CHAPTER 5

In vitro DNA damage protective activity and quantitative analysis of hydroethanolic extracts of three promising plant samples
Mitochondria and nuclei have their own DNA. Mitochondrial DNA is susceptible to oxidative damages induced by reactive oxygen species because of excessive generation of reactive oxygen species in this organelle. Many internal and external factors such as exposure to xenobiotics and ultra violet (UV) light generate free radicals such as •OH and H• which react with DNA. These radicals oxidize guanosine or thymine to 8–hydroxy–2–deoxyguanosine and thymine glycol, respectively, which changes DNA and leads to mutagenesis and carcinogenesis (Ames et al., 1993). 8–Hydroxy–2–deoxy guanosine has been used as a biological marker for oxidative stress (Kasai, 1997). Altered DNA can be repaired by DNA glycosylase (Halliwell, 1997). If oxidative stress is too great, the DNA repair system using glycosylase is not enough and mutagenesis and/or carcinogenesis can be induced.

Scientists have indicated that antioxidant supplied from daily diets rich in vegetables and fruits quench the reactive oxygen species or are required as cofactors for antioxidant enzymes which play significant roles in the prevention of DNA damage. Epidemiological studies also reported the relevance of antioxidative nutraceuticals to health issues and the prevention of diseases (Kaur and Kapoor, 2001). Health-conscious consumers have made antioxidative nutraceuticals the leading trend in the food industry worldwide in recent years.

As per our results of primary screening, B. oleracea var. gongylodes pulp extract (BOGPE), L. siceraria leaf extract (LSLE) and V. faba fruit extract (VFFE) were found to be effective scavengers for different free radicals with good amount of phenolic content. These extracts were selected further to study their protective effect against DNA damage protection and HPLC analysis.
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5.1 Materials and Methods:

5.1.1 Reagents: Gallic acid, caffeic acid, catechin, silymarin and epicatechin were purchased from Sigma Chemicals Co. (St. Louis, MO, USA), whereas agarose was obtained from GE Healthcare (USA). Calf thymus DNA was procured from Himedia Co. Ltd (India). All the other chemicals used throughout the study were commercial products of the highest purity grade and purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Solutions were prepared in de-ionized ultrapure water (Direct Q5, Millipore, Bangalore, India).

5.1.2 Collection of plant materials: As described in chapter 3.

5.1.3 Extraction and yield: As described in chapter 4.

5.1.4 Estimation of DNA damage caused by hydroxyl radicals: To evaluate the hydroxyl radical scavenging potential of the extracts, plasmid nicking assay was performed using calf thymus plasmid DNA according to the method of Lee et al. (2002) with some modifications. The nicking of DNA was introduced by two different methods; Fenton’s reagent induced damage and UV rays induced damage.

5.1.4.1 DNA damage induced by Fenton’s reagent: Briefly, each extracts (2-10 g/ml) and 500 ng plasmid DNA in 1x TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.2), were incubated for 10 min at room temperature followed by the addition of Fenton's reagent (30 mM H₂O₂, 50 μM ascorbic acid and 80 μM FeCl₃). The reaction mixture was incubated for 60 min at 30°C. The reaction mixture had final volume of 20.0 μl including phosphate-buffer saline (PBS), in 0.5 ml microcentrifuge tubes. After incubation, the samples were mixed with 3 μl of gel loading dye (0.15%)
bromophenol blue (BPB) and 80% (w/v) glycerol and immediately loaded into a 1.5%
agarose gel. The running buffer contained 40 mM Tris, 20 mM sodium acetate and
2 mM EDTA in which gel was electrophoresed in a horizontal slab gel apparatus for
1.5 h (60 V/30 mA). The gels were then photographed with Gel Doc system (Alpha
Innotech). Catechin and Silymarin (2.5 µg/ml) were used as positive controls.

5.1.4.2 DNA damage induced by UV rays: The methodology is same as given in
Fenton’s reagent experiment; except the nicking of DNA was introduced by adding 2
µl H₂O₂ (20.5 mM) in the presence of UV rays (302 nm).

5.1.5 HPLC Analysis: For HPLC analysis, 2.0 g of dried and powdered plant material
was extracted with EtOH:H₂O (1:1, 1 × 20 ml) for 2 hour at room temperature. The
plant extract was hydrolysed with 1.2 N HCl by refluxing on a water bath for 1 hour
according to Lin-Chin et al. (2000). The hydrolysed extract was filtered and
fractionated with ethyl acetate (EtOAc, 3 × 10 ml). Solvent from EtOAc soluble
fraction was removed under reduced pressure. The residue thus obtained was
dissolved in EtOH and subjected to HPLC for the qualitative and quantitative analysis
of phenolic contents. The HPLC system Shimadzu LC- 10A (Japan) was equipped
with dual pump LC-10AT binary system, UV detector SPD- 10A, Phenomenex Luna
RP, C 18 column (4.6 × 250 mm) and data was integrated by Shimadzu Class VP
series software. Separation was achieved with a two pump linear gradient program for
pump A (Water containing 1% acetic acid) and pump B (Acetonitrile). Initially started
with a gradient of 18% B changing to 32% in 15.0 min and finally to 50% in 40 min
followed by washing for 25 min. The flow rate was 1.0 ml/min. Result (mg/g DW)
were obtained by comparison of peak areas (254 nm) of the samples with that of
standards.
5.1.6 **Statistical analysis:** Data are expressed as mean±SD of three replicates.

5.2 **Results**

5.2.1 **Inhibition of \( \cdot OH \) radical induced strand breaks in plasmid DNA by UV radiation and Fenton’s reagent:** The protective activity of *B. oleracea* var. *gongylodes* pulp extract (BOGPE), *L. siceraria* leaf extract (LSLE) and *V. faba* fruit extract (VFFE) with standards silymarin and catechin against \( \cdot OH \) induced DNA damage was studied using an *in vitro* method employing calf thymus plasmid DNA. As shown in Fig. 5.1 the DNA damage was introduced by two different means: lane 3-7 showed time dependent DNA damage by 1, 3, 5, 8 and 10 min \( \cdot OH \) generated by UV + H\(_2\)O\(_2\), lane 8-9 show time dependent damage by Fenton’s reagent (45 and 60 min) while lane 10 t-BHP (250 \( \mu \)M) (60 min) and lane 2 H\(_2\)O\(_2\) (20.5 mM) (60 min) showed no damage, whereas lane 1 of each figure shows the control DNA (500 ng).

The results indicate that there is not any damage induced by H\(_2\)O\(_2\) itself in calf thymus DNA (Lane 2 of Fig. 5.1, 5.3 & 5.4), until the decomposition of H\(_2\)O\(_2\) is not induced either by UV or iron. But \( \cdot OH \) generated by decomposition of H\(_2\)O\(_2\) mediated either by UV mediated (Fig. 5.1, lane 3-7; Fig. 5.2, lane 2; Fig. 3 & 4, lane 3) or iron (Fig. 5.1, lane 8-9; Fig 5.5, lane 4) produced nicked DNA. This type of oxidative damage can be reduced in the presence of standard antioxidant, like silymarin (lane 7-10 in Fig. 5.2, lane 8-11 in Fig. 5.3 & 5.4 and lane 3 in Fig 5.5) and catechin (lane 11-12 in Fig. 5.2 & lane 12-13 in Fig. 5.3 & 5.4 while lane 2 in Fig 5.5) as well as plant extracts.

In the presence of BOGP, LSL and VFF extracts at 1.0-5.0 \( \mu \)g/ml to UV mediated calf thymus DNA damage showed significant reduction in damage of
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**Fig. 5.1** Time dependent calf thymus DNA (500 ng) damage caused by UV induced and Fenton’s reagent induced hydroxyl radicals. Lane 1: Calf thymus DNA + PBS; Lane 2: Calf thymus DNA + H$_2$O$_2$ (20.5 mM); Lane 3-7: Calf thymus DNA + H$_2$O$_2$ (20.5 mM) + UV (1, 3, 5, 8 and 10 min); Lane 8-9: Calf thymus DNA + Fenton’s reagent (45 and 60 min) and Lane 10: Calf thymus DNA + t-BHP (250 μM, 60 min).

**Fig. 5.2** Inhibitory effects of *B. oleracea var. gongylodes* pulp extract (BOGPE) on nicked DNA caused by UV induced hydroxyl radicals. Lane 1: Calf thymus DNA (500 ng) + PBS, Lane 2: Calf thymus DNA + H$_2$O$_2$ (20.5 mM) + UV (8 min), Lane 3-6: Calf thymus DNA + H$_2$O$_2$ (20.5 mM) + UV (8 min) + BOGPE (1, 1.5, 2.5 & 5.0 μg/ml); Lane 7-8: Calf thymus DNA + H$_2$O$_2$ (20.5 mM) + UV (8 min) + Catechin (2.0 & 2.5 μg/ml); Lane 9-12: Calf thymus DNA + H$_2$O$_2$ (20.5 mM) + UV (8 min) + Silymarin (1, 1.5, 2.5 & 5.0 μg/ml)
plasmid DNA as shown in Fig. 5.2, 5.3 & 5.4, respectively. LSLE and BOGPE showed partial protection at 1.0 μg/ml whereas VFFE showed complete protection of plasmid DNA at 1.0 μg/ml. The protective activity of VFFE was shown to be similar to the standards silymarin and catechin. The LSLE and BOGPE have shown significant protection at 2.5 μg/ml in comparison to standard (Fig. 5.2 & 5.3).

Similar results were found in the case of DNA damage mediated by Fenton’s reagent mixture. Protective activity of all three extracts VFF, LSL, BOGP and standard antioxidant silymarin and catechin are depicted in Fig. 5.5. *OH induced damage by Fenton’s reagent on calf thymus plasmid DNA is shown in lane 4 in Fig. 5.5. Oxidative DNA damage was found to be reduced in the presence of standard antioxidant silymarin (2.5 μg/ml) and catechin (2.5 μg/ml) depicted in Lane 2 & 3 of

![Image](image_url)

**Fig. 5.3** Inhibitory effects of *L. siceraria* leaf extract (LSLE) on nicked DNA caused by UV induced hydroxyl radicals. Lane 1: Calf thymus DNA (500 ng) + PBS; Lane 2: Calf thymus DNA + H₂O₂ (20.5 mM); Lane 3: Calf thymus DNA + H₂O₂ (20.5 mM) + UV (8 min); Lane 4-7: Calf thymus DNA + H₂O₂ (20.5 mM) + UV (8 min) + LSLE (1, 1.5, 2.5, & 5.0 μg/ml); Lane 8-11: Calf thymus DNA + H₂O₂ (20.5 mM) + UV (8 min) + Silymarin (1, 1.5, 2.5 & 5.0 μg/ml); Lane 12-13: Calf thymus DNA + H₂O₂ (20.5 mM) + UV (8 min) + Catechin (2.0 & 2.5 μg/ml)
Fig. 5.4 Inhibitory effects of *V. faba* fruit extract (VFFE) on nicked DNA caused by UV induced hydroxyl radicals. Lane 1: Calf thymus DNA (500 ng) + PBS; Lane 2: Calf thymus DNA + H₂O₂ (20.5 mM); Lane 3: Calf thymus DNA + H₂O₂ (20.5 mM) + UV (8 min); Lane 4-7: Calf thymus DNA + H₂O₂ (20.5 mM) + UV (8 min) + VFFE (1, 1.5, 2.5, & 5.0 µg/ml); Lane 8-11: Calf thymus DNA + H₂O₂ (20.5 mM) + UV (8 min) + Silymarin (1, 1.5, 2.5 & 5.0 µg/ml); Lane 12-13: Calf thymus DNA + H₂O₂ (20.5 mM) + UV (8 min) + Catechin (2.0 & 2.5 µg/ml)

Fig. 5.5 Inhibitory effects of different plant extracts on DNA nicking caused by Fenton’s reagent. Lane 1: Calf thymus DNA (500 ng) + PBS; Lane 2: Calf thymus DNA + Fenton’s reagent (60 min) + Catalase (2.5 µg/ml); Lane 3: Calf thymus DNA + Fenton’s reagent (60 min) + Silymarin (2.5 µg/ml); Lane 4: Calf thymus DNA + Fenton’s reagent (60 min); Lane 5-7: Calf thymus DNA + Fenton’s reagent (60 min) + LSLE (5.0, 2.5 and 1.0 µg/ml); Lane 8-10: Calf thymus DNA + Fenton’s reagent (60 min) + BOGPE (5.0, 2.5 and 1.0 µg/ml) and Lane 11-13: Calf thymus DNA + Fenton’s reagent (60 min) + VFFE (5.0, 2.5 and 1.0 µg/ml)
Fig. 5.5, respectively. Effect of LSL, BOGP and VFF extract is shown in lane 5-7, 8-10 and 11-13, respectively.

The protection was effective in both cases UV mediated as well as Fenton’s reagent induced damage. All three extracts showed significant antioxidant and DNA damage protection, but VFF extract showed maximum protection among them. All the extracts showed concentration dependent protection (1.0-5.0 μg/ml) and neutralized the oxidative stress produced by Fenton’s reagent / UV rays induced •OH.

5.2.2 HPLC analysis: The HPLC analysis for 4 phytochemicals of extract showed different concentrations of each phytochemical in each extract depicted in Table 5.1. Gallic acid was present in both extracts. The analysed value of gallic acid content in BOGP and V. faba fruit are (91.80±13.12 mg/100g) and (70.81±8.77 mg/100g), respectively, while catechin was present only in V. faba fruit. The quantity of catechin in V. faba fruit is 49.63±3.58 mg/100g. Other phytochemical; hydroxybenzoic acid (HBA) and caffeic acid (CA) were not present in both B. oleracea var. gongylodes pulp and V. faba fruit extract.

Table 5.1 Identification and quantification of specific phenols (mg/100g) of selected plant samples through HPLC

<table>
<thead>
<tr>
<th>Plant</th>
<th>Part</th>
<th>GA</th>
<th>CT</th>
<th>HBA</th>
<th>CA</th>
</tr>
</thead>
<tbody>
<tr>
<td>V. faba</td>
<td>Fruit</td>
<td>70.81±8.77</td>
<td>49.63±3.58</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B. oleracea var. gongylodes</td>
<td>Fruit pulp</td>
<td>91.80±13.12</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

GA = gallic acid, CT = catechin, HBA = hydroxybenzoic acid and CA = caffeic acid

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5.3 Discussion

Hydroxyl radicals generated by irradiation or Fenton reaction are known to cause oxidative breaks in DNA strands to yield its fragmented forms (Udovick et al., 1994). Prevention of DNA damage is one of the important mechanisms of protecting the system from the onslaught of genotoxic agents from exogenous sources. In the present study, the free radical scavenging effect of *V. faba* fruit extract (VFFE), *L. siceraria* leaf extract (LSEE), *B. oleracea var. gongylodes* pulp extract (BOGPE) were studied on calf thymus DNA damage. The protective effects of these extracts on the hydroxyl radical mediated DNA strand breakages by Fenton’s reagent and UV rays were investigated. First, the supercoiled plasmid DNA was subjected to oxidative damage by UV rays + H₂O₂ and ascorbate fortified Fenton’s reaction and protective effects were analyzed by agarose gel electrophoresis.

In the absence of vegetable extracts, exposure of -OH generating system (Fe²⁺ concentration and UV + H₂O₂) caused a complete fragmentation of DNA. Supplementation of extract (1.0-5.0 µg/mL) to the plasmid DNA during to the exposure could prevent it significantly. *V. faba* extract (1.0 µg /mL) provided an almost complete protection. In our study, the effect of plant extract was also studied on the plasmid DNA exposed to UV radiation and found that the nicking of DNA was reduced in the presence of plant extract.

Studies have suggested that plant extracts show protection against DNA damage caused by different oxidative stresses (Lee et al., 2002; Khanduja et al., 2006; Singh et al., 2011). According to Prakash et al. (2007a) and Singh et al. (2009b), red onion peel could protect pBR322 plasmid DNA against the damage caused by free
radicals. Some earlier report suggest that leaves of *M. oleifera* have strong antioxidants and a good scavengers of reactive metabolites (Iqbal and Bhanger, 2006; Chumark *et al.*, 2008; Singh *et al.*, 2009a; Sreelatha and Padma, 2010). Our study was also supported by Lee *et al.* (2001), Sharma *et al.* (2007) and Wang *et al.* (2007) who showed the inhibitory effect of plant extract on Fenton's reaction-mediated degradation of calf thymus DNA and found good hydroxyl radical scavenging potential.

Duthie *et al.* (1996) examine the effects of antioxidant supplements (a mixture of vit C, vit E and beta carotene) on oxidative DNA damage in human white blood cells and found a substantial decrease in DNA base oxidation. Boyle *et al.* (2000) determined that flavonoid glucosides were significantly elevated in plasma and decrease in the level of urinary 8-Hydroxy-2-deoxyguanosine (8-oxodG) following ingestion of an onion and tomato meal was observed. A previous intervention study had shown that consumption of carotenoid containing vegetable juices reduce oxidative DNA damage in lymphocytes (Pool-Zobel *et al.*, 1998). Cruciferous vegetable Brussels sprouts contain bioactive compounds which reflect the rate of guanine oxidation in DNA and decrease the level of 8-oxodG in urine (Deng *et al.*, 1998).

The BOGP and VFF extract were quantified with HPLC analysis and showed considerable variation for the presence of specific phytochemicals. Studies demonstrated that gallic acid possess many potential therapeutic properties including anti-cancer and antimicrobial properties (Agarwal *et al.*, 2006; Kaur *et al.*, 2009). Flavanols or flavan-3-ols are often commonly called catechins (Tsao, 2010). Catechin is a group that occupies an intermediary position in the tannin hierarchy as a family of
catechin tannins (Bhat et al., 1998). The presence of catechin in green tea and fermented tea is associated with health protective, antimicrobial properties and cancer preventive properties in animal models, due to its antioxidant activity. Once they were isolated and tested in animals, cell cultures and other types of research tests, catechins supported healthy gastrointestinal tract function, detoxification function and repair of damaged DNA (Uesato et al., 2000 & 2003; Sang et al., 2002; Morre et al., 2003). Another work had described the isolation of condensed tannins and anthocyanins phenolic acids from V. faba which are mainly responsible for the remarkable antioxidant and antimicrobial effect of this plant (Mergham et al., 2004; Akroum et al., 2009).

As we know that prevention is more effective strategy than treatment for chronic diseases, a constant supply of phytochemical containing plants with desirable health benefits beyond basic nutrition is essential to reduce the risk of chronic diseases, many of which involve DNA damage prevention in humans. Phytochemical from vegetables such as tannins, quercetin, anthocyanin and catechin are known to inhibit lipid peroxidation and scavenge free radicals which are known to be important in cellular pro-oxidant states (Sestili et al., 1998; Devi et al., 2012). These compounds may play the primary and most important role in DNA damage protection in our study. Dietary polyphenols have received tremendous attention among nutritionists, food scientists and consumers due to their roles in human health.

Research in recent years strongly supports a role for polyphenols in the prevention of degenerative diseases, particularly cancers, cardiovascular diseases and neurodegenerative diseases (Duthie and Brown, 1994; Hausladen and Stamler, 1999; Molan et al., 2012). Most of the evidence of the antioxidant activity of polyphenols is
based on *in vitro* and *in vivo* studies (Williams *et al.*, 2004; Tsao, 2010). Therefore, supplementation of these plants extracts may help to prevent oxidative damage associated diseases and illnesses. Hence, all the three extracts were further selected for *in vitro* hepatoprotective activity.