyanin or delphinidin extracts.[43] This finding explains the potential of natural colorants to prevent mutations and also the applicability of genotoxic evaluation for improving health.

Genotoxicity tests:

Ames test:

General Procedure



Problems

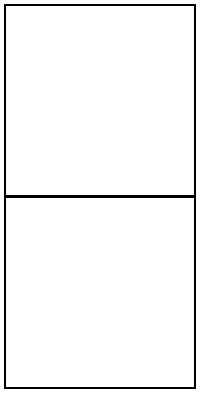
As Salmonella is a prokaryote, it is not a perfect model for humans.[44]

Micronucleus test

Micronulei are formed as a result of chromosomal breakage or spindle damage. During cell division fragments of chromosomes may not be included in the nuclei of the daughter cells and form single, or multiple micronuclei in the cytoplasm of these cells. These micronuclei are also known as Howell-Jolly bodies. Erythrocytes are chosen for examination, since micronuclei are not obscured by the main nucleus are easily detected [45, 46]. All the chromosomal damage detected in immature erythrocytes (less than 24 hours old) during recent exposure to the genotoxic drug.

Immature erythrocytes can be differentiated using a variety of staining techniques which depends on their relatively high content of residual DNA. Giemsa is used for mouse bone marrow/peripheral blood and this stains immature erythrocytes blue, and mature erythrocytes that have low nucleic acid content, appear pinkish-orange. Analysis of lymphocytes in the presence of cytochalasin-B (added 44 hours after the start of cultivation), which inhibits actins, made possible to

distinguish between mononucleated cells which did not divide and binucleated cells which completed nuclear division during *in vitro* culture.



The micronucleus assay in combination with fluorescence in situ hybridisation (FISH) with a probe labelling the (peri-)centromeric region of the chromosomes allows distuinguishing between micronuclei containing a whole chromosome (centromere positive micronucleus) and an acentric chromosome fragment (centromere negative micronucleus).[47]

Feutures of micronuclei:

- the diameter of the MN should be less than one-third of the main nucleus
- MN should be separated from or marginally overlap with main nucleus as long as there is clear identification of the nuclear boundary .[48]

Chromosome aberration test

Chromosome aberration is defined as any change in the normal structure and number of chromosomes.

Human lymphocytes has been shown feasible for the detection of chromosome damage. As peripheral lymphocytes are in the resting G_0 stage of the cell cycle, they have to be stimulated to divide by a specific antigen, like phytohaemagglutinin.[49]

Chromosomal aberrations can be divided into two main classes:

1. chromosome-type aberrations, involving both chromatids of a chromosome,
2. chromatid-type aberrations involving only one of the two chromatids.

Ionizing radiation induces chromosome-type aberrations (symmetric aberrations), like *dicentrics, inversions, ring chromosomes,* in the G0 or G1 stage of the cell cycle (i.e. prior to replication)

While chromatid type aberrations (asymmetric aberrations), like *breaks and gaps*, are produced during the S or G2 stage (i.e. during or after replication). Most *chemical mutagens* are *S-dependent clastogens* and therefore produce chromatid-type aberrations.

Several types of aberrations can be distinguished:

Structural aberrations:

- gaps
- breaks
- dicentric chromosomes
- ring chromosomes

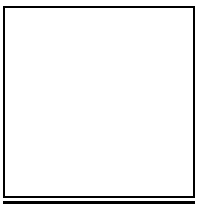
Numerical aberrations:

- polyploidy (4n)
- hypodiploidy (< 46chr)
- hyperdiploidy (> 46chr)

Characteristics of CA test:

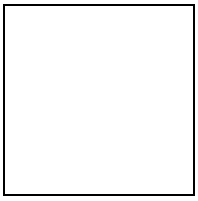
- Biomarker for cancer risk assessment.
- End-point: chromosome (and genome) mutations
- Discrimination between chromatid type aberrations, chromosome type aberrations

Chromosome abberations



Comet assay

The comet assay is also known as Single Cell Gel Electrophoresis assay which is a sensitive technique for the detection of DNA damage. It was first described by Singh *et al.* in 1988. This is a standard technique for evaluation of DNA damage/repair, and genotoxicity testing. The procedure involves the encapsulation of cells in a low-melting-point agarose suspension, lysis of the cells in neutral or alkaline (pH>13) conditions, and electrophoresis of the suspended lysed cells. This is followed by visual analysis with staining of DNA and calculating fluorescence to determine the extent of DNA damage.[50] This can be performed by manual scoring or automatically by an imaging software. An electric field is applied (typically 1 V/cm) for ~20 minutes. The slides are then neutralised to pH 7, stained with a DNA-specific fluorescent stain and analysed using a microscope with an attached CCD (charge coupled device - essentially a digital camera).



CONCLUSION

In the view of these results, it is possible that common mechanisms of action of the natural products and their extract, such as antioxidant activity, free radical scavenging and even gene regulation, could contribute to their direct and indirect anticlastogenic profile.In utilizing pharmacologically active herbs, both therapeutic and potential adverse effects must be taken into consideration. The dose and form of the plant to be administred is also taken in to account.Therefore it is essential to investigate the potential impact of these herbs on human health and to explain their mechanisms of action.

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