Genoprotective activity of hydro alcoholic extract of *Curcuma aromatica* and *Curcuma zedoaria* rhizomes and their comparison with Curcumin

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**Article history**
Received: 26 December, 2012
Accepted: 20 January, 2013
Available online: 21 March, 2013

**Keywords:**
Curcuma aromatica, Curcuma zedoaria, Curcumin, SOS Chromotest, DNA sugar damage assay, DNA-damage Protective activity, Plasmid nicking assay.

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**Abstract**
Food and Drug Administration (FDA) has approved curcumin as a food supplement for the cancer patients. Most of the anticancer drugs are also mutagenic in nature. In many literatures, it has been reported that curcumin has both genotoxic and mutagenic activity. In this case it is very much essential to get thorough information about the curcumin genotoxicity. Therefore, this research is designed to carry out the antioxidant and genotoxicity of curcumin. In this paper, genotoxicity of curcumin and for the plants that belongs to *Curcuma* species such as *Curcuma aromatica* salisb and *Curcuma zedoaria* (Christm.) Roscoe have been focused. Genotoxicity studies of the hydro alcoholic extract of *Curcuma aromatica*, *Curcuma zedoaria* and Curcumin were performed by SOS chromotest, DNA sugar Damage and Plasmid nicking assay and sequential analysis. Curcumin alone was found to be genotoxic even at the concentration of 250 µg/ml where as the hydro alcoholic extract of *Curcuma aromatica* and *Curcuma zedoaria* was found to be non- genotoxic even at the highest concentration i.e 1000 µg/ml.

**Citation:**

**1. Introduction**

1.1 Importance of DNA
Deoxyribonucleic acid is a genetic material that can able to store the information which has to be transferred from one generation to another. Continuously all the living organisms are exposed to various chemicals and environmental substances that can damage their DNA, RNA and proteins. The maintenance of DNA integrity in somatic cell is of paramount importance because damage to the DNA, which carries the genetic information, can have serious consequence for the organism. DNA damage is one of the causes for the reduction in homeostatic capacity related to age and increased incidence of cancer and other disease such as Alzheimer’s disease because of the dysfunctioning of cells and tissue. Cell has a variety of DNA- repair systems which are able to move or circumvent lesions that might otherwise interfere with DNA replication and transcription. DNA repair is not always perfect. Certain forms of DNA damage accumulate with age because of age related decrease in DNA repair efficiency. The extent to which the individuals are exposed to DNA- damaging agents and their activity of DNA- repair systems can lead to larger inter- individual differences in the rate of damage accumulation.
1.2 Role of antioxidant in DNA damage
Various enzyme systems such as superoxide dismutase (SOD), catalase and glutathione eliminate DNA damaging agents before they interact with the DNA. Superoxide dismutase converts the \( \text{O}_2^- \) radical into hydrogen peroxide which in turn converted into water by catalase, glutathione peroxidase and their level can differ between different species. There are several substances such as selenium, vitamin E, vitamin C, glutathione cysteine generated in the cells or present in the food that have the ability to scavenge free radicals. Cells are equipped with a battery of repair systems to remove DNA damage which can be divided into three categories. They are direct repair, excision repair and post replication repair (Mullaart et al., 1990). Varieties of biologically active compounds are present in plant foods and products include antioxidant properties. Increased dietary intake of natural phenolic antioxidants correlates with reduced coronary heart disease. In the prevention of cardiovascular disease, cancer, neurodegenerative diseases including Parkinson’s and Alzheimer’s disease, inflammation, problems caused by cell and cutaneous aging, food rich in antioxidants plays an essential role. Natural antioxidants, vitamins, minerals, soluble and insoluble fibers were found to present in fruits, vegetables and oil seeds. For application as nutraceuticals as well as food additives, natural antioxidants are in high demand because of consumer preferences (Kumar and Chattopadhyah, 2007).

1.3 Reason for screening these plants for this research
Curcuma species are perennial herbs belonging to the family Zingiberaceae. From that species Curcuma aromatica and Curcuma zedoaria has been selected for this study. Curcuma aromatica and Curcuma zedoaria are the plants used in Indian traditional medicines to cure various ailments. Moreover we have carried out many researches to find out various biological activities such as antioxidant (Srividya et al., 2013 submitted for publication) anti-diabetic (Srividya, et al., 2012), antihyperlipidemic (Srividya et al., un published) activity and they have shown prominent biological activities when compared to the standards used. Curcumin content was also estimated in these plants and it was found to be 6.1±2.13 %, 3.8±1.76 % for Curcuma aromatica and Curcuma zedoaria respectively.

1.4. Reported biological Activities for the selected compounds
Historically Curcuma aromaticia rhizome is used as tonic, carminatives astringent and bitters. It is also used in sprain and in snake bite. They are also used for treating skin eruption due to infection. It is found to possess essential oil namely α-curcumene and β-curcumene, d and p- methoxy cinnamic acid. The coloring matter is Curcumin (Naglaa M. Ebeed, 2010). Traditionally Curcuma zedoaria rhizome is used as carminative, stomachic, gastrointestinal stimulant, diuretic, expectorant demulcent, rubifacient and also used in flatulence and dyspepsia. Fresh root is used for checking leucorrhea discharge and as blood purifier. The ayurvedic pharmacopoeia of India indicated the use of the rhizome in goiter. Dried rhizome contain a number of terpenoids including curcumin, curcumenone, curdione, curcumenol, curzerenone, furanogeranone, germacrene, germacrene epoxide a volatile toil (1.0-1.5) resembling ginger oil and starch (50%). It is used in the treatment of goiter, and also used as antitumor, antiallergic and antimicrobial (Ansari et al., 2009; Khare, 2007). The curcumin, the most active components which make up to 2-5 % of spice. Curcumin (C\(_{15}\)H\(_{18}\)O\(_6\)) which is an orange yellow crystalline powder practically insoluble in water. Curcumin has been consumed as a dietary spice at dose up to 100 mg/day for centuries. Over the last five decades, extensive investigations about curcumin were carried and revealed the uses such as, reduction of the blood cholesterol (Rao et al 1970), prevention of LDL peroxidation (Ramire Z-Tortosa et al., 1999) inhibition of platelet aggregation (Srivastava et al., 1986), suppression of thrombosis (Srivastava et al., 1985), cures myocardial infarction (Dikshit, et al., 1995), suppress symptoms associated with type–II diabetes (Srinivasa, 1972), cures rheumatoid arthritis (Deodhar, et al., 1980), Multiple Sclerosis MS (Natarajan and Bright, 2002), Alzheimer’s disease (Lim, et.al., 2001), inhibits human immune deficiency virus (HIV) replication (Sui et al., 1993), enhances wound healing (Sindhu, et al., 2006), protects from liver injury (Morikawa et al., 2002), increases bile secretion (Ramprasad and Sirsi, 1956), protects from cataract formation (Awasthi et al., 2002), protects from pulmonary toxicity and fibrosis (Venkatesan and Chandrakasan, 1995). It has an anti- leshmaniasis (Saleheen et al., 2002), anti atherosclerotic (Huang, et al., 1992), antioxidant activities (Adames, et al., 2005) and anticancer properties (Huang, et al., 1988).
In literatures the adverse effects of widely used plants are not well documented. One might expect that plants which are used in long term by humans to have at least low toxicity. Through investigation it was reported that many plants that are used as food or drugs have mutagenic effects in the in vitro assay (Esameldin et al., 2005).

Purpose of this study was to investigate genotoxicity of plants used in Indian traditional medicines using in-vitro assays and this paper focuses on the assessment and comparison of genotoxicity effect of hydro alcoholic extract of *Curcuma aromatica*, *Curcuma zedoaria* with that of Curcumin by performing DNA-damage protective activity, SOS chromotest and DNA sugar damage.

2. Literature Review

2.1 Curcuma aromatica

Ahmad et al., (2012) carried out the phytochemical investigations of the chloroform extract of the rhizomes of *Curcuma aromatica*. Along with the known compounds they obtained three new phytoconstituents such as n- neneitriacontan-14-one, n- pentatriacontan-5-one, 11a-cyclopentyl-n-decan-1-ol (Curcumapentadecanol).

Pant et al., (2012) isolated three phytochemicals, curcumin, demethoxycurcumin and b-sitosterol-3-O-b-D-glucopyranoside from the ethyl acetate extract of rhizomes of *Curcuma aromatica*. They isolated b-sitosterol-3-O-b-D-glucopyranoside for the first time from this plant.

Al-Reza et al., (2010) evaluated the chemical composition of hydro distilled essential oil from leaves of *Curcuma aromatica* salisb by GC-MS method and it showed the presence of twenty three components. Antioxidant studies of this oil and methanolic extract was carried out by DPPH method, Superoxide radical scavenging assays. Methanolic extract was found to be superior in exhibiting the antioxidant activity. Total phenolic content was found to be higher in ethyl acetate extract when compared to other extracts.

Wu et al., (2000) revealed the inhibitory effects of *Curcuma aromatica* oil on cell proliferation of hepatoma in mice by DNA image cytometry and immune histochemical staining of proliferating cell nuclear antigen method. Index of hepatic carcinoma was found to be lower after the administration of *Curcuma aromatica* oil.

Snu- YaoTsai et al., (2011) isolated the essential oils from the dried rhizomes of *Curcuma aromatica* salisb, *Curcuma longa*, *Curcuma sichuanensis* by using simultaneous steam distillation and solvent extraction apparatus. It showed the presence of curcumon and 1,8- cineole in *Curcuma aromatica*, α- tumerone, humulene oxide and selinene in *Curcuma longa*, and α- tumerone, selinene and cadinene in *Curcuma sichuanensis*. Antioxidant activity of *Curcuma aromatica* was found to be least when compared to *Curcuma sichuanensis* and *Curcuma longa*.

2.2 Curcumin

Hideji Itohawa et al., (2008) evaluated curcumin derivatives for their bioactivity and structural activity. They also reviewed the anti-inflammatory, antioxidant, anti- HIV, chemo preventive and anti-prostate cancer effects of curminoids.

Chun Zhao et al., (2012) studied the antidepressant effect of curcumin that are mediated by the percentage HT system and also the effect of curcumin on the behavioral consequences of methamphetamine (METH). Curcumin enhanced the behavioral effect of METH.

Ming-Xiang Yea et al., (2012) investigated the mechanism of curcumin as a chemo sensitizer in lung cancer. Curcumin sensitized various chemotherapeutic agents in human breast, colon, pancreas, gastric, liver, brain and hematological malignant disorders in both in-vivo and in-vitro models in cancer cell lines by inhibiting HIF-1 expression and activating caspase-3.

Santosh et al., (2012) explored the delivery of curcumin, a broad-spectrum anticancer drug in the form of liposomal nanoparticles to treat osteosarcoma because of its insoluble in nature. For the treatment of cancers of different tissue origin, Curcumin loaded γ-cyclodextrin liposome is considered to be a potential delivery vehicle.
Sindhwani et al., (2011) reported the cytotoxic effects of curcumin on bladder tumor cell lines as well as its effects on the intra-vesicle implantation of tumor cells in C3H mice with the intention of developing an effective nontoxic intra-vesicle agent that might be used immediately after bladder tumor resection to prevent the implantation of tumor cells.


Mahendra P. Singh et al., (2011) tested the genotoxic and apoptotic potential of monocyclic aromatic hydro carbons such as benzene, toluene and xylene which are extensively used for various industrial and household purposes by using the Drosophila model. They also evaluated effect of quercetin and curcumin in attenuating these chemical’s induced toxicity. Exposure to benzene increased apoptotic makers and genotoxicity with concentration and time dependent manner in organism. Along with the quercetin or curcumin, cytochrome P450 activity, GST levels, oxidative stress parameters, genotoxic and apoptotic endpoints were found to decrease.

Loganathan Palanikumar et al., (2011) evaluated the Clastogenic activity of curcumin and aloin at different doses such as 5, 10, 20, 30, 50 and 100 μg/ml. The mitotic index was significantly lowered at higher concentrations in plant test systems.

European Food Safety Authority, (2010) provided a scientific opinion for re-evaluating the safety of curcumin (E 100). Intake of curcumin from the normal diet amounts to less than 7% of the ADI of 3 mg/kg bw/day. Somaya Youssef Mostafa Hamoudah et al., (2010) investigated the protective role of ginger and curcumin powders against some toxicological effects of thermo oxidized frying cotton oil in male albino rats. The addition of ginger or curcumin to diet of OFO fried cotton oil produced improvement in the liver function, decreased the glucose level, increased the level of total antioxidants, reduced the frequencies of chromosomal aberrations and showed improvement in hepatic pathological changes. Ginger and curcumin can protect against toxicity of frying oil.

2.3. Curcuma zedoaria
Yogamaya Dhal et al., (2012) identified the presence of antioxidant enzyme like catalase, guaiacol, peroxidase, glutathione peroxidase and superoxide dismutase in the leaf extract of Curcuma zedoaria (Christm.) Roscoe and also carried out antioxidant activity with that of enzymatic leaf extract. The maximum antioxidant activities were found in catalase, glutathione peroxidase and superoxide dismutase enzyme where as the guaiacol peroxidase showed poor antioxidant activity as compared to ascorbic acid.

Vitor Hugo Farina et al., (2012) evaluated the effect of Curcuma zedoaria and Camellia sinensis on halitosis (bad breath) which is produced due to volatile sulphur compounds. Both the plant extracts showed the immediate inhibitory effects but no residual inhibitory effects on VSC.

Tauheeda Riaz et al., (2011) evaluated in vitro antioxidant activities and also screened the phytochemical constituents in organic and aqueous fractions of Curcuma zedoaria (Christm.) Roscoe. Chloroform fraction showed potent antioxidant activity and ethyl acetate fraction showed moderate antioxidant activity.

Fátima Navarro et al., (2002) carried out phytochemical analysis and analgesic activity of hydro alcoholic extract of Curcuma zedoaria rhizome grown in Brazil and isolated curcumenol which is the main active principle showed potent and dose-related

2.4. Objectives of this study
1. To establish the safety evaluation of the botanical that belongs to the Curcuma family at the cellular level.

2. To investigate the circumstances under which phytochemicals from the Curcuma species exhibit either beneficial or harmful effect.

3. To provide meaningful and significant testing data to the herbalists and practitioners of alternative medicine.

4. To encourage the botanical drug sponsors to obtain genotoxic information early in their
product development by performing the genotoxicity studies.

With the intake of plant materials, epidemiological studies support that the chemo preventive effects in which phytochemicals exhibit genotoxic/mutagenic effect by themselves or by potentiating the effect of other xenobiotics. To investigate the circumstance under which phytochemicals used in traditional medicine as potential prophylactic agents either exhibit beneficial or harmful effects is very much important. The presence of phyphenolic components including tannins, catechin, flavonones, isoflavones are responsible for the possible genotoxic effects of plant extracts. Genotoxicity might be related to hydrogen peroxide formation arising from auto oxidation of phenolic molecules. Flavonoids inhibit topoisomerase I and II enzyme which will interfere with the replication and transcription process, inhibiting the relegation of DNA double strand breaks and enhancing the formation of cleavable DNA-enzyme complexes. Phenolic rich extracts could lead to accumulated DNA breaks and mutation, thus contributing significantly to genotoxicity. The extract which exhibits potent antioxidant and free radical scavenging properties ascribed to its polyphenolic richness more particularly to its flavonoid content.

Through scientific research, there have been many validations of traditional medicines. Direct use of isolated bioactive compounds in medicine, ethno medical information’s has contributed to heath care worldwide. In the literature, the adverse effects of widely used plants are not well documented. One might expect that plants used in traditional medicine to have low toxicity based on their long term use. In in-vitro assay, recent investigations have revealed that many plants used as food or in traditional medicine have mutagenic effects. Long term use of plants has raised concern about the potential mutagenic hazard (Esameldin et al., 2003). When compared to the individual chemical constituents, the genotoxic information obtained from studies using a whole herbal or multi- component herb product is relatively lacking. Therefore, it is important to evaluate the genotoxic properties of the plant extracts.

Curcumin is an ingredient that is obtained from the Curcuma species. The plant that belongs to the Curcuma family contains curcumin as one of the constituents but its proportion gets vary from one species to another. Curcumin is reported to produce chromosomal aberration and genotoxicity. Because of the poor bioavailability of Curcumin, it is expected to find out an alternate from the plant source itself to increase the bioavailability. At the same time presence of other constituents in the plant extract could able to protect the cells from genotoxicity effect. Now, FDA has approved curcumin as the food supplements for the cancer patients. Based up on the research literatures Curcumin is found to be genotoxic. In this circumstance it is necessary to undergo through investigations related to curcumin genotoxicity and in case if it has to be replaced by some other plant source, we have carried out the research with Curcuma aromatica and Curcuma zedoaria rhizomes which found to possess 6.14±2.13 % and 3.84±1.76 % of curcumin respectively.

3. Materials and Methods

Curcuma aromatica, Curcuma zedoaria (PSS Herbs Pvt. Ltd, Kerala, India), Curcumin was sponsored by Sami Lab Pvt. Ltd, Bangalore, Mitomycin-C (Sigma Life Sciences) Cyclophosphamide monohydrate (Fluka), Metabolic activation system (S9 Molecular Toxicology, INC, Boone. NC 28607, USA).

3.1 Plant collection and authentication

The rhizomes of Curcuma aromatica salisb and Curcuma zedoaria (Christm.) Roscoe were purchased from PSS Herbs Pvt. Ltd, Kerala, India in the month of July 2012 and authenticated by Dr. S. Rajan, Botanist, Udhagamandalam. Curcumin was sponsored by Sami Labs Pvt. Ltd, Bangalore. This research was carried out in Department of Pharmaceutical Biotechnology, JSS College of Pharmacy, Rocklands, Ootacamund, Nilgiri district, Tamil Nadu, South India

3.2 Preparation of the powder

The rhizomes of Curcuma aromatica salisb, Curcuma zedoaria (Christm.) Roscoe are shade dried, milled, and coarse powder is separated.

3.3 Preparation of extract

50% hydro alcoholic extracts are prepared for Curcuma aromatica salisb, Curcuma zedoaria (Christm.) Roscoe rhizomes and these prepared extracts are used for these genotoxicity studies.

3.4. DNA Sugar Damage (Miral Dizdaroglu., 1991; Montserrat Casadevall, et.al., 1999)

Variety of DNA lesions such as single strand breaks (SSB), alkali- labile sites, DNA-Protein
cross links, DNA- amino acid cross links and DNA adducts occurs due to genotoxicity of various compounds which as well established as carcinogens. To form with 4'- hydroxylated AP site or an SSB with 3' phosphoglycolate and 5' phosphate ended fragment and a base propenal, there is a partitioning of degradation products along with two pathways. In such a case deoxy ribose degradation products such as base propenals should be released up on reacting with DNA and readily detected by using thiobarbituric acid (TBA) assay. The release of free bases and deoxyribose fragments are caused in the presence of nucleotides or the complex which has been formed between the test compound and DNA.

3.4.1 Principle for estimating the DNA sugar damage

A pink chromogen is formed when Malondialdehyde which is formed by the oxidative degradation of deoxyribose reacts with thiobarbituric acid. At acidic pH, at temperature close to 100° C, this reaction is quantitative.

Calf thymus DNA → Fenton’s reagent → 2-Deoxyribose → Oxidative degradation → Malondialdehyde (MDA) + TBA → a pink chromogen → Measured at 532 nm.

After treatment MDA – like products were not released free in solution but remains attached to the DNA. Prior to TBA assay, DNA was precipitated by using acid. All the TBA reactive products were recovered as resuspended DNA pellets. For the release of MDA and the formation of the TBA adduct a recombination of acid' a heat condition was required. During TBA assay, hydroxyl radicals, which are capable of oxidizing the DNA sugar moiety release the MDA- like product. For hydroxyl radicals, indeed the colour reaction with TBA has been described as a simple test tube assay. Deoxyribose fragments are called “MDA- like substance” due to their ability to react with TBA in a fashion similar to MDA and the formation of such species is considered as a marker for the oxidation at C4'. Hydroxyl radicals can be ruled out as the oxidizing species leading to the formation of SSB and AP sites. Upon the oxidation of C4' the carbon centered radical intermediate is generated which can either hydroxylated to yield an abasic site or it can react with molecular oxygen to produce a peroxy radical species which may yield a SSB.

3.4.2 Procedure

Total volume of 1.24 ml contain 0.5 ml of calf thymus DNA (1 mg/ml of 0.15 N NaCl), 0.5 ml of phosphate buffer (0.1M pH 7.4) and 0.2 ml of Ascorbic acid (1mM) and 0.04 ml of FeCl₃ (100 µM) concentration. The reaction mixture was incubated for 1 hour at 37° C in a water bath shaker. After incubation TBA (1ml) was added to the reaction mixture and then it was kept in boiling water bath for 15 minutes. TBA reacting species so generated forms an adduct which shows the characteristic absorption at 535 nm.

3.5. SOS Chromo test (Manon Bombardier, et al., 2001; Skandrani, et. al., 2010)

For detecting DNA damaging agent the SOS chromo test assay is a bacterial test which involves a set of functions known as the SOS response. Lac Z is the structural gene for β-galactosidase under the control of SfiA gene. In cell division inhibition, a SOS function which is involved helps to device a simple and direct colorimetric assay of the SOS response to DNA damage. The SOS chromo test employs error prone DNA repair pathway E. coli PQ-37 also known as the SOS response, a complex regulatory net work that is induced by DNA damaging substances. As a measure of genotoxicity, the bacteria E. coli PQ-37 with the sample and subsequent determination of β-galactosidase (β-gal) and alkaline Phosphatase (AP) was performed.

3.5.1 Procedure

E. coli PQ-37 strain was grown in nutrient broth and incubated for 24 hours at 37° C. 500 µl of the suspension of microorganism was mixed with 50 µl of extract which contains various concentration of plant extract such as (1000, 500, 250 µg/ml) along with 450 µl of ultra pure water in RIA tubes. The tubes were then incubated at 37° C for 2 hours and then centrifuged at 7000 rpm for 20 minutes. The supernatant was discarded and bacterial pellets were resuspended with 200 µl of (100 µg/ml concentration of SOS chromogen (p-nitrophenyl phosphate (PNPP) for the detection of Alkaline Phosphatase. The tubes were incubated for 10 minutes and the absorbance was measured at 405 nm.

For the estimation of β-galactosidase, 500 µl of suspension of microorganism was mixed with 50 µl of the plant extract which contains different concentration of plant extract such as (1000, 500, 250 µg/ml) along with 450 µl of ultra pure water in RIA tubes. The tubes were then incubated at 37° C for 2 hours and then centrifuged at 7000 rpm for 20 minutes. The
supernatant was discarded and bacterial pellets were resuspended in 200 µl of 100 µg/ml concentration of SOS chromogen (5-Bromo- 4-chloro- 3- idoly b-D-galactopyranoside). The tubes were incubated for 60 minutes and the absorbance was measured at 620 nm.

Control was maintained with E. coli-PQ37 culture without plant extract.

Each extract was tested in four replicates. 1 ml of extract + 1 ml of E.coli PQ 37

<table>
<thead>
<tr>
<th>Incubate 2 hours at 37 0 C</th>
<th>centrifuge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pellets + P. Nitrophenyl phosphate (100 µg/ml)</td>
<td>405 nm</td>
</tr>
</tbody>
</table>

Pellets + 5-Bromo-4-chloro-3-idoly b-D-galactopyranoside (100 µg/ml) | 620 nm |

Alkaline Phosphatase is known as reduction factor which is represented by RF.

β-Galactosidase is known as induction factor which is represented by IF.

Correction Induction factor = CIF= IF/RF

RF= X OD\textsubscript{405} / X OD\textsubscript{405} C

IF= X OD\textsubscript{620} / X OD\textsubscript{620} C

CIF= IF/RF

X= Mean of four OD readings of test compound

C= control

RF and IF- value accounts for the background activity of the control.

The ratio of IF to RF units yield an estimate of β-gal activity corrected for toxicity a normalized induction factor of 1.2 or more was considered to present significant genotoxic activity.

Assay variance was determined using ranges standard deviations and coefficient variations.

Quercetin was used as positive control:

Plasmid DNA+ Fenton’s reagent + quercetin 250 µg/ml—loaded in the lane 1 and 15

Plasmid DNA+ Fenton’s reagent + quercetin 500 µg/ml—loaded in the lane 2 and 16

Plasmid DNA + Fenton’s reagent+ Quercetin 1000 µg/ml—loaded in the lane 3 and 17

Plasmid DNA+ Fenton’s reagent+ Curcuma aromatica 250 µg/ml- loaded in the lane 4 and 18

Plasmid DNA+ Fenton’s reagent+ Curcuma aromatica 500 µg/ml- loaded in the lane 5 and 19

pBR\textsuperscript{322} was purchased from Chromos biotech, Bangalore. Different concentration of 50 % hydro alcoholic extract of Curcuma aromatica, Curcuma zedoaria and Curcumin were prepared. 10 µl of the each concentration of the extract was incubated with 5 µl of DNA for 10 minutes at room temperature followed by 10 µl of Fenton’s reagent for 45 minutes at 300 C. Blank was prepared by mixing 5 µl of DNA with 20 µl of PBS.

3.6.1 Preparation of Gel

0.8 % of agarose was prepared by boiling 600 mg of agarose powder in 40 ml of 1 x TAE buffer till it looks clear. Mean while the comb was adjusted in such a way that the comb on the left side is about 2 cm from the cathode (negative rod), when the gel temperature was around 50° C, gently poured into the gel tray without creating any air bubble and kept it undisturbed till the gel solidified. Once the gel gets solidified poured the 1x TAE buffer slowly into the gel tank till the buffer level stands 0.5-0.8 cm above the gel surface and comb is gently lifted without damaging the gel. 10 µl of plasmid DNA was mixed with 10 µl of gel loading dye, 10 µl of this mixture was added to each well. The gel was run at 50 v for 1½ hour or until the dye moves to the 1/3 of the gel length. The gel was stained with 0.6µl/ml ethidium bromide for 30 minutes. After 30 minutes, stained gel was detained using the destaining solution. After destaining the gel was placed under UV illuminator and photographed.

Plasmid nicking assay (Kumar A., Chattopadhyah S., 2007; Singh, et. al., 2009; Verma, et.al., 2010)

In the plasmid nicking, assay, addition of Fenton’s mixture to the plasmid DNA increases the formation of single and double stranded nick and linear forms of DNA due to the attack of OH. Radicals generated in the reaction mixture on the nitrogenous bases or deoxyribosyl back bone.

pBR\textsuperscript{322} was prepared by mixing 5 µl of DNA with 20 µl of PBS.
Plasmid DNA + Fenton’s reagent + Curcuma aromatica 1000 µg/ml loaded in the lane 6 and 20.

Plasmid DNA + Fenton’s reagent + Curcuma zedoaria 250 µg/ml loaded in the lane 7 and 21

Plasmid DNA + Fenton’s reagent + Curcuma zedoaria 500 µg/ml loaded in the lane 8 and 22

Plasmid DNA + Fenton’s reagent + Curcuma zedoaria 1000 µg/ml loaded in the lane 9 and 23

Plasmid DNA + Fenton’s reagent + Curcumin 250 µg/ml loaded in the lane 10 and 24.

Plasmid DNA + Fenton’s reagent + Curcumin 500 µg/ml loaded in the lane 11 and 25

Plasmid DNA + Fenton’s reagent + Curcumin 1000 µg/ml loaded in the lane 12 and 26

Plasmid DNA alone was used as control loaded in the lane 13 and 27.

Plasmid DNA treated with Fenton’s reagent was used as negative control loaded in the lane 14 and 28.

3.7. Sequencing method
To confirm the mutagenicity of Curcuma aromatica, Curcuma zedoaria, and Curcumin, sequential analysis were carried out by treating the pBR322 with 1000 µg/ml concentration of the testing materials.

4. Results

4.1. SOS Chromotest
For the detection of alkaline Phosphatase activity P- nitrophenyl phosphate (PNPP) was used as the standard SOS chromogen and the plates were incubated for 10 minutes and it absorbance were measured at 405 nm. Alkaline Phosphatase activity reflects the reduction factors which is represented as RF. For β-galactosidase (β- gal) activity 5- bromo-4- chloro-3-indolyl b- D-galactopyranoside was used as the standard SOS chromogen and the plates were incubated for 60 minutes and its absorbance were measured at 620 nm. β- galactosidase activity reflects the induction factors IF. The ratio of the induction factor and reduction factor is the correction factor and their results were tabulated in table no. 1, 2, 3 and 4. The absorbance both at 405 nm and 620 nm was found to increase with the concentration of hydro alcoholic extract of Curcuma aromatica, Curcuma zedoaria and Curcumin. RF values were found to increase with the concentration hydro alcoholic extract of Curcuma aromatica, Curcuma zedoaria and Curcumin. Induction factor were also found to increase with the concentration of hydro alcoholic extract of Curcuma aromatica, Curcuma zedoaria and Curcumin.

4.1.1 For Curcuma aromatica the corrected induction factor produced at the concentration of 250 and 500 µg/ml was found to be more or less equal such as 0.9711 and 0.9403 where as the correction induction factor at the concentration of 1000 µg/ml was found to be 1.0309. All these values were found to be less than 1.2 which is considered as an index to represent genotoxicity. The hydro alcoholic extract of Curcuma aromatica was considered to be non- genotoxic.

4.1.2 For Curcuma zedoaria the corrected induction factor was found to be 0.4299, 0.8943 and 1.1975 for 1000, 500 and 250 µg/ml concentration respectively. The corrected induction factor was found to be more at the concentration of 250 µg/ml. The corrected induction factor found to decrease with the increase in the concentration of the hydro alcoholic extract Curcuma zedoaria from 500 µg/ml to 1000 µg/ml. All these values were found to be less than 1.2 which is considered as an index to represent genotoxicity. From the values it was considered that hydro alcoholic extract of Curcuma zedoaria as non- genotoxic.

4.1.3 For Curcumin the corrected induction factor was found to be 1.3308, 1.3725 and 1.1975 for 1000, 500 and 250 µg/ml. Curcumin was found to be genotoxic even at the concentration of 250 µg/ml onwards and corrected induction factor was found to increase with the increase in the concentration from 500 µg/ml to 1000 µg/ml. Curcumin was considered to be genotoxic because all the values obtained were found to be more than 1.2 which is considered as an index to represent genotoxicity.

4.2. DNA Sugar Damage
Among the selected test compounds hydro alcoholic extract of Curcuma aromatica and Curcumin, protected the sugar moiety in DNA at 250 µg/ml onwards where as Curcuma zedoaria protected the DNA only at 1000
Table 1: Absorbance of the extract at 405 nm and 620 nm

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/ml)</th>
<th>Absorbance (405 nm)</th>
<th>Sample</th>
<th>Concentration (µg/ml)</th>
<th>Absorbance (620nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcuma aromatica</td>
<td>1000</td>
<td>0.676</td>
<td>Curcuma aromatica</td>
<td>1000</td>
<td>0.317</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.671</td>
<td></td>
<td>500</td>
<td>0.287</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.609</td>
<td></td>
<td>250</td>
<td>0.269</td>
</tr>
<tr>
<td>Curcumin</td>
<td>1000</td>
<td>0.603</td>
<td>Curcumin</td>
<td>1000</td>
<td>0.365</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.567</td>
<td></td>
<td>500</td>
<td>0.354</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.514</td>
<td></td>
<td>250</td>
<td>0.280</td>
</tr>
<tr>
<td>Curcuma zedoaria</td>
<td>1000</td>
<td>1.897</td>
<td>Curcuma zedoaria</td>
<td>1000</td>
<td>0.371</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.880</td>
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<td>500</td>
<td>0.358</td>
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<tr>
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<td>250</td>
<td>0.652</td>
<td></td>
<td>250</td>
<td>0.350</td>
</tr>
<tr>
<td>Blank</td>
<td></td>
<td>0.897</td>
<td>Blank</td>
<td></td>
<td>0.408</td>
</tr>
</tbody>
</table>

Table 2: Determination of the Repression factor for the SOS Chromotest

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/ml)</th>
<th>Absorbance (405 nm)</th>
<th>RF values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcuma aromatica</td>
<td>1000</td>
<td>0.676</td>
<td>0.7536</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.671</td>
<td>0.7480</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.609</td>
<td>0.6789</td>
</tr>
<tr>
<td>Curcumin</td>
<td>1000</td>
<td>0.603</td>
<td>0.6722</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.567</td>
<td>0.6321</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.514</td>
<td>0.5730</td>
</tr>
<tr>
<td>Curcuma zedoaria</td>
<td>1000</td>
<td>1.897</td>
<td>2.1148</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.880</td>
<td>0.9810</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.652</td>
<td>0.7268</td>
</tr>
<tr>
<td>Blank</td>
<td></td>
<td>0.897</td>
<td></td>
</tr>
</tbody>
</table>

Table 3: Determination of Induction Factor for the SOS chromotest

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/ml)</th>
<th>Absorbance (620nm)</th>
<th>IF values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcuma aromatica</td>
<td>1000</td>
<td>0.317</td>
<td>0.7769</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.287</td>
<td>0.7034</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.269</td>
<td>0.6593</td>
</tr>
<tr>
<td>Curcumin</td>
<td>1000</td>
<td>0.365</td>
<td>0.8946</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.354</td>
<td>0.8676</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.280</td>
<td>0.6862</td>
</tr>
<tr>
<td>Curcuma zedoaria</td>
<td>1000</td>
<td>0.371</td>
<td>0.9093</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.358</td>
<td>0.8774</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.350</td>
<td>0.8578</td>
</tr>
<tr>
<td>Blank</td>
<td></td>
<td>0.408</td>
<td></td>
</tr>
</tbody>
</table>

Table 4: Correction Induction Factor for the SOS chromotest

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (µg/ml)</th>
<th>IF Value</th>
<th>RF Value</th>
<th>CIF = IF/RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcuma aromatica</td>
<td>1000</td>
<td>0.7769</td>
<td>0.7536</td>
<td>1.0309</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.7034</td>
<td>0.7480</td>
<td>0.9403</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.6593</td>
<td>0.6789</td>
<td>0.9711</td>
</tr>
<tr>
<td>Curcumin</td>
<td>1000</td>
<td>0.8946</td>
<td>0.6722</td>
<td>1.3308</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.8676</td>
<td>0.6321</td>
<td>1.3725</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.6862</td>
<td>0.5730</td>
<td>1.1975</td>
</tr>
<tr>
<td>Curcuma zedoaria</td>
<td>1000</td>
<td>0.9093</td>
<td>2.1148</td>
<td>0.4299</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.8774</td>
<td>0.9810</td>
<td>0.8943</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.8578</td>
<td>0.7268</td>
<td>1.1802</td>
</tr>
</tbody>
</table>

µg/ml. Quercetin which is taken as the control showed the protection at 250 µg/ml. Curcumin showed the maximum protection activity among all the tested material. Results are tabulated in table No.5

4.3 Plasmid nicking assay or DNA-damage protective activity

In this method plasmid DNA pBR322 was purchased from Chromos biotech and it was used to carry out the plasmid nicking assay or DNA-damage protective activity of the test compounds. Quercetin at all the tested concentration such as 250, 500 and 1000 µg/ml showed the
damage to the plasmid and it might have occurred due to the incision in the DNA.

*Curcuma aromatica* and *Curcuma zedoaria* protected the DNA from the concentration of 250 µg/ml to 1000 µg/ml.

Curcumin caused slight damage when compared to quercetin as well as negative control in all the tested concentration such as 250, 500 and 1000 µg/ml. Results are photographed in figure No.1.

**Table 5: DNA sugar damage**

<table>
<thead>
<tr>
<th>SAMPLE NO</th>
<th>250µg/ml</th>
<th>500µg/ml</th>
<th>1000µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>0.031</td>
<td>0.031</td>
<td>0.031</td>
</tr>
<tr>
<td>Curcuma zedoaria</td>
<td>-0.063</td>
<td>0.003</td>
<td>0.025</td>
</tr>
<tr>
<td>Curcuma aromatica</td>
<td>0.037</td>
<td>0.079</td>
<td>0.223</td>
</tr>
<tr>
<td>Curcumin</td>
<td>0.110</td>
<td>0.124</td>
<td>0.284</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.028</td>
<td>0.082</td>
<td>0.090</td>
</tr>
</tbody>
</table>

Curcumin caused slight damage when compared to quercetin as well as negative control in all the tested concentration such as 250, 500 and 1000 µg/ml. Results are photographed in figure No.1.

**Figure 1:** DNA damage protective activity of *Curcuma aromatica*, *Curcuma zedoaria* and Curcumin.

![Figure 1: DNA damage protective activity of *Curcuma aromatica*, *Curcuma zedoaria* and Curcumin.](image)


**Table 6: Results for the sequencing**

<table>
<thead>
<tr>
<th>Name of the sample</th>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid 322</td>
<td>Forward primer</td>
<td>5'-GCA GGA AAG AAC ATG TGA GCA AAA GGC CA -3'</td>
</tr>
<tr>
<td></td>
<td>Reverse Primer</td>
<td>5'CAA AAT CCC TTA ACG TGA GTT TTG GT G3'</td>
</tr>
<tr>
<td>PBR 322 treated with <em>Curcuma aromatica</em> at the concentration of 50 µg/ml</td>
<td>Forward primer</td>
<td>5'-GCA GGA AAG AAC ATG TGA GCA AAA GGC CA -3'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of the sample</th>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Forward primer</td>
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</tr>
<tr>
<td></td>
<td>Reverse Primer</td>
<td>5'CAA AAT CCC TTA ACG TGA GTT TTG GT G3'</td>
</tr>
<tr>
<td>PBR 322 treated with <em>Curcuma aromatica</em> at the concentration of 50 µg/ml</td>
<td>Forward primer</td>
<td>5'-GCA GGA AAG AAC ATG TGA GCA AAA GGC CA -3'</td>
</tr>
</tbody>
</table>
5. Discussion

Several substances in plants express cytotoxic and genotoxic activities and show correlation with the incidence of tumors. Therefore, understanding the health benefits and or potential toxicity of the plants is important (Yen, et al., 2002). Although plant extracts have been used in the treatment of diseases according to knowledge accumulated over centuries, it is also known that many plants synthesize toxic substances, which in nature acts as defense against infections, infects and herbivores. Some substances present in some medicinal plants are potentially toxic and carcinogenic and it has also been reported that some traditional medicines may have genotoxic potentials. Assessment of the...
potential genotoxicity of traditional medicine is indeed an important issue as damage to the genotoxic materials may lead to critical mutation and therefore also to an increased risk of cancer and other disease. Major bioactive phytochemicals that have been associated with many plants are different types of saponins and flavonoid.

In overall safety evaluation of the botanical, a modest trend towards increasing the inclusion of information, a genotoxicity appeared in peak in the last four years because of significant awareness of the impact of genotoxicity (Kuei-Meng Wu, et. al., 2010). In in-vitro assay, recent investigations have revealed that many plants used as food or in traditional medicine have mutagenic hazard (Esameldin, et al., 2003). The isolated compounds from the plants such as Quercetin, furoquinoline, alkaloids and isothiocyanates were considered to be mutagens. It is very difficult to speculate the compounds that are responsible for mutagenic response detected with plant extracts because they are complex mixtures of organic compounds (Esameldin, et al., 2003). Short term in-vitro and in vivo studies as well as long term carcinogenicity studies, with chemically treated animals confirmed that phytochemicals could also possess antimutagenic and anticarcinogenic effect. Epidemiological studies also supported that the chemo preventive effect in which phytochemicals exhibit genotoxic/ mutagenic effect by themselves or potential the effect of other xenobiotics (Volker Mersch-Sundermann, et al., 2006).

5.1 SOS Chromotest
In the SOS Chromotest, it was ascertained that different concentrations of selected compounds for this study such as hydro alcoholic extract of Curcuma aromatica, Curcuma zedoaria and Curcumin added to the indicator bacteria were not genotoxic as the induction factor induced by the tested doses was below 1.2. The compounds are classified as non-genotoxic, if the induction factor (IF) remains <1.2 and genotoxic if IF exceeds 1.2 (Manon Bombardier, et. al., 2001; Skandranri, et.al., 2010). For Curcuma aromatica the corrected induction factor produced at the concentration of 250 and 500 µg/ml was found to be more or less equal such as 0.9711 and 0.9403 where as the correction induction factor at the concentration of 1000 µg/ml was found to be 1.0309. For Curcuma zedoaria the corrected induction factor was found to be 0.4299, 0.8943 and 1.1975 for 1000, 500 and 250 µg/ml concentration respectively. For Curcumin the corrected induction factor was found to be 1.3308, 1.3725 and 1.1975 for 1000, 500 and 250 µg/ml. Curcumin alone was found to be genotoxic even at the concentration of 250 µg/ml where as the hydro alcoholic extract of Curcuma aromatica and Curcuma zedoaria was found to be non-genotoxic even at the highest concentration i.e 1000 µg/ml.

5.2. DNA sugar damage
By normal cellular metabolism and by exogenous source such as genotoxic compounds and ionizing radiations, reactive oxygen derived species including free radical are formed in living cells which causes oxidative damage to DNA, resulting in the formation of modified bases and sugars, DNA protein cross links, strand break, base free site and tandem lesions such as 8,5’ cyclopurine-2’ deoxyribonucleosides and clustered damage sites. Among the free radical, the hydroxyl radical is highly reactive and reacts with DNA by addition to double bond of heterocyclic DNA bases and by abstraction of an H atom from the methyl group of thymine and from each of the C-H bonds of 2’ deoxyribose (Winofred, et. al., 2006).

Concomitant damage to the sugar and base moieties of the same nucleotides is shown by the formation of 8,5’ cyclopurine-2’ deoxyribonucleoside that has been identified in mammalian cells that are exposed to free radical- generating system In base- excision repair (BER) of oxidative DNA sugar damage, numerous DNA glycosylases which are substrate specificities are involved. Major products of oxidative damage to DNA bases are substrates of known glycosylases. Some enzyme exhibit cross reactivity specific for both pyrimidine- purine derived lesions and some enzymes have more substrates than others. Eukaryotic counterparts possess narrow specificity than prokaryotic enzyme (Miral Dizdaroglu., 1991). Malondialdehyde which is the product of lipid peroxidation forms adduct with cellular DNA (Maria Eurica Fracasso, et. al., 2006). This study showed that Curcuma aromatica, Curcumin which gave the protection for the DNA sugar bone where as Curcuma zedoaria damaged the sugar backbone at the concentration of 250, 500 µg/ml.

5.3. Plasmid nicking assay or DNA-damage protective activity
By damaging the cellular antioxidant defence mechanism, can induce oxidative damage to vital cellular molecules including DNA, proteins
and lipids. Genomic DNA is the most important target in the living cells. Damage suffered by DNA includes strand break and cross link of the intra and inter strand type. Malondialdehyde which is the product of lipid peroxidation forms adduct with cellular DNA (Maria Eurica Fracasso, et. al., 2006). Drugs can cause cellular damage through metabolic activation of these compounds to highly reactive substances such as oxygen species which are derived from the metabolism of oxygen that includes superoxide radicals, hydroxyl radicals, hydrogen peroxide radicals which are often generated or obtained as byproducts of biological reactions or from exogenous factors. Some of these reactive oxygen species play a positive role in cell physiology as well as cause great damage to the cell membrane and DNA including oxidation that cause membrane lipid peroxidation, decreased membrane fluidity and DNA mutation which leads to cancer and other degenerative disease (Wan Ibrahim, et.al., 2010). When singlet oxygen together with small contribution of hydroxyl radical-mediated reactions through initially generated superoxide radical produced/ induced the oxidative damage to DNA (Betina Kappel Pereira, et. al., 2009). In this study Quercetin was used as a positive control. Quercetin at all the tested concentration such as 250, 500 and 1000 μg/ml showed the damage to the plasmid and it might have occurred due to the incision in the DNA. Curcuma aromatica and Curcuma zedoaria protected the DNA from the concentration of 250 μg/ml to 1000 μg/ml. Curcumin caused slight damage when compared to quercetin as well as negative control in all the tested concentration such as 250, 500 and 1000 μg/ml. In the sequential analysis method with pBR322, it was clear that no mutation has been taken place after the treatment with hydro alcoholic extract of Curcuma aromatica, Curcuma zedoaria and Curcumin with this particular primer. This is confirmed that our test compounds are not mutagenic in nature as far as these primers are concerned at the concentration of 50 μg/ml with all these results, it is giving the conclusion that curcumin which is commonly used in many food as spices and coloring agent especially in almost all the Indian foods. Normally all the Indians will consume nearly 1 gm of curcumin in their diet every day and it is considered as a good home remedy for skin infections and wound healing purpose and also to improve the complexion. Because of it continuous use, it is necessary to confirm its safety for long term use.

**Conclusion**

In SOS chromotest, Curcumin alone was found to be genotoxic even at the concentration of 250 μg/ml where as the hydro alcoholic extract of Curcuma aromatica and Curcuma zedoaria was found to be non-genotoxic even at the highest concentration i.e 1000 μg/ml. Curcuma aromatica, Curcumin which gave the protection for the DNA sugar bone where as Curcuma zedoaria, damaged the sugar backbone at the concentration of 250, 500 μg/ml. This study confirms that higher concentration of the drug is required for the DNA Protection activity. This might be due to incomplete neutralization of hydroxyl radical that has been produced due to biological reaction.

Quercetin at all the tested concentration such as 250, 500 and 1000 μg/ml showed the damage to the plasmid and it might have occurred due to the incision in the DNA

Curcuma aromatica and Curcuma zedoaria protected the DNA from the concentration of 250 μg/ml to 1000 μg/ml.

Curcumin caused slight damage when compared to quercetin as well as negative control in all the tested concentration such as 250, 500 and 1000 μg/ml.

In the sequential analysis method with pBR322, it was clear that no mutation has been taken place after the treatment with hydro alcoholic extract of Curcuma aromatica, Curcuma zedoaria and Curcumin with this particular primer. This is confirmed that our test compounds are not mutagenic in nature as far as these primers are concerned at the concentration of 50 μg/ml with all these results, it is giving the conclusion that curcumin which is commonly used in many food as spices and coloring agent especially in almost all the Indian foods. Normally all the Indians will consume nearly 1 gm of curcumin in their diet every day and it is considered as a good home remedy for skin infections and wound healing purpose and also to improve the complexion. Because of it continuous use, it is necessary to confirm its safety for long term use.

**Present and future Work**

Our research work is now focused to carry out the same studies in the presence of S9 (metabolic activation factor) and our future work will be in-vivo genotoxicity studies by using the animal models as well as to perform the sequencing with other primers to confirm the mutagenicity of curcumin as well as anti-mutagenicity of hydro alcoholic extract of Curcuma aromatica and Curcuma zedoaria.

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