Antimutagenic and Antioxidant Action of Naphthoquinone Rich Foods and Underlying Mechanism

By

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As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by Sanjeev Kumar entitled "**Antimutagenic and antioxidant action of naphthoquinone rich foods and underlying mechanism**" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

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List of Publications arising from the thesis

Journal

1. **Sanjeev Kumar**, Chatterjee, S., Tripathi, J., & Gautam, S. Purification and characterization of the principal antimutagenic bioactive as ethoxy-Substituted phylloquinone from spinach (*Spinacea oleracea* L.) based on evaluation in models including human lymphoblast $TK^{+/-}$ cells. *Journal of Agricultural and Food Chemistry* **64**, 8773-8782 (2016).
2. **Sanjeev Kumar**, Gautam, S., & Sharma, A. Antimutagenic and antioxidant properties of plumbagin and other naphthoquinones. *Mutation Research* **755**, 30-41 (2013).
3. Gautam, S., Saxena, S., & **Sanjeev Kumar**. Fruits and vegetables as dietary sources of antimutagens. *Journal of Food Chemistry and Nanotechnology* **2**, 96-113 (2016).

BARC News letter

Sanjeev Kumar, Gautam, S., & Sharma, A. Structure based variation in the antimutagenic / antioxidant properties of different naphthoquinones. *BARC News letter* Founder's Day Special Issue, 236-242 (2014).

Conference/Symposium

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Sanjeev Kumar

Dedicate to the memory of my

beloved Parents

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CONTENTS

Homi Bhabha National Institute

SYNOPSIS OF Ph. D. THESIS

1. Name of the Student: Mr. Sanjeev Kumar

2. Name of the Constituent Institution: BARC

3. Enrolment No. : LIFE01201204003

4. Title of the Thesis: Antimutagenic and antioxidant action of naphthoquinone rich foods and underlying mechanism

5. Board of Studies: Life Sciences

Living organisms are exposed to various genotoxic agents such as insecticides, herbicides, pesticides, viruses, toxigenic microbes including fungi and radiation (ultraviolet and X-rays) sources leading to permanent inheritable alteration in DNA nucleotide sequence, i.e., mutation [1]. This may cause chronic diseases such as atherosclerosis, cardiovascular diseases (CVD), neoplastic inductions, cancer, mortality and other degenerative diseases [2]. Dietary intervention has been postulated to minimize the onset of such diseases mostly due to the occurrence of natural antimutagens [3]. Many foods especially green vegetables and certain herbals are rich source of naphthoquinones such as phylloquinone, lapachol, plumbagin, juglone and alkanin [4]. Besides, certain fungi, lichens, and sea urchins also produce naphthoquinones [5]. It has two different isomers, 1,4-naphthoquinone and 1,2-naphthoquinone. Several 1,4-naphthoquinone derivatives have been reported from different plants. These are produced by higher plants as secondary metabolites through various pathways which include acetate, malonate, shikimate, succinyl CoA and mevalonate [6]. These compounds have been reported for health protective potential such as antioxidant, antitumor, antimicrobial, anti-inflammatory and anti-atherosclerosis [7, 8, 9]. However, compared to other phytochemicals such as

phenolics, flavonoids and anthocyanins, naphthoquinones are less explored in terms of bioactivity. Therefore, in the present work, it was intended to study the health protective functional properties such as antimutagenic activities of naphthoquinone rich vegetables such as spinach, lettuce, iceberg lettuce, cabbage, broccoli, and French bean. Naphthoquinone from most potent vegetable was selected for characterization of bioactive compound. Further study on underlying mechanism was addressed using purified and identified bioactive compound.

OBJECTIVES:

- Extraction of naphthoquinones from its rich dietary sources including vegetables.
- Analysis of health protective functional properties (antimutagenic and antioxidant) of the naphthoquinones.
- Purification, identification and characterization of bioactive naphthoquinone showing highest antimutagenicity from the most potent source.
- Understanding the molecular mechanism contributing to antimutagenicity by the bioactive naphthoquinone.

ORGANIZATION OF THESIS:

The thesis is divided into five chapters which include introduction and review of literature (Chapter 1), materials and methods (Chapter 2), results (Chapter 3 with two sub-chapters 3.1 and 3.2) followed by discussion in Chapter 4. At the end summary and conclusions followed by references placed in the order of citation in the text.

Chapter 1: Introduction and review of literature

This chapter provided overview with some details on relevant information on the thesis title. The topics covered are: functional relevance of food, type of phytochemicals, naphthoquinones (occurrence, structure, biosynthesis, and

bioactivities), sources of dietary antimutagens with emphasis on naphthoquinone, mutations and their role in human diseases, and reported mechanism of antimutagenicity. The scope of the thesis was concluded at the end of this chapter.

Chapter 2: Materials and methods

In this chapter details of experimental procedures and techniques are provided. Naphthoquinone rich vegetables spinach (*Spinacia oleracea* var. semisavoy), lettuce (*Lactuca sativa*), iceberg lettuce (*Lactuca sativa* var. Iceberg), cabbage (*Brassica oleracea* var. capitata), broccoli (*Brassica oleracea* var. *italica*) and French bean (*Phaseolus vulgaris*) were selected for the study. Quinones and phenolics extraction procedure from naphthoquinone rich vegetables is discussed. Antimutagenic potential is assessed by *rpoB* based rifampicin sensitive (Rif^S) to resistance (Rif^R) assay in wild type *E. coli* (MG1655) and thymidine kinase base $tk^{+/-}$ to $tk^{-/-}$ assay in human lymphoblast cell line (TK) upon induced mutagenicity through known mutagens [ethyl methanesulfonate (EMS) and 5-azacytidine (5-AZ)]. From quinone extract of the most potent vegetable, antimutagenic compound was characterized by spectrophtometric, thin layer chromatography (TLC) using fluorescent plate (λ_{Ex}: 254 nm), high performance liquid chromatography (HPLC), fourier transform infrared (FTIR) spectroscopy, biochemical tests (-OH substitution; and analysis of 2, 3 position for presence or absence of functional group), nuclear magnetic resonance (NMR) spectroscopy (^1H and ^{13}C), and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS). Mechanism of antimutagenicity of bioactive has been addressed by analyzing antimutagenicity in various experimental conditions: a) bioactive compound simultaneously added with mutagen to the culture; b) bioactive compound co-incubated with mutagen for 4 h and then added to the culture; and c) bioactive compound added after 4 h of mutagen treatment. Further studies were conducted on assessment of direct interaction of mutagen and antimutagen, interaction of antimutagen with DNA [spectrophotometric analysis, circular dichroism (CD) spectroscopy, dye binding assays [methyl green, ethidium bromide, and 4′,6-diamidino-2-phenylindole (DAPI)], protein expression studies using two dimensional (2-D) gel electrophoresis, MALDI TOF MS/MS and gene knockout

studies. To assess the role of antioxidant capacity in observed antimutagenicity different radical scavenging assays using TSP and quinone extracts, TLC purified compounds and standard naphthoquinones were performed. Besides, DCFDA staining of EMS treated cells was also performed to assess the induction of oxidative stress in the cells.

Chapter 3: Results

3.1. Screening of various selected foods rich in naphthoquinone for antimutagenicity and characterization of bioactive from the most potent food

The dry matter content (w/w) in selected vegetables ranged between 5.6% (lettuce) to 11.5% (broccoli). Yield of naphthoquinone rich extract ranged between 1.5 (mg/g) (cabbage) to 3.4 (mg/g) (broccoli)on dry weight basis. Yield of phenolic rich extract ranged between 0.5 (mg/g) in iceberg lettuce to 5 (mg/g) in broccoli on dry weight basis. Naphthoquinone rich extract of spinach displayed highest (72±6%) antimutagenic activity against ethyl methanesulfonate (EMS) in *rpoB* (β-subunit of RNA polymerase) based rifampicin resistance ($Rif^S \rightarrow Rif^R$) assay in *E. coli*. Antimutagenicity of naphthoquinone from other vegetables ranged between 2±1 (lettuce) to 21±3% (iceberg lettuce).Such variation in antimutagenicity was not observed in case of phenoic rich extracts where most of the vegetables displayed high level of antimutagenicity, i.e., 63±6% in French bean to 78±4%in lettuce, however, in spinach its contribution to antimutagenicity was comparatively at lower level, i.e., 35±4%. *In vitro* antioxidant activities measured in terms of radical scavenging ($DPPH^{\cdot}$and $ABTS^{\cdot+}$) assays of phenolics and quinones extracts of these vegetables indicated that quinones have lesser antioxidant capacity compared to phenolics even at 1000 fold higher concentrations. Therefore, observed antimutagenic activity of quinones does not seem to be primarily due to radical scavenging but possibly due to involvement of other mechanism(s). The quinones extract from spinach displaying high antimutagenicity was resolved by thin-layer chromatography (TLC). It got resolved into 4 bands of quinones named as C1, C2, C3, and C4. Amongst these C1 matched the standard phylloquinone when detected on TLC at 254 nm. Its yield was found to be 64±7 μg/g dry weight. C4 compound fluoresced on TLC plate at 366 nm

and its yield on dry weight basis was found to be 396±14 μg/g. This compound displayed highest antimutagenicity. The R_f value of C4 did not match with any of the naphthoquinone standards analyzed. The purity of compound (C4) was confirmed by HPLC analysis using different wavelength of absorbtion. C4 compound was characterized by biochemical and spectroscopic analyses as ethoxy-substituted phylloquinone (ESP), i.e., 2-ethoxy-3-(3,7,11,15-tetramethyl hexadec-2-ethyl) naphthaquinone-1,4-dione.

3.2. Comparative bioactivity evaluation of ESP with other naphthoquinones and understanding the underlying mechanism

The simultaneous treatment or co-incubation of ESP (from spinach) and EMS resulted in similar level of antimutagenicity (56±6%). This indicated that the compound ESP does not directly inactivate the mutagen (EMS) through any type of complex formation or modification. In the post-treatment condition too, ESP displayed significant reduction (44±4%) in mutagenicity. Similar, findings were observed in thymidine kinase based $tk^{+/-}$ to $tk^{-/-}$ assay in human lymphoblast cell line. To further investigate the direct interaction between ESP and EMS, they were co-incubated at equimolar concentration (8 mM) for 2 h and analyzed using TLC. At 366 nm, R_f or fluorescence intensity upon co-incubation of ESP and EMS was not found to be affected andat 254 nm, no compound was detected at same or different R_f on fluorescent plate. This indicated absence of any modification or complex formation of ESP. DCFDA staining was carried out to study the antioxidant capacity of the ESP. The EMS did not show any significant induction of the oxidative stressin human lymphoblast (TK 6) cell line. However, low level of oxidative stress was observed in *E. coli* due to EMS treatment which was not found to be suppressed in the presence of ESP. In *E. coli* cells treated with H_2O_2 (positive control) oxidative stress was quite high. Untreated cells served as negative control. As ESP does not possess significant antioxidant capacity, observed antimutagenicity cannot be attributed through antioxidant mechanism. Interaction of ESP with DNA was also studied. During spectrophotometric analysis, a concentration dependent increase in absorbance at 260 nm was observed which indicated that ESP interacted with calf thymus DNA. The

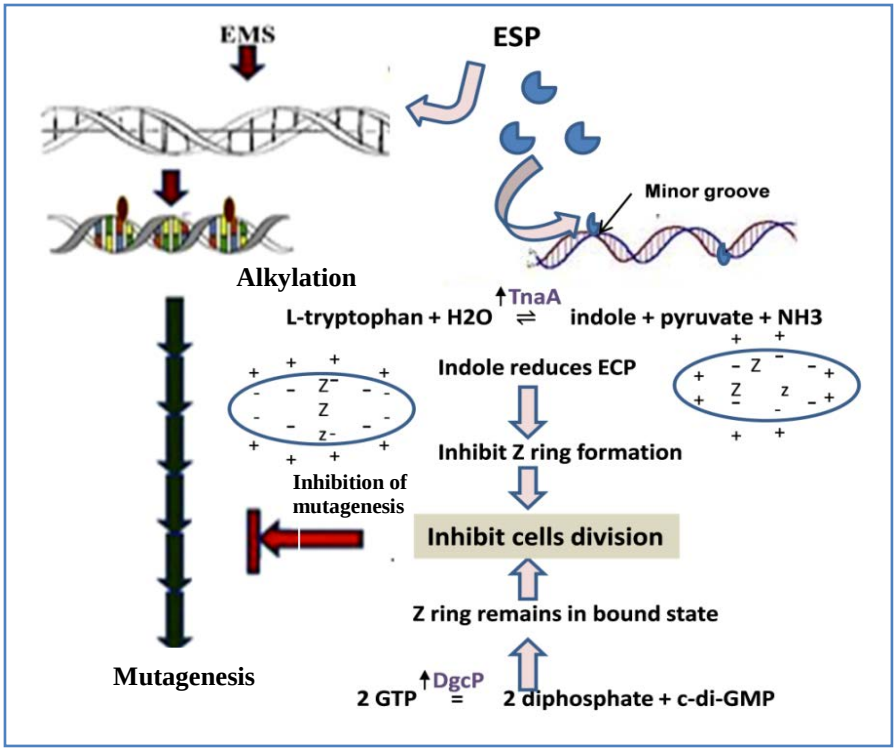
absorbance of ESP per se was found to be quite low at this wavelength. In circular dichroism spectroscopy, there was a significant change in elipcity (millideg) at both the negative 245 and positive 275 nm wavelengths with the increase in ESP concentration. This too indicated physical interaction of ESP with DNA. In methyl green and ethidium bromide (EtBr) binding assays, increase in ESP concentration was not found to change the absorbance or fluorescence intensity. This indicated that ESP was not a major groove binder or DNA intercalating agent. However, in DAPI binding assay, a significant reduction in the fluorescence intensity observed which indicated that the ESP binds to the minor groove.

To understand the underlying molecular mechanism, 2-dimentional protein gel electrophoresis was performed using untreated and treated *E. coli* cells. Among these, proteins showing prominent difference in expression were further selected for identification using MALDI-TOF MS/MS. Some of the up-regulated proteins included *tnaA* (tryptophanase; synthesizes indole from L-tryptophan), *dgcP* (diguanylate cyclase; synthesis of c-di-GMP), *selA* (L-seryl-tRNA (Sec) selenium transferase) and *rpoH* (RNA polymerase sigma factor H). Gene knockout studies confirmed role of *tnaA* and *dgcP* genes in observed antimutagenicity of ESP. Other naphthoquinones like plumbagin and juglone also displayed antimutagenicity but this activity was quite less in menadione or phylloquinone. Quinones displayed quite low antioxidant capacity and oxidative stress was not induced significantly in EMS treated cells.

Chapter 4: Discussion

Among the naphthoquinone rich foods, spinach naphthoquinone extract displayed highest antimutagenicity. The yield was found to have weak correlation with antimutagenicity indicated the significance of phytoconstituent in this activity. From the quinone extract of spinach antimutagenic compound was characterized as an ethoxy-substituted phylloquinone (ESP). This novel compound possessed $-OCH_2CH_3$ instead of $-CH_3$ in case of phylloquinone at the 2^{nd} position. During mechanistic study, ESP was found to bind to the minor groove of DNA which could be one of the mechanisms for its antimutagenicity as minor groove is often recognized by

transcription factors and such interaction may lead to change in gene expression profile. The proteomic analysis and knockout studies indicated role of *tnaA* and *dgcP* in observed antimutagenicity. The gene *tnaA* encodes for tryptophanase which is involved in biosynthesis of indole. This is known to inhibit cell division in *E. coli*by reducing electrochemical potential (ECP) across the cytoplasmic membrane leading to inhibition of FtsZ ring which is a prerequisite for the division. The *dgcP* gene is involved in c-di-GMP formation which can bind with another diguanylate cyclase (YfiN) and exposes binding sites for FtsZ and ZipA and thus retains the Z ring at themidcell and prevents cell division. The up-regulation of these genes may retard the cell growth and provide additional time period for proof reading leading to DNA repair. High antimutagenicity in ESP, plumbagin and juglone, but comparatively less in phylloquinone and menadione indicated that -OH / $-OCH_2CH_3$ substitutions contribute to the antimutagenicity of naphthoquinones. In general, quinones displayed poor antioxidant activity but high antimutagenicity indicating the lack of correlation between these activities.



Chapter 5: Summary and conclusions

Naphthoquinone extract from spinach displayed high antimutagenic activity as compared to other naphthoquinone rich vegetables. Naphthoquinone extract was resolved to four different compounds in TLC.A fluorescent compound (C4) displayed highest antimutagenicity and found to have high purity level when analyzed using HPLC. The compound was characterized as an 2-ethoxy-3-(3,7,11,15-tetramethyl hexadec-2-ethyl) naphthaquinone-1,4-dione or in short ethoxy-substituted phylloquinone (ESP). ESP was not found to interact directly to the mutagen. Induction of oxidative stress or its reduction was not found to be the major contributing factor to the observed antimutagenicity. A strong DNA binding activity was observed at minor groove. The 2-D proteomics profile, mass spectrometry and gene knockout studies confirmed the role of *tnaA* and *dgcP* genes reported to inhibit cell division in observed antimutagenicity of ESP. Besides, other naphthoquinones like plumbagin and juglone also displayed antimutagenicity. This indicated that functional groups like -OCH_2CH_3/ -OH are important for antimutagenicity of naphthoquinones. Findings of this study provided fundamental information pertaining to functional and nutraceutical potential of dietary ingredients.

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Publications:

Journal

1. **Sanjeev Kumar**, Chatterjee, S., Tripathi, J., & Gautam, S. Purification and characterization of the principal antimutagenic bioactive as ethoxy-Substituted phylloquinone from spinach (*Spinacea oleracea* L.) based on evaluation in models including human lymphoblast $TK^{+/-}$ cells. *Journal of Agricultural and Food Chemistry* **64**, 8773-8782 (2016).

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Sanjeev Kumar

Signature of Student:

Date: 6/9/18

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S. No.	Name	Designation	Signature	Date
1.	Dr. P. S. Variyar	Chairman		6/9/18
2.	Dr. S. Gautam	Guide/ Convener		6.9.18
3.	Dr. H. S. Misra	Member		07/09/18
4.	Dr. J. S. Melo	Member		6/9/18
5.	Dr. S. S. Kumar	Member		7/9/18

ABBREVIATIONS

µL	**Microliter**
mL	**Milliliter**
µg	**Microgram**
mg	**Milligram**
g	**Gram**
µM	**Micromolar**
mM	**Millimolar**
nm	**Nanometer**
mm	**Millimeter**
s	**Second**
min	**Minute**
h	**Hour**
^{o}C	**Degree Celsius**
OD	**Optical density**
λ_{Ex}	**Excitation wavelength**
λ_{Em}	**Emission wavelength**
λ_{max}	**Absorbance maxima**
LB	**Luria Bertani broth**
LA	**Luria Bertani agar**
EMS	**Ethyl methanesulfonate**
5-AZ	**5-Azacytidine**
Rif^{R} assay	**Rifampicin resistance assay**

TK 6	**Thymidine kinase 6**
CHAT	**Cytidine hypoxanthine aminopterin thymidine**
CHT	**Cytidine hypoxanthine thymidine**
RPMI medium	**Roswell Park Memorial Institute medium**
CCD	**Charge-coupled device**
DMB	**1,3-dimethylbarbituric acid**
TLC	**Thin layer chromatography**
HPLC	**High performance liquid chromatography**
FTIR	**Fourier transform infrared spectroscopy**
NMR	**Nuclear magnetic resonance**
MHz	**Megahertz**
ppm	**Parts per million**
MALDI-TOF MS	**Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry**
HCCA	**α-Cyano-4-hydroxycinnamic acid**
ESP	**Ethoxy-substituted phylloquinone**
$DPPH^{\cdot}$ radical	**1,1-Diphenyl-2-picrylhydrazyl radical**
$ABTS^{\cdot+}$ radical	**2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) radical**
DCFDA	**2', 7' dichlorofluorescin diacetate**
CT DNA	**Calf thymus DNA**
PBS	**Phosphate buffer saline**
EtBr	**Ethidium bromide**
DAPI	**(4', 6-Diamidino-2-phenylindole dihydrochloride)**
MG	**Methyl green**

CD	**Circular dichroism**
mdeg	**Millidegrees**
2-D	**2-dimentional protein gel electrophoresis**
c-di-GMP	**Bis-(3′-5′)-cyclic dimeric guanosine monophosphate**
GTP	**Guanosine triphosphate**

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Chapter 1:

Introduction

&

Review of Literature

1.1. Food and their functional relevance

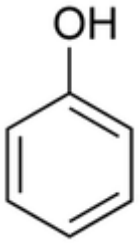
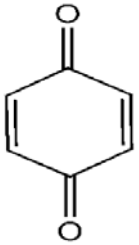
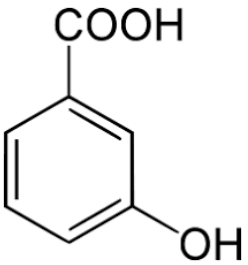
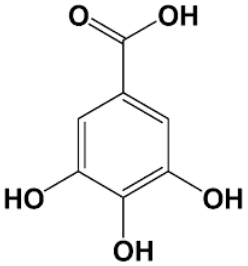
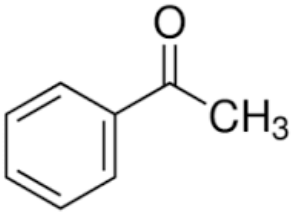
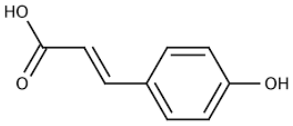
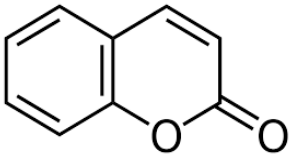
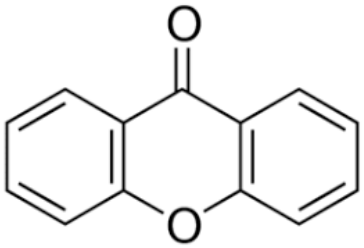
Hippocrates, often referred as the 'Father of Medicine', emphasized on ***"Let food be your medicine and medicine be your food".*** Besides serving the calorific needs, foods have often been investigated for its nutraceutical potential and pharmaceutical relevance [1, 2, 3]. Most foodsbelieved to be of health promoting nature, possess various bioactive phytochemicals. Its primary validation is based upon studies involving *in vitro* cell-culture systems, animal models, and finally human beings [4]. Among foods, vegetables and fruits are primarily considered to be rich in nutraceuticals due to phytoingredients such as phenolics, flavonoids, and naphthoquinones. Many of these have been reported to help in preventing or curing different chronic diseases [5, 6].

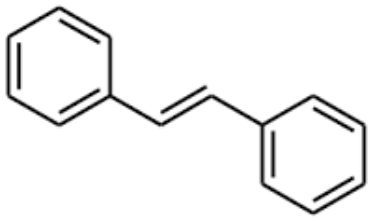
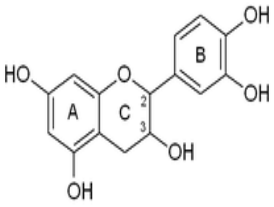
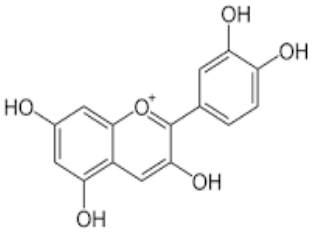
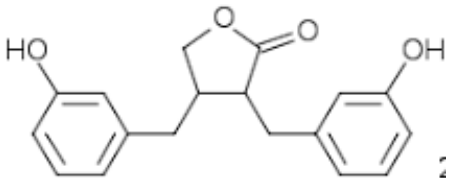
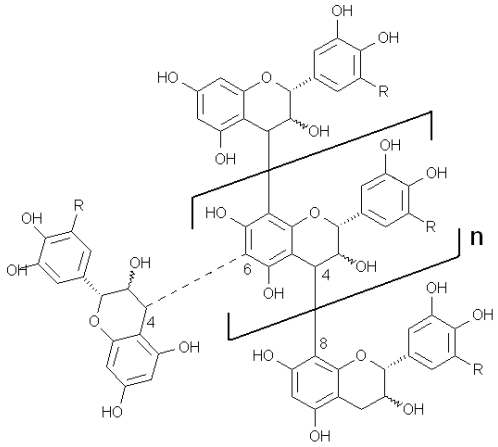
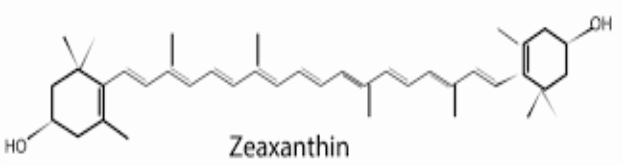
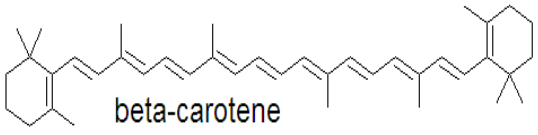
1.2. Phytochemicals: importance and their types

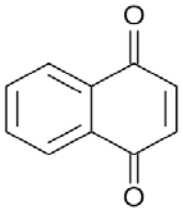
The term 'Phytochemicals' has evolved from the Greek word 'phyto', meaning plant. These are biologically active, naturally occurring compounds in plants and classified as primary or secondary constituents, depending on their role in plant metabolism [7]. Primary constituents include the common sugars, amino acids, proteins, purines and pyrimidines, whereas, the secondary constituents are simple phenols, phenolic acids, flavonoids (anthocyanins, flavones, flavanones, isoflavones, and flavanols), coumarins, stilbenes as well as hydrolysable and condensed tannins, lignans, and lignins (Table 1). Secondary constituents have been reported to be involved during response to conditions such as infection, wounding, and exposure to UV radiation in plants [8]. Among secondary metabolites, phenolics share common structural feature: an aromatic ring with at least one hydroxyl substituent [9, 10]. Phenolics have been reported for numerous health protective such as antioxidant, antitumor, antimicrobial,

anti-inflammatory, antirheumatic, analgesic, estrogenic and antimutagenic properties as well as astringent and styptic uses [11]. The basic flavonoid is 15 carbon flavan nucleus atoms arranged in three rings (C6-C3-C6), which are labeled A, C, and B (Table 1). Flavonoids have been shown to have antioxidant activity, coronary heart disease (CHD) prevention, hepatoprotective, anti-inflammatory, anticancer and antiviral activities [12]. Anthocyanins are flavonoids with positive charge at C-ring (Table 1). It have also been reported for anti-inflammatory, prevention of capillary fragility, antitumor, hepatoprotective, antimutagenic, antioxidant bioactivities as well as inhibiting lipoprotein oxidation and platelet aggregation [13]. Another secondary metabolite naphthoquinones are two ring (C6 and C4) structures with quinone group at 1^{st} and 4^{th} positions (Table 1).

Table 1. Class of phytochemicals based upon chemical structure.

Class	Basic skeleton	Common example
Simple phenols Benzoquinones	C_6	 Hydroxybenzene  Benzoquinone
Phenolic acids	C_6- C_1	 Hydroxybenzoic acid  Gallic acid
Acetophenones Tyrosine derivatives	C_6-C_2	 Acetophenones
Hydroxycinnamic acid Coumarins	C_6-C_3	 Hydroxycinnamic acid  Coumarin
Xanthones	C_6- C_1-C_6	 Xanthones

Stilbenes	C_6- C_2-C_6	 (E)-Stilbene
Flavonoids: Flavanol / Anthocyanins	C_6- C_3-C_6	 Catechin  Cyanidin
Lignans	$(C_6$- $C_3)_2$	
Condensed tannins (proanthocyanidins)	$(C_6$-C_3-$C_6)_n$ Linear (4→8 bounds) Branched (4→6 bounds)	
Carotenoids (xanthophylls/ carotenes)	Isoprene derivative [CH_2=C(CH_3)CH=CH_2]	 Zeaxanthin  beta-carotene
Naphthoquinones	C_6-C_4	

	 1,4-naphthoquinone

Note: Structures taken from Google images (https://en.wikipedia.org; https://socratic.org; http://www.chm.bris.ac.uk; https://commons.wikimedia.org; https://www.glentham.com; https://pubs.rsc.org). These are required for the basic understanding of the problem addressed in the current study.

1.3. Naphthoquinones and their functional role

Naphthoquinones are one of the groups of secondary metabolites. It has two different isomers, 1,4-naphthoquinone and 1,2-naphthoquinone in which the former occurs naturally. Several 1,4-naphthoquinone derivatives have been reported from different plants and herbals such as phylloquinone (vit. K), ubiquinone, plastoquinone, plumbagin, juglone, lapachol, alkanin etc [14]. Phylloquinone (vit. K), ubiquinone, and plastoquinoneare prevalent in green vegetables [15]. In plants, they may occur in dimeric, trimeric, reduced and glycosidic forms. Most of them are coloured compounds and their colours usually vary between yellow, orange and brown.

1.3.1. Occurrence

Naphthoquinones are widespread in nature and produced by certain fungi (e.g. *Fusarium* spp., *Marasmius* spp., *Verticillium* spp.), Actinomycetes (Streptomyces), lichens, sea urchins, algae and higher plants [15]. Many foods especially green vegetables and certain herbals are rich source of naphthoquinones. Botanical families such as Avicenniaceae, Bignoniaceae, Boraginaceae, Droseraceae, Ebenaceae, Juglandaceae, Nepenthaceae and Plumbagnaceae include plants rich in naphthoquinones.

1.3.2. Structure

1,4-naphthoquinone is the backbone structure for various naturally occurring or synthetic naphthoquinones. Figure 1 depict naturally occurring naphthoquinone where 1,4-naphthoquinone and menadione are synthetic naphthoquinones [14]. Vitamin K1 or Phylloquinone is 1,4-naphthoquinone, with 2-methyl and 3-phytyl (isoprenoid side chain) substituent. Plumbagin possesses 5-hydroxyl and 2-methyl functional groups on the 1,4-naphthoquinone nucleus, whereas juglone has only the 5-hydroxyl and

menadione only the 2-methyl group. Lapachol is 1,4-naphtho-quinone with 2-hydroxy and 3-(3-methyl-2-butenyl) groups.

1.3.3. Biosynthesis

Naphthoquinones are biosynthesized via a variety of pathways which includes acetate, malonate, shikimate, succinyl CoA and mevalonate [16]. In plants, phylloquinone (2-methyl-3-phytyl-1,4-naphthoquinone or vitamin K 1) is a vital redox co-factor required for electron transfer in photosystem I. A closely related form called menaquinone [2-methyl-3-(all-trans-polyprenyl)-1,4-naphthoquinone or vitamin K2] is synthesized by red algae, diatoms, and bacterial species [17]. In vertebrates, vitamin K is required for blood coagulation, vascular and bone metabolism [18]. The biosynthetic pathway of phylloquinone is shown in Figure 2. The immediate precursor of the redox active naphthoquinone ring of phylloquinone is chorismate. It is first isomerized to serve as a substrate for an atypical multi-functional enzyme, termed PHYLLO that catalyzes addition, elimination and aromatization. The product from PHYLLO, o-succinylbenzoate, is activated by ligation with CoA and cyclized, yielding the 1,4-dihydroxy-2-naphthoate (DHNA) CoA thioester. DHNA-CoA is subsequently hydrolyzed, and DHNA is prenylated and methylated [19].

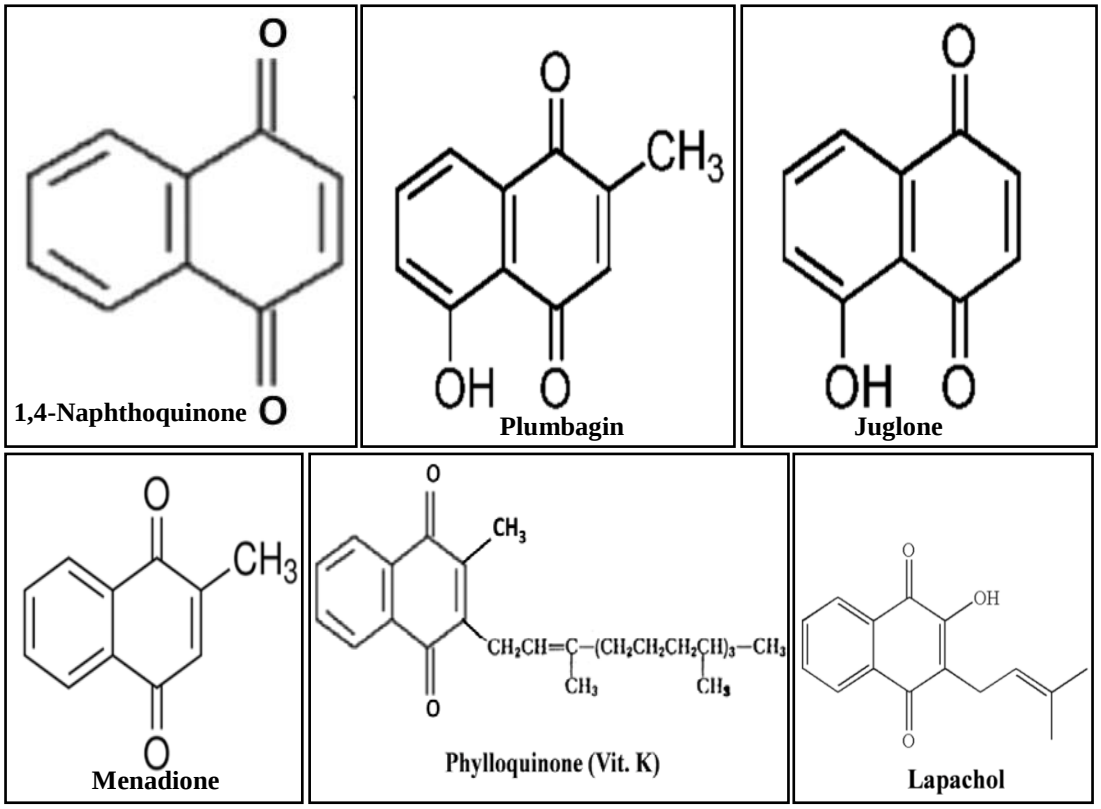
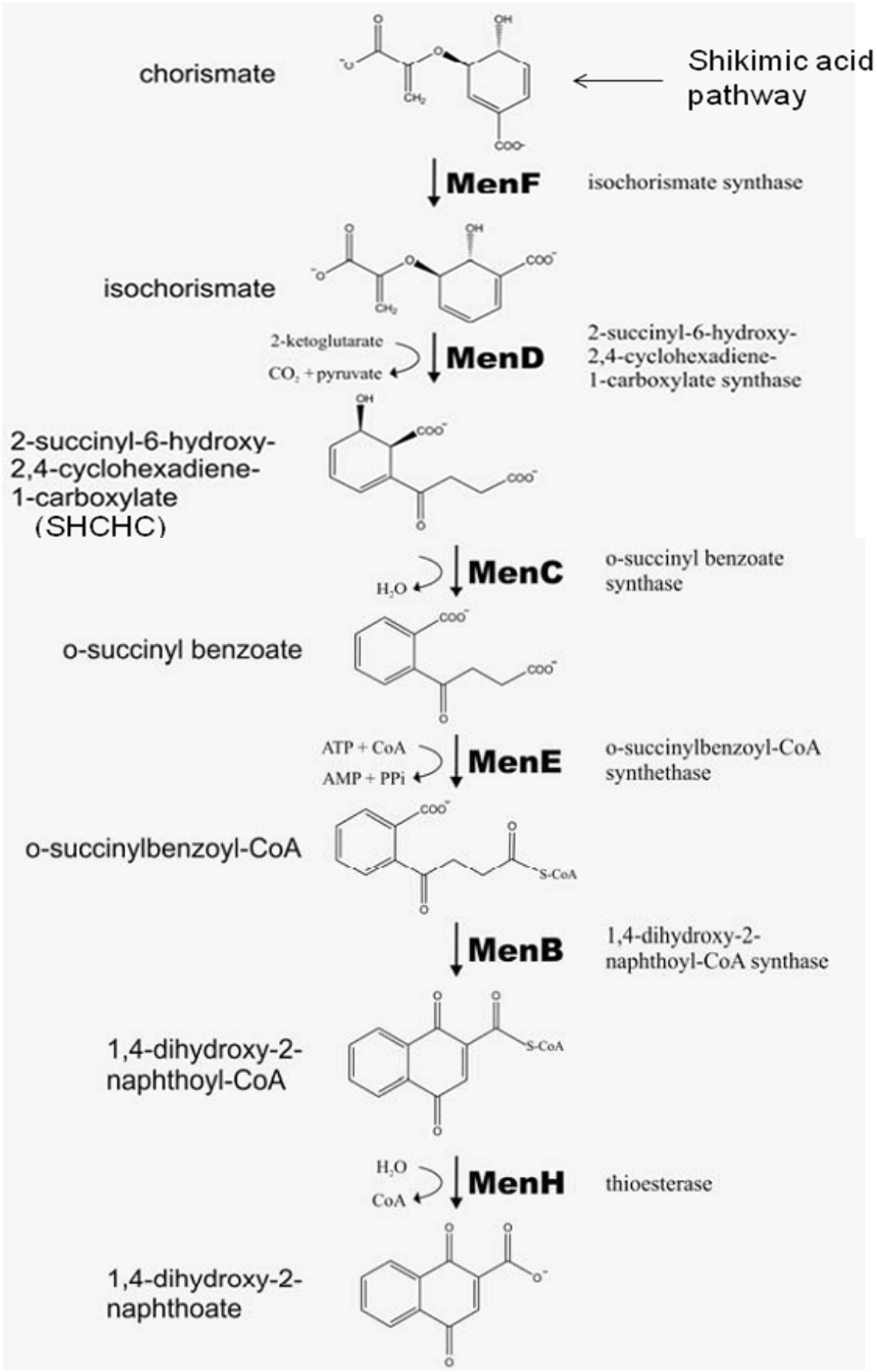


Figure 1. Structure of various 1,4-naphthoquinone derivatives.

Note: Structures taken from Google images (https://en.wikipedia.org; https://www.researchgate.net; http://www.fao.org). These are required for the basic understanding of the problem addressed in the current study.


chorismate
Shikimic acid pathway
MenF
isochorismate synthase
isochorismate
2-ketoglutarate
CO2 + pyruvate
MenD
2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase
2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate (SHCHC)
H2O
MenC
o-succinyl benzoate synthase
o-succinyl benzoate
ATP + CoA
AMP + PPi
MenE
o-succinylbenzoyl-CoA synthethase
o-succinylbenzoyl-CoA
S-CoA
MenB
1,4-dihydroxy-2-naphthoyl-CoA synthase
1,4-dihydroxy-2-naphthoyl-CoA
H2O
CoA
MenH
thioesterase
1,4-dihydroxy-2-naphthoate

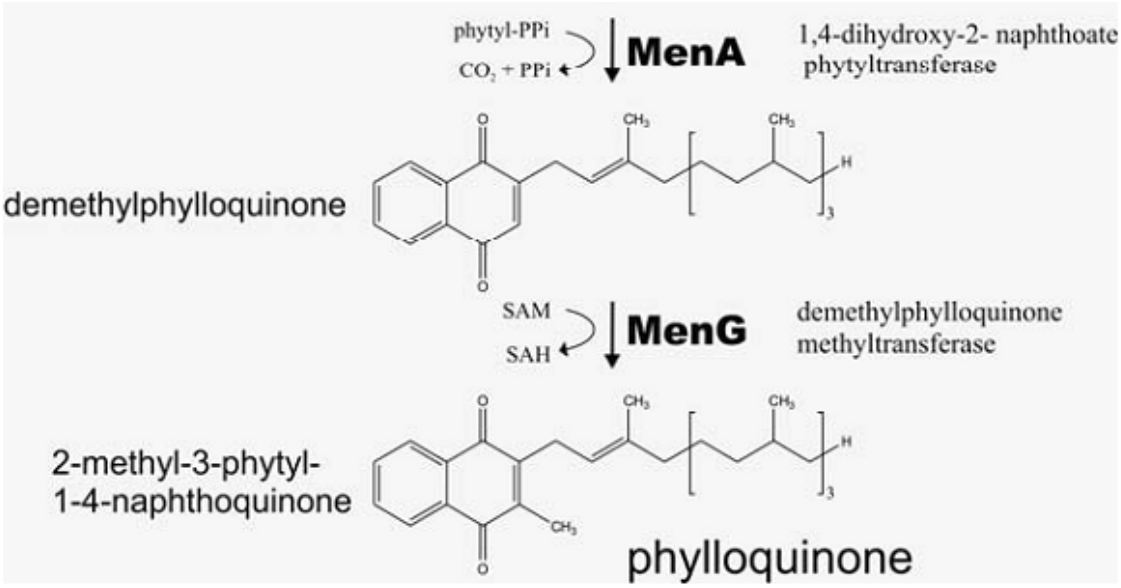


Figure 2. Pathway of phylloquinone (vitamin K) biosynthesis.

Note: Structures taken from Google images (https://edoc.ub.uni-muenchen.de). These are required for the basic understanding of the problem addressed in the current study.

1.3.4. Bioactivities

Recently, interest of many investigators in naphthoquinone compounds is due to their broad-range of biological activities such as antibacterial, fungicidal, antiparasitic, insecticidal, antitumor, antimutagenic, antioxidant, allelopathic anti-inflammatory, and anti-atherosclerosis [15, 20, 21, 22, 23, 24]. Because of these properties many naphthoquinone rich plants are used in folk medicines by natives in Asia. Especially Chinese and South Americans have been using aerial as well as subterranean parts of these plants for medicinal uses since more than 100 years. Table 2 depicts the bioactivities of some common natural and synthetic naphthoquinones.

1.4. Mutations and their role in human disease

Mutations cause permanent inheritable alteration in DNA nucleotide sequence leads to alteration the code in a gene. This is due to change, removal or insertion of one or more bases in a gene resulting in an altered or inactive gene product [25]. Living organisms are exposed to various genotoxic agents such as insecticides, herbicides, pesticides, viruses, toxigenic microbes including fungi and radiation (ultraviolet and X-rays) sources [26]. Dietary heterocyclic aromatic amines have also been reported from protein rich foods such as meat and fish which can induce damage to mammalian cells [27]. Besides, mutations can also occur spontaneously due to errors in DNA replication, repair, and recombination [28]. In general, mutations are detrimental because in most cases they cause defects in cellular functions.

Table 2. Functional properties of (a) natural (b) synthetic naphthoquinones.

(a) Naturally occurring naphthoquinones

Name	**Source**	**Functional application**
Phylloquinone (vit. K_1)	Green plants	Blood coagulation, calcium homeostasis [29]
Plumbagin	Chitrak	Antitumor, antimicrobial, anti-inflammatory, anti-atherosclerosis [20, 23]
Juglone	Black walnut	Antimicrobial, insecticidal, anthelmintic, antitumor [20]
Lapachol	Lapaco tree	Antitumor [22]
Alkannin	*Alkanna tinctoria*	Antioxidant, antitumor, antimicrobial, antithrombotic [30]
Lawsone	Rose balsam	Antimicrobial, antiviral, antitumor, antiparasitic [14]
Tetra-, Penta-, Hexa-hydroxylated naphthoquinone	Sea urchins	Antioxidant [15, 24]

(b) Synthetic naphthoquinones

Name	**Functional application**
Menadione (vit. K_3)	Treatment of hypoprothrombinemia [31]
Naphthazarine	Anticancerous [32]

Some mutation related diseases are inherited from the parents and present in individual at birth, whereas others are related to acquired mutation caused by gene(s) that occur either randomly or due to some environmental exposure. Such mutations are not inherited from a parent. However, this may result in innate metabolic defects in cellular systems, triggering morbidity that possibly are translated and manifested in chronic diseases such as atherosclerosis, cardiovascular diseases (CVD), neoplastic inductions, cancer, mortality andother degenerative diseases [25, 33]. Many of these diseases involve mutation in single gene or multiple genes. However, recently rare genetic variants of TREM2 gene with increased risk of Alzheimer's disease, fronto-temporal dementia, amyotrophic lateral sclerosis, Parkinson's disease and Nasu-hakola disease has been reported [34].

Mutagens work through different mechanism such as alkylation [e.g. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), ethyl methanesulfonate (EMS), and methyl methanesulfonate (MMS)], substitution by base analogs [e.g. 5-bromouracil (5-BrU), 2-amino-purine (2-AP) and 5-azacytidine (5-AZ)], and intercalation [e.g. acridines (proflavine and quinacrine), and ethidium bromide (EtBr)] (Table 3).

As a consequence of mutagenic exposure base substitution or frame shift mutation may occur in the genome [35]. Base substitutions are those mutations in which one base pair is replaced by another. Base substitutions can be divided into transitions and transversions. A transition is the replacement of a purine base by purine (A to G or G to A) as shown in Figure 3or pyrimidine by pyrimidine (C to T or T to C). In transversion replacement is of pyrimidine by purine (C to A, C to G, T to A, T to G) or purine by pyrimidine (A to C, A to T, G to C, G to T). Base-pair additions or deletions or intercalation of mutagen causes frame shift mutations.

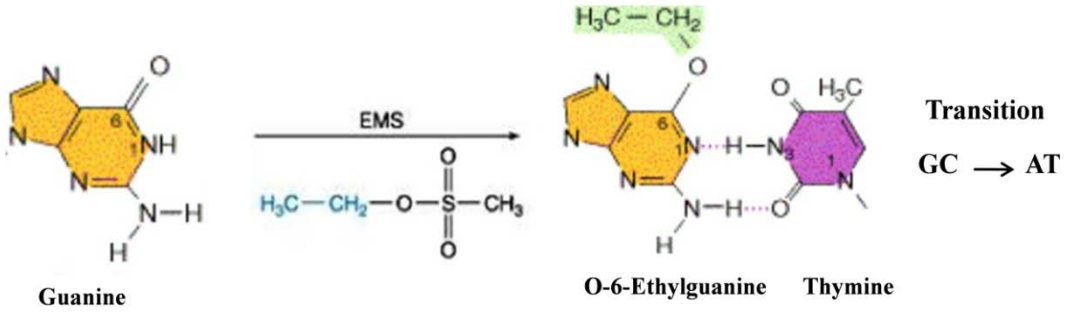


Figure 3: Mechanism of ethyl methanesulfonate (EMS) mutagenesis

Note: Drawings taken from Google images (https://www.slideshare.net). These are required for the basic understanding of the problem addressed in the current study.

Table 3. General mutagens and possible mode of action [36].

Mutagens	**Mode of action**
Methyl methanesulfonate	Alkylation leading to mispairing
Ethyl methanesulfonate	Alkylation leading to mispairing; may induce DNA strand and lesions
N-methyl-N'-nitro-N-nitrosoguanidine	Alkylation leading to mispairing
9-aminoacridine	Intercalation and frameshift mutation
Acridine	Intercalation and frameshift mutation
Ethidium bromide	Intercalation and frameshift mutation
Quinacrine	Intercalation and frameshift mutation
4-nitro-o-phenylenediamine	Frameshift mutations
2-amino-purine	Base substitution
5-bromouracil	Base substitution
5-azacytidine	Base substitution
4-nitroquinoline-N-oxide	Base substitution

1.5. Dietary sources of antimutagens

As per the World Health Organization (WHO), cancer prevention is closely linked to diet, and around one-third of all cancer deaths are preventable [37]. Dietary intervention has been postulated to minimize the onset of such diseases mostly due to the occurrence of natural antimutagens [38]. There are continued efforts all over the world to explore the rich biodiversity of edible and medicinal plants as well as other edible non-toxic plants in pursuit of the most effective phytoantimutagens. Several studies have been carried out in last four decades in order to identify compounds that might protect humans against DNA damage and its consequences as phytochemicals that reduce mutagenesis may offer preventive potential.

1.5.1. Fruits

Among fruits, *Aronia melanocczrpa* (Black chokeberry), Java plum (*Syzygium cumini*), apple (*Malus domestica*) cv. 'Granny Smith', chloroform extracts of guava (*Psidium guajava*), hexane fraction of *Randia echinocarpa*, ethanolic extract of *Eugenia stipitata*, and aqueous fruit extracts /juice of copaiba (*Copaifera langsdorfi*), pomegranate, murici (*Byrsonima crassifolia*), noni (*Morinda citrifolia*), mangaba (*Hancornia speciosa*), jackfruit (*Artocarpus heterophyllus*), grape (*Vitis vinifera*), date palm (*Phoenix dactylifera*), and elephant apple (*Dillenia indica*) have been reported to display high antimutagenicity [25].

1.5.2. Vegetables

Among vegetables such as beets, chives, horse radish, rhubarb, onions, spinach including cruciferous vegetables displayed strong antimutagenic activity whereas, moderate antimutagenicity was found with tomatoes and green beans. Some vegetables such as Asparagus, carrots, fennel leaves, parsley, radish and green pepper

were not found to display any antimutagenicity [39]. In another study, most antimutagenic vegetables were cauliflower, cabbage, pepper (bell-red, hot-red Jalapino, and hot Arbol), eggplant (Tis, small-violet and green-yellow-striped), garlic, onion (red), Bean (lima, clustered and yardlong), Zucchini, squash, gourd (bottle), cucumber (Madras), pea (green), drumstick, and Indian gooseberry against ultraviolet induced mutagenesis [40]. Antimutagenicity has reported to be affected by several factors such as variety, cultivation mode, and solvent used for bioactive extraction.

1.5.3. Bioactives from fruits and vegetables

Tomato, carrot and green pepper non-polar compounds such as carotenoids, xanthophylls or carotenol esters have been reported as possible antimutagen [41, 42]. Several mid polar compounds have also reported been reported for antimutagenicity. Thirteen flavonoids and related compounds were reported from spinach where only 5,6,3',4'-tetrahydroxy-7-methoxyfavonol 3-O-disaccharide was reported as potent antimutagen. Besides, polyphenolic from French bean was reported for antimutagenicity against 1-NP, B[a]P and aflatoxin B1 [43, 44]. Anthocyanin-rich water fraction (polar) and ethyl acetate (mid polar) fraction from Andean purple corn were analyzed against Trp-P-1 in Ames test where quercetin derivative containing ethyl acetate fraction was found to be more potent [45]. Anthocyanins of Black chokeberry, procyanidin B1 of apple cv. 'Granny Smith', anthocyanin (petunidin-3, 5-diglucoside) of Java plum and anthocyanin (peonidin-3 glucoside) of red rose petal have been reported as antimutagens in various foods [25, 46, 47, 48].

1.6. Diets as antioxidant

Increased levels of ROS such as superoxide anion radical ($O_2^{-\cdot}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\cdot}OH$) and decreased ROS

scavengers and antioxidant enzymes are associated with DNA damage leading to mutations and human diseases including cancers [49]. Antioxidants are compounds capable to either delay or inhibit the oxidation processes by direct scavenging of free radicals such as reactive oxygen species (ROS) or enhancing antioxidant enzyme at cellular level [50]. Plant-based diet such as fruits, vegetables, spices and herbals may reduce the risk of oxidative stress-related diseases [51]. They contain phytochemicals such as phenols, flavonoids, anthocyanins, carotenoids, and vitamin C which have antioxidant activity [52]. Most spices, herbs and their formulations have been reported as antioxidant rich products [50]. Besides, fruits (e.g. Indian gooseberry, bilberries, zereshk, strawberry, artichoke, pomegranate, and black olives), nuts (e.g. walnuts, pecans, Chestnuts, peanuts, and pistachios), vegetables (e.g. curly kale, and chilly) and chocolate constitute common foods with high antioxidant values [53].

1.7. Possible mechanism of antimutagenicity

Several mechanisms have been proposed for antimutagenicity of extract/ compound which is also depicted in figure 4.

1.7.1. Inactivate the mutagen directly by modification or complex formation

This is based upon direct chemical interaction between an antimutagenic compound and a mutagen before it induces DNA damage. Example: cysteine interaction with 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone and gallic acid interaction with electrophilic mutagens [54, 55].

1.7.2. Antimutagenicity through radical scavenging

Mutagens may act through generation of reactive oxygen species (ROS) and in these cases scavenging of the ROS could be the principal mechanism of antimutagenicity as reported in case of lipoic acid against mitomycin C [56, 57].

1.7.3. Blocking of mutagen and DNA interaction

Antimutagens (e.g. β-amino ketones) work by blocking the mutagen binding to DNA and thus may prevent MNNG and 9-aminoacridine (9-AA) induced mutation [58].

1.7.4. Modulation of replication or DNA repair enzymes activities

Repression of replication and cell division can provide additional period for DNA repair and consequently may lead to reduced SOS induced error prone pathway for translesion synthesis and subsequent reduced mutagenesis [59]. Increase in biosynthesis or activity of DNA repair enzymes or suppression of error prone repair pathway(s) could be another mechanism for antimutagenicity as reported in case of vegetables and honey against UV and EMS exposure [47, 60].

1.7.5. Prevention of mutagenic transformation

Antimutagens may interference with the cytochrome P450-mediated metabolism of mutagens and prevent transformation to active mutagenic metabolites as reported in case of quercetin against pro-mutagens [55, 61].

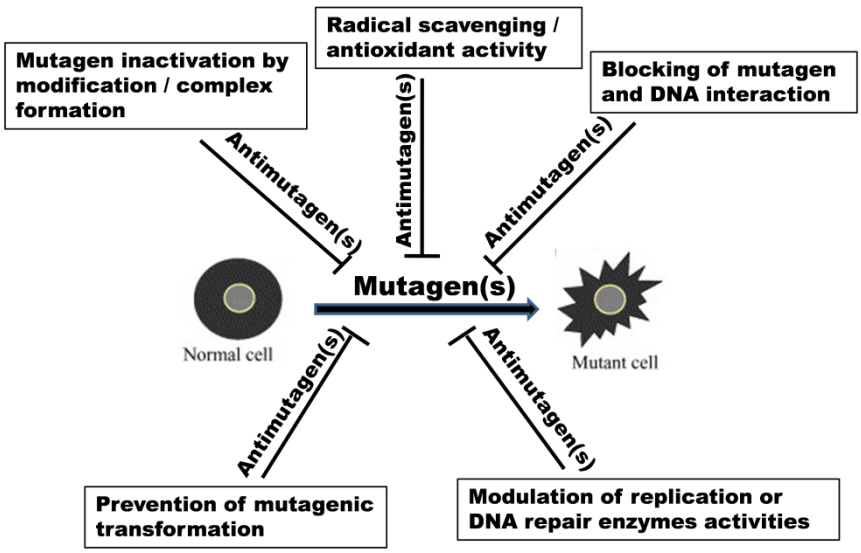


Figure 4. Possible mechanism of antimutagenicity

1.8. Statement of the problem

Naphthoquinones are known to have high nutraceutical potential but somehow less explored in terms of antimutagenicity compared to other group of phytochemicals. Thus natural source of naphthoquinones (which is widely available and consumed) needs to be explored for this bioactivity.

1.9. Aims and Objectives

1.9.1. Extraction of naphthoquinones from its rich dietary sources including vegetables

A. Selection of naphthoquinone rich vegetables (spinach, lettuce, iceberg lettuce, cabbage, broccoli and French bean).

B. Extraction of naphthoquinones and total soluble phenolics from these vegetables for comparative evaluation of bioactivity.

1.9.2. Analysis of health protective functional properties (antimutagenic and antioxidant) of the naphthoquinones

A. Determination of animutagenicity and antioxidant capacity using these extracts.

B. Selection of naphthoquinone (instead of phenolic) extract of vegetable showing highest antimutagenicity for further bioactive characterization.

1.9.3. Purification, identification and characterization of bioactive naphthoquinone showing highest antimutagenicity from the most potent source

A. Performing chromatographic and spectroscopic techniques, and biochemical tests for purification, identification and characterization of naphthoquinone bioactive from the most potent source.

B. Elucidating structure of bioactive naphthoquinone.

1.9.4. Understanding the molecular mechanism contributing to antimutagenicity by the bioactive naphthoquinone

A. To understand the molecular mechanism, assessment of antimutagenicity under various conditions, and evaluation of possibility of direct interaction with mutagen, interaction with DNA, protein profiling using 2-D and MALDI TOF MS/MS analysis, and antioxidant capacity.

B. Development of model based upon current findings to explain the possible mechanism of antimutagenicity of bioactive compound.

Chapter 2

Materials & Methods

2.1. Selection of foods (vegetables) for the proposed study

Most commonly grown fresh green vegetables such as spinach (*Spinacia oleracea* var. Semisavoy), lettuce (*Lactuca sativa*), iceberg lettuce (*Lactuca sativa* var. Iceberg), cabbage (*Brassica oleracea*), broccoli (*Brassica oleracea* var. *italica*) and French bean (*Phaseolus vulgaris*) are reported to have high naphthoquinone content were selected for the proposed study. These vegetables were procured from the agricultural field of Nashik, Maharashtra, India. The plant material was thoroughly washed 3 times with tap water to remove any debris/dust and then washed with distilled water. The cleaned material was dried in shed to remove residual water and weighed using a fine balance before and after lyophilization (Alpha 2-4 freeze-dryer, Martin Christ, Osterode, Germany) to determine their dry weights (g%).

2.2. Solvent extraction for the proposed bioactivity analysis

Various solvent extracts were freshly prepared and used for the bioactivity analysis.

2.2.1. Aqueous and methanolic extraction

This was performed by the method described earlier [46]. One gram of the lyophilized spinach powder was homogenized in Milli-Q water or methanol (20 mL) using a Polytron homogenizer (Kinematica, Lucerne, Switzerland). The suspension was filtered through Whatman no. 42 filter paper. The suspension was centrifuged (10000 x*g*) for 20 min, and supernatant was vacuum dried (Concentrator 5301, Eppendorf, Hamburg, Germany) at ambient temperature (26 ± 2°C). The dried residue was dissolved in Milli-Q water and filtered through a 0.45 μm (Millex-HV, Millipore, Ireland) filter.

2.2.2. Total soluble phenolic (TSP) extraction

This was performed by the method described earlier [46]. TSP were extracted in acidified water [0.01% (v/v) hydrochloric acid (HCl)] followed by solid-phase extraction (SPE) using a C-18 Sep-Pak cartridge (Waters Corp., Milford, MA, USA) at the ambient temperature. The cartridge was preconditioned with methanol (24 mL) and later with the same volume of acidified Milli-Q water (0.01% HCl). The acidified extract (6 mL) was applied on this column and then washed with acidified water (24 mL; 0.01% HCl) to remove sugars, acids, and other water-soluble compounds. TSP were subsequently eluted with ethyl acetate (24 mL) and vacuum-dried at ambient temperature. Later, this was dissolved in Milli-Q water and filtered through a 0.45 μm filter. Quantification of TSP was performed using the Folin-Ciocalteu colorimetric method and expressed as gallic acid equivalents (mg GAE/g).

2.2.3. Quinone extraction

This was performed by the method described earlier [62, 63]. The lyophilized samples were ground to a fine powder with a mortar and pestle, and 0.5 g was transferred to a centrifuge tube. A 2-propanol/hexane (15 mL; 3:2 v/v) and H_2O (32 mL) were added, followed by sonication (30 s), mixing using vortex (10 min), and centrifugation (5000 x*g*; 5 min; 4°C). The upper layer containing phylloquinone and other quinones was dried under vacuum (Concentrator 5301, Eppendorf) at ambient temperature. Hexane (10 mL) was added to dissolve the residue. Later, the hexane extract was processed by SPE using silica gel columns (Waters Corp.). The SPE column was preconditioned with hexane/diethyl ether (8 mL; 93:3 v/v) followed by the same volume of 100% hexane. The extract (2 mL) was applied directly on the reconditioned column, followed by washing with 100% hexane (8 mL). The quinone-containing fraction was

eluted from the SPE columns with hexane/diethyl ether (8 mL; 93:3 v/v). The eluent was treated with charcoal for overnight at 4°C to remove residual chlorophyll and centrifuged twice (10000 x*g*; 10 min; 4°C). The supernatant was collected and evaporated to dryness at ambient temperature. The qunonic nature of extract was tested by dissolving in methanol and addition of equal volume of concentrated sulphuric acid leading to formation of red colour [64].

2.3. Antimutagenicity analysis

Extracts from vegetables were screened for antimutagenicity against ethyl methanesulfonate (EMS) and 5-Azacytidine (5-AZ) induced mutagenicity using various assays as detailed below [46, 65].

2.3.1. Rifampicin resistance (Rif^R) assay

Escherichia coli MG1655 strain (genotype: F^- λ^- ilvG-rfb-50rph-1) was gifted by Dr. M. Z. Humayun, University of Medicine and Dentistry of New Jersey (UMDNJ), USA. It is a rod-shaped, Gram-negative and facultative anaerobic bacterium that is commonly found in the lower intestine of warm-blooded animals.

Principle: Rifampicin resistance (Rif^R) assay is based on acquisition of rifampicin resistance by *E. coli* MG1655 cells upon mutagen exposure (Figure 5). The *rpoB* gene encodes the β-subunit of RNA polymerase (subunits: α_2, β, β', and ω) which has many hot spots for mutations (Figure 5A) [46, 66]. Around 69 hot spots are located within I-III and N-terminal cluster (N) clusters. Mutation(s) in this gene reduces its binding to rifampicin resulting in acquisition of rifampicin resistance by *E. coli* mutants (Figure 5B) [46, 66].

A)

B)

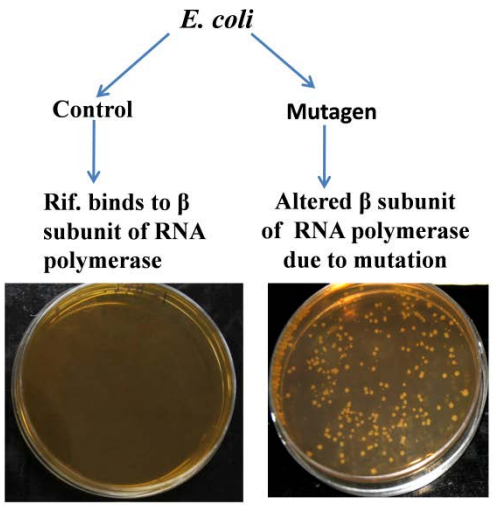


Figure 5. *E. coli* based rifampicin resistance (Rif^R) assay. A) β subunit of RNA polymerase having 4 clusters containing hot spots for mutations (http://fire.biol.wwu.edu); B) Mechanism of mutagen induced rifampicin resistance phenotype.

The presence of antimutagen(s) prevents mutagen induced mutation and thus, there is reduction in rifampicin resistant *E. coli* mutants on rifampicin selective plate. This assay is advantageous due to its simplicity in deployment, the ability to provide a wide spectrum of forward mutations and low level of spontaneous mutations (approximately $1/10^8$ cells).

Method: For determining antimutagenicity against EMS, *Escherichia coli* MG1655 cells were grown overnight at 37°C in Luria-Bertani (50 mL; LB) medium on a rotary shaker (150 rpm). One milliliter of this culture was inoculated in LB (50 mL) and grown for approx. 3 h in similar condition till the $OD_{600\ nm}$ reached to 0.5. This culture aliquot (10 mL) was taken in sterile polypropylene tubes and centrifuged (7500 x*g*; 10 min). The pellet was resuspended in LB (5 mL) and placed on ice for 10 min. One milliliter of cell suspension was transferred in a fresh microfuge tube and mixed with extract or purified compound and 14 µL of EMS (133 mM) and incubated for 45 min on a rotary shaker (75 rpm) at 37°C. The cells were centrifuged (7500 x*g*; 10 min), washed twice with LB, and resuspended (1 mL) of LB. An aliquot (250 µL) of this was further inoculated in LB (4.75 mL) and incubated on a rotary shaker (150 rpm) at 37°C for 16 h. Later, theserially diluted culture was spread plated on LA-rifampicin (100 µg/mL) plates for scoring Rif^R mutants and LA plates for enumerating viable cells.

For determining antimutagenicity against 5-AZ, *E. coli* MG1655 cells were grown overnight at 37°C in LB (50 mL) medium on a rotary shaker (150 rpm) and serially diluted to ~1000 cfu/mL in saline (0.85%). Furthermore, 100 µL of this cell suspension was inoculated in LB broth (2.5 mL), which contained 5-AZ (0.4 mM) and

extract or purified compound, and incubated on a rotary shaker (150 rpm) at 37°C for 16 h. Later, the serially diluted culture was spread plated on LA-rifampicin (100 μg/mL) plates for scoring rifampicin resistant (Rif^R) mutants and on LA plates for enumerating viable cells.

Mutation frequency was calculated as the ratio of total number of Rif^R mutants per mL to the total number of viable cells in the same culture volume. Spontaneous mutation frequency was determined by incubating the cell suspension in the absence of mutagen.

2.3.2. Human lymphoblast gene (*tk*$^{+/-}$) mutation assay

Principle: The autosomal and heterozygous nature of the thymidine kinase gene in the TK6 lymphoblast cell line allows the detection of cells deficient in thymidine kinase enzyme following mutation from $tk^{+/-} \rightarrow tk^{-/-}$ upon mutagen exposure [65]. The basis of this antimutagenic assay is shown below (Figure 6). The culture was cleansed of pre-existing mutants using aminopterin to eliminate the TK-deficient cells, where aminopterin inhibits the endogenous *de novo* biosynthesis of nucleotides.

Method: In brief, CHAT, i.e., cytidine (0.01 mM), hypoxanthine (0.2 mM), aminopterin (0.2 μM), and thymidine (0.0175 mM) were added to TK6 culture to ensure optimal growth of TK-proficient ($tk^{+/-}$) cells. After 2 days, the cells were centrifuged, resuspended in CHT (without aminopterin), grown for 1 day, and diluted with Roswell Park Memorial Institute (RPMI; 10%) medium for further growth. The exponentially growing cells ($tk^{+/-}$) having a density of ~2×10^5 cells/mL were treated with mutagen EMS (0.5 mM) or 5-AZ (0.3 μM)with or without extract or compound (0.5 or 1 mg/mL) for 4 h at 37°C in a humidified (5% CO_2) incubator.

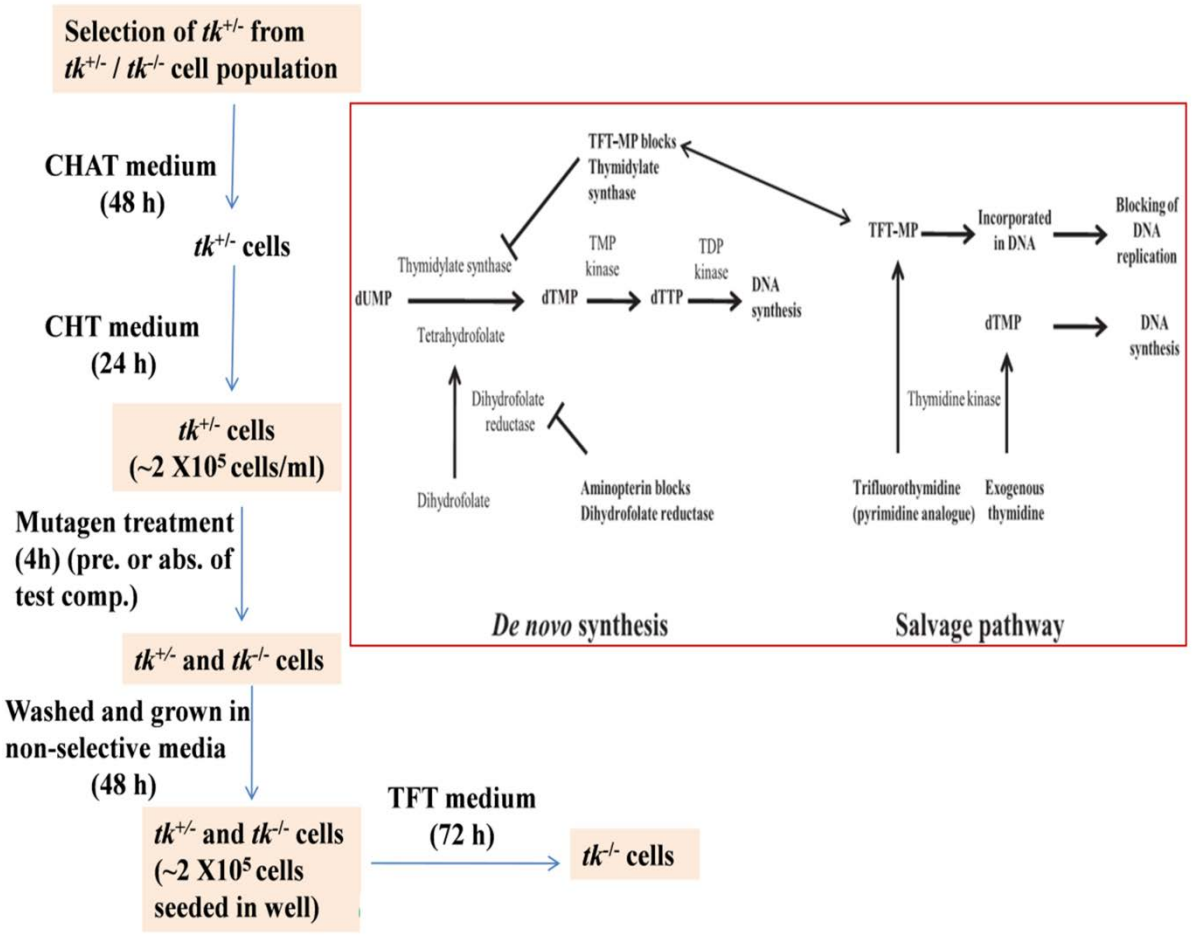


Figure 6. Human lymphoblast TK 6 gene ($tk^{+/-}$) mutation assay [Schematic representation: *de novo* and salvage pathway of pyrimidine and purine biosynthesis (https://www.sciencedirect.com)].

After the treatment, cells were washed twice with phosphate buffer saline (PBS; 10 mM; pH ~7.4) and counted. The cultures were adjusted at a density of ~2 × 10^5 cells/mL and grown for 2 days in non-selective conditions to allow expression of the $tk^{-/-}$ phenotype. After that, ~2 × 10^5 cells from each treatment were grown in selective medium containing trifluorothymidine (TFT; 5 µg/mL) for 3 days, and cell counting was performed as described below. The mutant $tk^{-/-}$ cells lack the thymidine kinase and thus survived the cytotoxic effect of trifluorothymidine (a pyrimidine analogue) (Figure 6) and formed aggregates of viable cells in the suspension cultures which was visualized under an inverted microscope, equipped with a charge-coupled device (CCD camera) (Axiovert 40 CFL, Carl Zeiss, Oberkochen, Germany). These cells were detected by trypan blue exclusion test, where trypan blue stains the dead cells only, and live cells remain unstained due to their intact cell membrane. The live cells were enumerated using a hemocytometer, where the number of cells counted per cubic millimeter (mm) was multiplied by the dilution factor to get the number of cells per millilitre (mL). The result was expressed as the relative number of mutant ($tk^{-/-}$ phenotype) cells per ~10^5 seeded cells as

$$\text{Relative number of mutants} = \frac{\text{number of mutants in test}}{\text{number of spontaneous mutants}}$$

The cytotoxicity of the compound against TK6 cells was also evaluated by trypan blue staining, where the cells were treated with the purified compounds (1 mg/mL) for 4 h and further grown for 2 days. Later, live cells were enumerated using a hemocytometer.

2.3.3. Ames test

Principle: The Ames test uses genetically engineered strains of *S. typhimurium* having mutation(s) in genes involved in histidine biosynthesis [67]. These strains are auxotrophic mutants and require histidine for growth. The method tests capability of the test compound to prevent mutagen induced reversion to a prototrophic state, i.e., cells can grow on a histidine-free medium.

Method: The Ames test was performed using *Salmonella typhimurium* TA102 (genotype: *hisΔ (G)8476galE503rfa1027/pAQ1/pKM101*) and TA100 (genotype: *hisG46galbio ch1005rfa1004 uvrBpKM101*) strains against EMS and 5-AZ induced mutagenesis for bioactive compound as described earlier [67]. The cultures were grown overnight in nutrient broth (50 mL; NB) on a rotary shaker (150 rpm) at 37°C, centrifuged (8000 x*g*; 10 min) and resuspended in fresh NB (50 mL). Cell suspension (1 mL) was transferred to microfuge tubes, mixed with test compounds, and incubated for 10 min. Later, mutagen like EMS (66.5 mM) or 5-AZ (0.4 mM) was added and incubated for 20 min, respectively. Treated cell suspensions were added to top agar (2 mL) containing histidine (0.05 mM) and biotin (0.05 mM), mixed by vortex, poured on minimal glucose plates (0.5% glucose and 1× VB salt), incubated (37°C; 48 h), and examined for revertant colonies. The antimutagenic potential (AP) was determined as AP = 100 − [(T/M) × 100], where T and M are the numbers of revertant colonies/plate in the presence of mutagen and test compound and in the presence of mutagen alone, respectively.

2.4. Isolation, purification and characterization of bioactive compound from most potent vegetable

2.4.1. Thin Layer Chromatography (TLC) for separation of quinones

Principle: TLC is a chromatography technique used to separate non-volatile analytes. It is performed on sheet of aluminium, glass, or plastic coated with a thin layer of adsorbent material such as cellulose, silica gel, or aluminium oxide (alumina) known as the stationary phase. In this technique, sample has to be applied on the plate, a solvent or solvent mixture (mobile phase) is drawn up via capillary action, analytes ascend the TLC plate at different rates and separation is achieved. The distance travelled by the substance being considered is divided by the total distance travelled by the mobile phase. This ratio is called the retention factor or R_f.

Method: Quinone extract and naphthoquinone standards were spotted on 0.25 mm thick TLC silica gel plates (gel 60, Fluorescent F254; Merck, Darmstadt, Germany), developed using an optimized solvent system comprising petroleum ether, chloroform, and acetone (5:1:0.106) and visualized under ultraviolet (UV; 254 and 366 nm) ray (CAMAG, Muttenz, Switzerland). Each resolved spot was quantified by TLC-densitometry on a dual-wavelength Flying spot scanning densitometer (Shimadzu CS-9310PC, Kyoto, Japan). Preparative TLC was also carried out similarly on 0.5 mm thick silica plates using the same solvent system. The TLC bands were scraped and suspended in solvent consisting of n-hexane and diethyl ether (93:3). The suspension was centrifuged (15000 x*g*; 40 min) to remove the silica particles and vacuum-dried.

2.4.2. Antimutagenicity analysis of TLC resolved bands

Antimutagenicity of TLC separated compounds was performed using various assays as detailed above (**2.3**).

2.4.3. Characterization of potent bioactive quinone

2.4.3.1. Spectrophotometric analysis

Principle: The basic principle of spectrophotometry is absorbance or transmittance of light by each compound over a certain wavelength range. The intensity of light that passes through a sample solution is measured. As per Beer-Lambert law, absorption of light (A) is proportional to both concentration of absorber (c) and path length or thickness (l).

$$A = \varepsilon_\lambda \, l \, c \text{ where } \varepsilon_\lambda \text{ is a molar absorbance coefficient}$$

T (transmittance) = I/I_o where, I_o is the intensity of incident radiation and I is the incidence of transmitted radiation. Intensity is number of photons interacting in unit time (seconds).

$$A = \log (1/T) = \log (I_o/I)$$

This measurement can also be used to measure the concentration of a known chemical substance.

Method: Spectrophotometric analysis of TLC-purified bioactive compound and some naphthoquinone (lapachol and phylloquinone) standards was performed using an UV-vis spectrophotometer (JASCO V-530, Japan). The compounds were scanned for spectrum from 200-600 nm.

2.4.3.2. Spectrofluorometeric analysis

Principle: A fluorometer or fluorimeter is a device used to measure fluorescence, i.e., intensity and wavelength distribution of emission spectrum post excitation by a certain spectrum of light. The wavelength of absorbed radiation must be lower (higher energy) than the emitted (fluoresced) wavelength. The difference between these two wavelengths is known as the Stokes shift. These parameters are used to identify the

presence of specific molecules in a medium. Fluorescence spectra provide information about events that occur in less than 10^{-8} s. Modern fluorometers are capable of detecting fluorescent molecule concentrations as low as 1 part per trillion.

The intensity of fluorescence (I_f) is related to the intensity of the incident radiation (I_o): $I_f = 2.3I_o \varepsilon_\lambda clQ$,

where c is the concentration of the fluorescing solution (molar), l is the light path in fluorescing solution (cm), and ε_λ is the molar extinction coefficient for the absorbing material at wavelength λ ($dm^3mol^{-1}cm^{-1}$). Q (quantum efficiency) = quanta fluoresced/quanta absorbed.

However, quenching phenomenon occurs in certain cases when energy emitted as fluorescence is lost to other molecule due to collisional interaction.

Method: Fluorometric analysis of bioactive compound which was fluorescent in nature performed using a spectrofluorometer, and emission wavelength was determined at optimal excitation wavelength (JASCO FP 6500, Japan).

2.4.3.3. High Performance Liquid Chromatography (HPLC)

Principle: High performance liquid chromatography was formerly referred to as high-pressure liquid chromatography. It is a technique used to separate, identify, and quantify each analyte in a mixture. It relies on pumps to pass a pressurized liquid solvent containing the sample mixture through a column filled with a solid stationary phase. The component(s) in the sample interacts slightly differently with the stationary phase material, resulting in different retention time (t_R) and leading to the separation of these components as they flow out the column. Adsorption, partition, ion-exchange, exclusion and affinity chromatography development has resulted in

faster and better resolution in HPLC. Various detectors are in common use, such as UV/Vis, photodiode array (PDA) or fluorescence detectors.

Method: The TLC-eluted bioactive fraction was further subjected to HPLC analysis (Waters) using a reverse-phase C-18 analytical column (250 × 4.6 mm; pore size = 10 μm) having a photodiode array (PDA) detector. An isocratic solvent system of methanol/aqueous acetic acid (5%) (80:20, v/v) with a flow rate 0.8 mL/min was used [68]. The chromatogram was recorded and analyzed using Empower software. Peaks were monitored at various wavelengths to confirm the purity of the bioactive compound.

2.4.3.4. Fourier Transform Infrared Spectroscopy (FTIR)

Principle: FTIR is a vibrational spectroscopic technique used to obtain an infrared spectrum of asymmetrical molecule. The infrared region has insufficient energy to effect nuclear or electronic transitions but involve the phenomena such as chemical bond vibration as well as bending deformations due to change in dipole moment or charge displacement. The scattered radiation has a frequency different from the incident radiation and constitutes spectra.

Method: The bioactive compound and some quinone standards were analyzed by FTIR scanning in the wavenumber range of 4000-400 cm^{-1} using a FTIR spectrometer (model FTIR-660 plus, JASCO, Japan). The compound was pressed directly to the attenuated reflectance crystal KBr supplied with the FTIR instrument. The analysis was performed to assess the functional groups of bioactive.

2.4.3.5. Biochemical test (for -OH substitution)

Principle: The -OH substitution test is based on the formation of coloured complex with Fe (III) with hydroxylated compounds (Figure 7) [69].

Method: TLC-purified bioactive compound was analyzed for hydroxyl substitution by spraying anhydrous ferric chloride in methanol (1 g/100 mL) on a TLC plate and heated at 110ºC for 10 min as per the method described earlier [70]. Some naphthoquinone standards (1,4-naphthoquinone, juglone, menadione, plumbagin, lapacol and phylloquinone) were included in this test as control.

2.4.3.6. Biochemical test (analysis of 2, 3 positions for presence or absence of functional group)

Principle: 2, 3 substitutions test was conducted by the method described earlier whereby 1,3-dimethylbarbituric acid (DMB) reacts with the second or third position of the quinone moiety to produce a pink adduct (Figure 8) [71].

Method: One mL of known standard naphthoquinones (1,4-naphthoquinone, juglone, menadione, plumbagin, lapacol and phylloquinone) and the bioactive compound (2.5 mM) dissolved in methanol were mixed with freshly prepared DMB (1 mL; 0.05 M) in methanol. Later, Milli-Q water (4 mL) was added to attain 50% (v/v) concentration. The mixture was diluted to 10 mL with methanol/water (50%, v/v) and heated at 50°C for 1 h. After cooling, the absorption was measured at the specific wavelength of each naphthoquinone using a spectrophotometer (Hitachi High-Technologies Corp., Tokyo, Japan). Naphthoquinones without DMB served as corresponding blank.

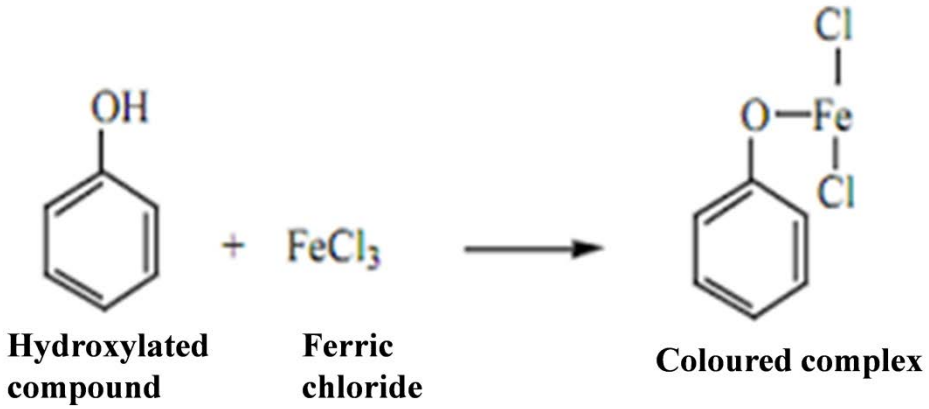


Figure 7. Biochemical test for structure determination (-OH substitution) of C4 compound (Reaction of hydroxylated compound with ferric chloride produces coloured complex).

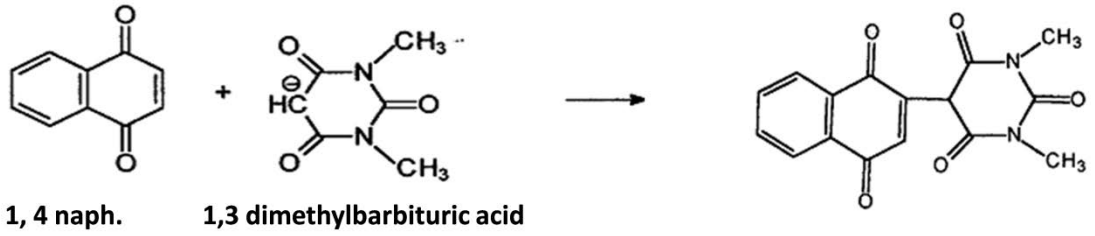


Figure 8. Biochemical test for structure determination (analysis of 2, 3 positions for presence or absence of functional group) of C4 compound (Reaction of 1,4-naphthoquinone with 1, 3 dimethylbarbituric acid produces pink adduct).

Note: Structures taken from Google images (http://1chemistry.blogspot.com; https://www.sciencedirect.com). These are required for the basic understanding of the problem addressed in the current study.

2.4.3.7. Nuclear Magnetic Resonance (NMR) analysis

Principle: NMR is a phenomenon in which nuclei absorb and re-emit electromagnetic radiation in a magnetic field. This energy is at a specific resonance frequency depending on the strength of the magnetic field and the magnetic properties of the isotope of the atoms. The resonance condition in NMR is satisfied in an external magnetic field of several hundred millitesla, with absorbance occurring in the region of radiowave (40-1000 MHz). Electrons, similar to the nucleus, are charged and rotate with a spin to produce a magnetic field opposite to the magnetic field produced by the nucleus. Thus, this electronic shielding reduces the magnetic field at the nucleus. As a result, the energy gap is reduced, and the frequency required to achieve resonance too get reduced. This shift in the NMR frequency due to the electronic molecular orbital coupling to the external magnetic field is called chemical shift. Thus, NMR is able to probe the chemical structure of molecules, which depends on the electron density distribution in the corresponding molecular orbitals. In general, the chemical shift reference standard for ^{1}H and ^{13}C is tetramethylsilane (TMS), whose chemical shift is considered to be 0.0 ppm. All isotopes that contain an odd number of protons and/or neutrons have an intrinsic magnetic moment and angular momentum, in other words a non-zero spin, while all nuclides with even numbers of both have a total spin of zero. The most commonly studied nuclei are ^{1}H and ^{13}C. In ^{13}C NMR, only the ^{13}C isotope of carbon is detected, whose natural abundance is only 1.1%. The main carbon isotope, ^{12}C, is not detectable by NMR since it has zero net spin.

Method: The proton (^{1}H) and carbon (^{13}C) NMR spectra were recorded with a Bruker AC-500 MHz Fourier transform (FT)-NMR spectrometer using $CDCl_3$; TMS was

used as an internal standard (Bruker, Fallanden, Switzerland). The usual abbreviations employed are d, doublet; dd, double doublet; t, triplet; q, quartet; m, multiplet; J, coupling constant (in hertz); and δ, chemical shift in parts per million.

2.4.3.8. Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Principle: Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass is spectrometric technique where samples for MALDI are mixed with a suitable matrix material and applied to a metal plate. The matrix absorbs a pulsed laser and converts it to heat energy. A small part of the matrix heats rapidly and is vaporized, together with the sample and triggers ablation and desorption of the sample and matrix material. Charged ions of various sizes are generated on the sample slide. A potential difference V_0 between the sample slide and ground attracts the ions. The velocity of the attracted ions v is determined by the law of conservation of energy. As the potential difference V_0 is constant with respect to all ions, thus ions with smaller m/z value (lighter ions) and more highly charged ions move faster through the drift space till they reach the detector. Consequently, the time of ion flight differs according to the mass-to-charge ratio (m/z) value of the ion. Mass spectrometry that exploits this phenomenon is called Time of Flight Mass Spectrometry.

Method: MALDI-TOF MS analysis was performed for bioactive compound and phylloqinone standard. These compounds were mixed with α-Cyano-4-hydroxycinnamic acid (HCCA) (5 mg/mL) matrix, 0.1% trifluoroacetic acid (TFA) and 50% acetonitrile (CAN) (1:1) in 1:2 ratio and the resulting 2 µL was spotted onto the MALDI plate and allowed for air drying. Later, the Ultraflex II system (Bruker

Daltonics, Bremen, Germany) equipped with a solid state YAG laser at 337 nm and 200 Hz were employed. Flex analysis 3.0 (Bruker Daltonik) software was used for data analysis.

2.4.4. Stability of bioactive compound

The bioactive compound was purified from raw as well as boiled (90ºC for 15 min) spinach using preparative TLC as discussed earlier (**2.4.1**). The compound was found to be stable and no significant reduction in quantity based upon TLC analysis and in terms of bioactivity was observed. Besides, bioactive stored at -20ºC in dried condition up to 4 months was also found to be stable. Thus, this was dissolved in DMSO and added just before conducting the experiments.

2.5. Comparative *in vitro* antioxidant activity evaluation of various extracts and characterized bioactive compound

Antioxidant activity of compound was performed by 1,1-Diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) radical scavenging activity and 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) ($ABTS^{\cdot+}$) radical scavenging activity [50]. These assays were performed with different extracts from spinach such as total soluble phenolic (TSP) extract, quinone extract, TLC purified quinones, and standards such as phylloquinone, plumbagin, juglone and menadione.

2.5.1. $DPPH^{\cdot}$ radical scavenging activity

Principle: In principle, $DPPH^{\cdot}$ is a stable free radical and converts to 1,1-diphenyl-2-picryl-hydrazine (reduced form) in the presence of hydrogen-donating substance (Figure 9). The $DPPH^{\cdot}$ solution is violet in colour that turns to yellow when the odd

electron of $DPPH^{\cdot}$ radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H.

Method: The $DPPH^{\cdot}$ solution (0.25 mM) was prepared in absolute ethanol. Samples dissolved in methanol were added at above concentrations and volume made up to 500 µL using methanol. Further, $DPPH^{\cdot}$ solution (500 µL) was mixed thoroughly and allowed to stand for 30 min at ambient temperature. The absorbance was measured at 517 nm. $DPPH^{\cdot}$ radical scavenging was determined using following equation:

$$DPPH^{\cdot} \text{ radical scavenging (\%)} = [1 - \{(T1 - B2) / B1\}] \times 100$$

where T1, B1 and B2 are the absorbance of the sample, sample blank and $DPPH^{\cdot}$ blank, respectively.

2.5.2. $ABTS^{\cdot+}$ radical scavenging activity

$ABTS^{\cdot+}$ (purple coloured) is a cationic radical. Antioxidant transfers electron and convert it to a colourless reduced form (Figure 10).

Method: For $ABTS^{\cdot+}$ stock preparation, 10 tablets (total 10 mg ABTS) was dissolved in 13 mL water. A 10 mL of this solution was mixed with 10 mL of freshly prepared ammonium persulphate (APS; 1.12 mg/mL) solution and incubated in dark for overnight. The $ABTS^{\cdot+}$ stock solution (700 µL) was diluted (~70 fold) for preparing working solution using Milli-Q water to achieve absorbance in the range of 0.75-0.85 at 734 nm. **S**amples at the different concentrations were added to $ABTS^{\cdot+}$ (850 µL) working solution and volume made up to 1 mL using methanol. Later, it was allowed to stand for 15 min and absorbance was measured at 734 nm.

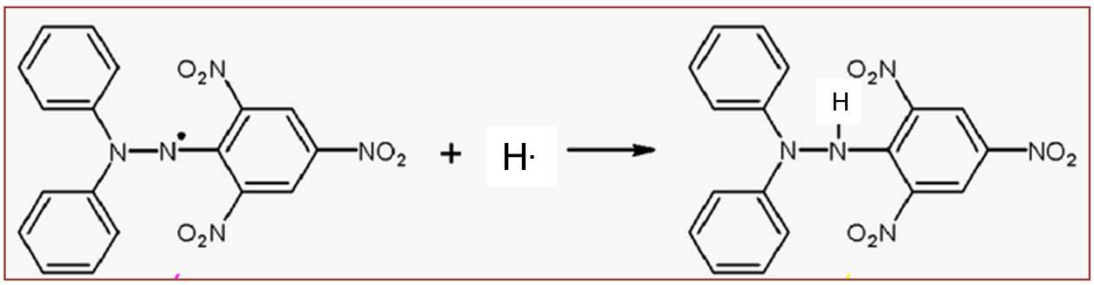


Figure 9. Mechanism of DPPH$^{\cdot}$ radical scavenging by antioxidants

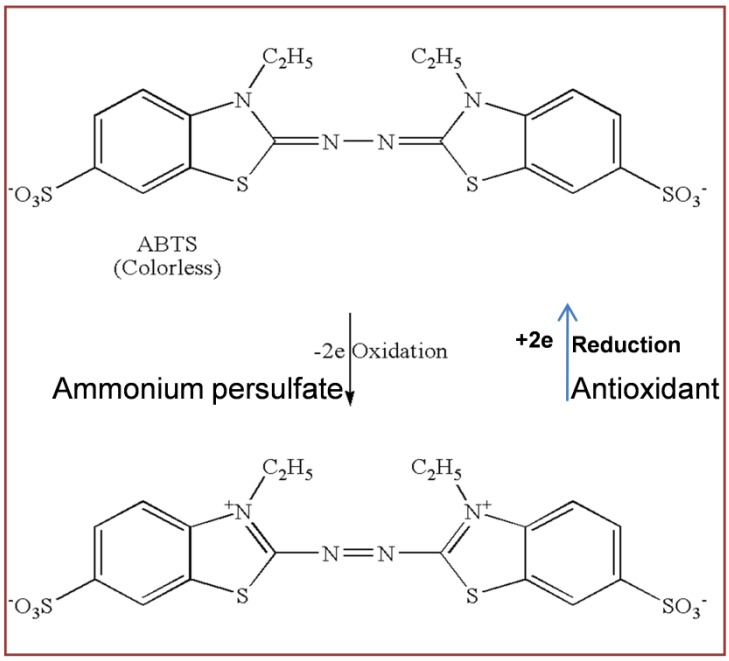


Figure 10. Mechanism of ABTS$^{\cdot+}$ radical generation and scavenging by antioxidants

Note: Structures taken from Google images (https://en.wikipedia.org; https://pubs.rsc.org). These are required for the basic understanding of the problem addressed in the current study.

2.6. Antimutagenicity of spinach bioactive vs. other standards

The antimutagenic activity of bioactive was compared to standard naphthoquinones such as phylloquinone, plumbagin, juglone, and menadione using *rpoB* based rif^R assay at 0.1- 1 mM concentrations as per the protocol discussed above **(2.3)**.

2.7. Mechanism of antimutagenicity

2.7.1. Antimutagenicity at different treatment conditions

Antimutagenicity of bioactive was determined at various conditions as detailed below using *rpoB* based Rif^R assay in *E. coli* MG1655 and TK 6 gene mutation assay in human lymphoblast cells against EMS induced mutagenesis. The EMS concentration was optimized for these assays (133 and 0.5 mM, respectively). A 0.5 mM concentration of the bioactive compound was used for both these assay.

a) EMS and bioactive compound added simultaneously in the cell system

b) EMS and bioactive compound added in the cell system after incubating for 4h outside

c) Bioactive compound added after EMS treatment for 40 min and 4 h in Rif^R and TK 6 gene mutation assay

Untreated and EMS treated cells served as controls.

2.7.2. Analysis of direct interaction of bioactive and mutagen

The TLC detectable concentration of bioactive compound (8 mM) was mixed with EMS at the same concentration (i.e., 8 mM) in absolute methanol and incubated for 2

h. Bioactive compound alone kept under similar condition served as control. These samples were subjected to TLC analysis, visualized under UV (at two wavelengths 254 and 366 nm), and quantified as detailed earlier.

2.7.3. Role of antioxidant activity of spinach bioactive for its antimutagenicity

Principle: The cell permeant reagent 2',7' dichlorofluorescin diacetate (DCFDA or H_2DCFDA), is a fluorogenic dye that measures reactive oxygen species (ROS) within the cell. After diffusion in to the cell, DCFDA / H_2DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, later this is oxidized by ROS into fluorescent compound 2', 7' dichlorofluorescein (DCF) (Figure 11).

Method: Human lymphoblast TK6 cells were treated with 0.5 mM concentration of EMS, stained using DCFDA (20 µM) and analyzed under a fluorescent microscope. Similarly, *E. coli* MG1655 cells were also treated with EMS (133 mM) alone, and EMS (133 mM) and bioactive (0.5 mM) followed by DCFDA (20 µM) staining and visualization under microscope. Cells treated with H_2O_2 (25 and 50 mM) and bioactive (0.5 and 1 mM), separately, served as controls. The level of oxidants in these treatment conditions were analyzed using fluorometer where excitation and emission wavelengths were 488 and 525 nm, respectively.

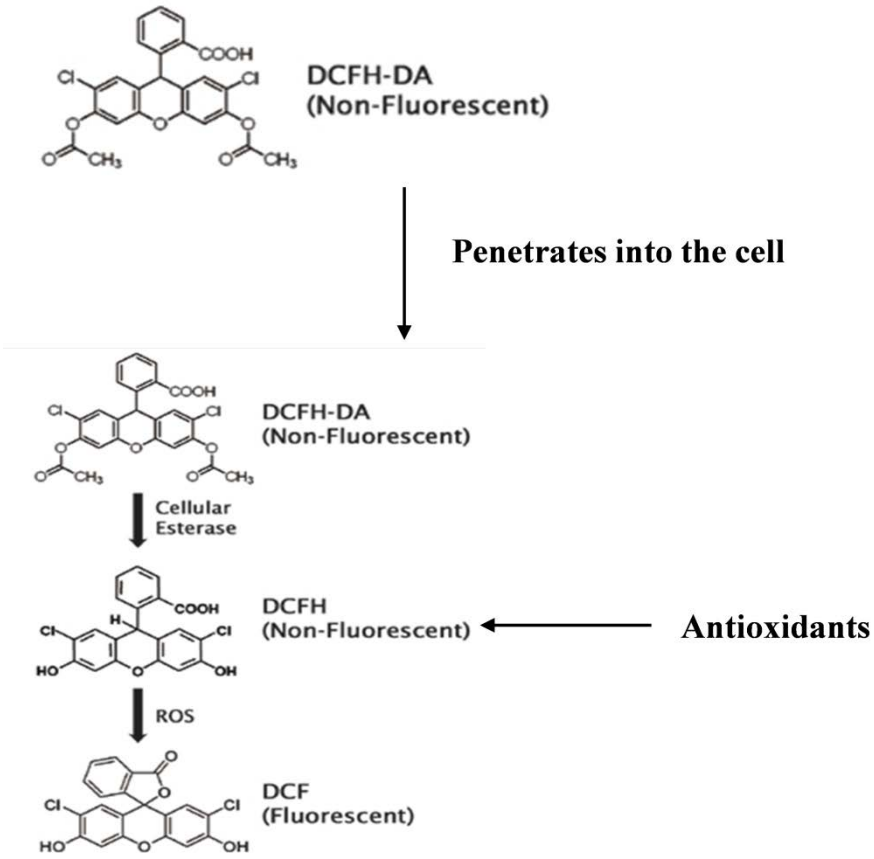


Figure 11. 2', 7' dichlorofluorescin diacetate (DCFDA) assay for *in vivo* ROS quantification

Note: Structures taken from Google images (https://www.cellbiolabs.com). These are required for the basic understanding of the problem addressed in the current study.

2.7.4. Analysis for interaction of bioactive with DNA

2.7.4.1. Spectrophotometric analysis

Calf thymus DNA (CT DNA; optimized concentration 120 µM) was treated with bioactive compound (10 - 60 µM) and absorbance spectrum (230 - 450 nm) was recorded using a spectrophotometer. CT DNA (120 µM) and bioactive compound (60 µM) alone served as controls.

2.7.4.2. Circular dichroism

Principle: The asymmetry in the chiral molecule or centres interacts differently with circularly polarized light (right and left handed). The resultant beam after having passed through the sample is recombination of right (R) and left (L) components to give an emergent beam of elliptically polarized light.

$\theta = 2.303\Delta A = 33\ \Delta A$ degree

where ΔA is the difference in the absorption between R and L components.

Method: CD measurements were performed using a JASCO-810 automatic recording spectropolarimeter at 25°C. The CT DNA (50 µM) solution in phosphate buffer (pH 7.4) was titrated with bioactive compound (40 and 60 µM), and CD spectra were recorded (230 to 320 nm) under nitrogen atmosphere (1-mm cuvette, scan rate 100 nm ⁄ min, response time 1s). Each spectrum was recorded thrice, and the results were expressed as average ellipticity in millidegrees (mdeg). CT DNA (50 µM) and bioactive compound (60 µM) alone served as controls.

2.7.4.3. DAPI (4', 6-Diamidino-2-phenylindole dihydrochloride) binding assay

DAPI binding assay were performed where CT DNA (120 µM in 10 mM phosphate buffer, pH 7.4) was incubated in presence of bioactive compound (10-40 µM) for 15 min followed by incubation with DAPI (15 mM) for the same period and recording the spectrum (λ_{Ex}: 338 nm; λ_{Em}: 450 nm) using a fluorometer [72]. This assay was conducted to determine the possibility of the bioactive compound to bind the minor groove as DAPI is a minor groove binder of DNA. Buffer, DAPI, bioactive compound and CT DNA alone served as controls. This assay was also performed by addition of DAPI 15 min prior to the bioactive compound (40 µM) to confirm the result.

2.7.4.4. Methyl green binding assay

CT DNA (120 µM in 10 mM phosphate buffer, pH 7.4) was incubated in presence of bioactive compound (40 and 60 µM) for 15 min followed by addition of methyl green (20 µM) for the same period. Later, spectrum (230-700 nm) was determined using a spectrophotometer. This assay was performed to determine the possibility of the bioactive to bind the major groove by blocking methyl green, a known major groove binder of DNA. Buffer, methyl green, bioactive and CT DNA alone served as controls.

2.7.4.5. Ethidium bromide (EtBr) binding assay

This was performed by incubating the CT DNA (120 µM in 10 mM phosphate buffer, pH 7.4) in presence of bioactive (10-40 µM) for 15 min followed by incubation with EtBr (10 mM) for the same period and recording the spectrum (λ_{Ex}: 480 nm; λ_{Em}: 610 nm) using a fluorometer [73]. This assay was performed to determine the possibility

of the bioactive to intercalate with the DNA by blocking EtBr. Buffer, EtBr, bioactive compound and CT DNA alone served as controls.

2.7.5. Two dimensional (2-D) gel electrophoresis

To understand the mechanism of antimutagenicity by the bioactive compound, gene expression profile was studied using two dimensional (2-D) gel electrophoresis. **Principle:** This technique combines isoelectric focusing (IEF) and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The first dimension (IEF) is carried out in polyacrylamide gels containing ampholytes in narrow tubes (~1-2 mm diameter) where denatured proteins resolve according to isoelectric points and later the extruded gel is placed along the stacking gel, and subjected to electrophoresis. In SDS-PAGE, proteins are separated based upon difference in molecular weight. As it is unlikely that two proteins will be similar in both these properties, i.e., isoelectric point and molecular weight, thus molecules are more effectively separated in 2-D electrophoresis.

Method:

2.7.5.1. Protein extraction

The overnight grown *E. coli* culture (2 mL) control as well as treated (EMS alone and, EMS and bioactive compound) was centrifuged at 7500 x*g* for 7 min and washed with chilled PBS (phosphate buffered saline, 10 mM, pH 7.4). To this 500 µL of trizol was mixed to lyse the cells. Later, chloroform (200 µL) was added, vigorously mixed for 5 s, and allowed to stand for 5 min (25°C). This was centrifuged at 12000 x*g* at 4°C for 15 min. Aqueous supernatant was discarded, and ethanol (300 µL) was added to the reddish brown pellet and centrifuged at 5000 x*g* at 4° C for 5 min. Further,

isopropanol (1.5 mL) was added to the supernatant, allowed to stand for 20 min at 25°C and centrifuged at 12000 x*g* at 4°C. Pellet was washed with 95% ethanol, air dried and rehydration buffer (200 µL) was added to dissolve the protein (as provided by the manufacturer, Bio-Rad Laboratories, USA). Protein was estimated by Bradford's method, using a protein estimation kit (Bangalore Genei, Bangalore, India), and bovine serum albumin (BSA) as a standard [50].

2.7.5.2. Rehydration of protein

The protein sample (90 µg) was loaded from each of the treatment conditions on the IPG strip (7 cm; pH gradient: 4-7) and allowed to be absorbed for 30 min. Later 1 mL of mineral oil was added and left for 12-16 h at ambient temperature.

2.7.5.3. Isoelectric Focusing

Isoelectric focusing was performed using an IEF tray of same as the size of rehydration tray. Using forceps the paper wicks were placed on both the ends of the channel covering the wire electrodes. An 8 µL of nano pure water was added to wet the wick. The IPG strip (7 cm) was hold for 7 to 8 s to allow the mineral oil to drain, and then transferred to the corresponding channel in the focusing tray (gel side down). IPG strip was covered with the fresh mineral oil and lid was placed onto the tray. The protocol was programmed to attain 8000 V-h (pH gradient 4-7) and electrophoresis was initiated. After completion, the IPG strip was removed and transferred (gel side up) into a new clean and dry rehydration tray. Before the second dimension run, the IPG strip was stored at -70°C overnight

2.7.5.4. SDS-PAGE

The equipment was assembled and sealed using 1.5 % agar. Resolving gel (12%) was added and covered with butanol (70%) to increase the rate of polymerization. Butanol

was removed and washed off. Stacking gel (5%) was added and again layered with butanol (70%). Again, the butanol was removed and washed off. The strip was kept at room temperature for some time to thaw properly and later equilibrated using equilibration buffer 1 for 10 min on a shaker (as provided by the manufacturer, Bio-Rad Laboratories, USA). Buffer 1 was drained, buffer 2 was added and the strip was kept on a shaker for 10 min. The IPG strip was rinsed with 1X tris glycine buffer. The strip was put on the SDS-PAGE gel and a layer of agarose with a trace of tracking dye (bromophenol blue) was overlaid and run at 200 V.

2.7.5.5. Silver staining

After the completion of the run, the gel was incubated with fixative solution for 1 h in shaking condition and was washed with Milli-Q water for 3 times (15 min each). Freshly prepared pre-treating solution (20 mg/100 mL $Na_2S_2O_3$) was added for 1.5 min. The gel was later washed with water for 4 times (30 s each). Cold impregnating solution (200 mg/100 mL $AgNO_3$; 100 µl/100 mL formaldehyde) was added and kept on shaker incubator for 20 min. The gel was rinsed with excess Milli-Q water for 4 times (30 s each). Developing solution (6 g/100 mL Na_2CO_3; 100 µL/100 mL formaldehyde; and 0.4 mg $Na_2S_2O_3$) was added till the spots were developed followed by washing of the gel. The gel was immediately transferred to the stopping solution and the spots on the gel were documented and compared (PDQuest, Bio-Rad Laboratories, USA).

2.7.6. In-gel digestion and Matrix-Assisted Laser Desorption/Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

The differentially expressed proteins were excised from gel and transferred in microfuge tube. A 30 µL of freshly prepared $K_3[Fe(CN)_6]$ and $Na_2S_2O_3$ (1:1; v/v) in

water was added and incubated for 30 min at ambient temperature (26±2°C) for destaining. The gel was washed with water and 50 mM NH_4HCO_3 /acetonitrile (1:1; v/v) for 15 min. The liquid was removed and acetonitrile was added to cover the gel. After that, acetonitrile was removed and gel was dried using vacuum centrifuge. The gel was swelled in 10 mM dithiotreitol / 50 mM NH_4HCO_3 by incubating for 45 min at 56°C. Then the temperature was brought back to ambient, the liquid was removed and freshly prepared 55 mM iodoacetamide (in 50 mM NH_4CO_3) was added. This was incubated for 30 min in dark at ambient temperature (26 ± 2°C). Later liquid was removed and washed twice with 50 mM NH_4CO_3 and acetonitrile (1:1; v/v) for 15 min each. Acetonitrile was added to cover the gel and once the gel shrunk it was removed. This was vacuum centrifuged for drying. For in-gel digestion, 4.5 µL of trypsin reaction buffer (25 mM NH_4CO_3 and 9% acetonitrile) was added to the gel. A 7 µL of the trypsin enzyme stock [20 µg trypsin / 120 µL NH_4CO_3 (25 mM)] was diluted with 63 µL of NH_4CO_3 (25 mM) and 5.5 µL of that was added to the gel. This was incubated over night at 37°C for digestion. The resulting peptide was extracted using 100 µL of 0.1% TFA, 0.1% TFA in 50% acetonitrile, and acetonitrile, sequentially, pooled and vacuum dried.

MALDI-TOF MS analysis was performed by mixing these peptides with (A) α-cyano-4-hydroxycinnamic acid (HCCA) (5 mg/mL), and (B) 0.1% TFA and 50% acetonitrile (1:1), in 1:2 ratio. The 2 µL aliquot was spotted onto the MALDI plate [(MTP 384 ground steel (Bruker Daltonics, Germany)] and allowed for air drying. Later, MALDI TOF/TOF was performed using ULTRAFLEX III instrument (Bruker Daltonics, Bremen, Germany) equipped with smart laser beam (100 µJ at 337 nm) operating at 200 Hz. External calibration was performed with standard peptide (PEPMIX Mixture)

of masses ranging from 1046 to 3147 Da (supplied by Bruker, Germany).FLEX ANALYSIS SOFTWARE (Version 3.3) in reflectron ion mode with an average of 500 laser shots was used for analysis (mass detection range between 500 to 5000 m/z). The masses obtained were submitted for Mascot search in "CONCERNED" database for identification of the protein.

2.7.7. Knockout studies using relevant *E. coli* strains

Keio collection includes single-gene deletions in *Escherichia coli* K-12 where open-reading frame coding regions were replaced with a kanamycin cassette. Such mutants were obtained for 3985 genes. These mutants provide a resource for analyses of unknown gene functions and gene regulatory networks [74]. Thus, the study using relevant *E. coli* knockout strains (Keio collection, Japan) was conducted to determine the role of certain up-regulated proteins(s) (due to the presence of bioactive compound) in observed antimutagenicity. This helped in developing the possible hypothesis explaining the underlying mechanism of observed antimutagenicity by the spinach bioactive napthoquinone.

2.7.8. Statistical analysis

Experiments pertaining to antimutagenicity were performed twice with ten replicates, whereas all other experiments were conducted twice with three replicates. One-way ANOVA was used to determine the variation due to treatments and the results were expressed in terms of mean and standard deviations (SD). The analyses were performed using BioStat 2009 Professional 5.8.0.0 software (AnalystSof Inc., Vancouver, BC, Canada) at $p \leq 0.05$.

Chapter 3

Results

3.1. Screening of various selected foods rich in naphthoquinone for antimutagenicity and characterization of bioactive from the most potent food

3.1.1. Quinone extract from spinach displayed maximum antimutagenicity

Naphthoquinone rich foods (vegetables) such as spinach, lettuce, iceberg lettuce, cabbage, broccoli and French bean were analyzed for antimutagenicity against ethyl methanesulfonate (EMS) induced mutagenesis using rifampicin resistance (Rif^R) assay in *E. coli* MG1655. The various extracts such as aqueous, methanolic, total soluble phenolic and quinonic extracts were used in this assay. Aqueous (at the optimized concentration of 2 mg/ml) extract displayed antimutagenicity in the range of 58% (in broccoli) to 73% (in French bean) whereas, in methanolic extracts, antimutagenicity varied between 41% (in cabbage) to 88% (in French bean). In most of these vegetables except spinach, phenolics were found to be primarily responsible for the observed antimutagenic activity (i.e., in the range of 63-78%) (Table 4). In case of spinach, quinone rich extract displayed significantly higher antimutagenicity (72%) than the phenolic rich extract (35%) (Table 4). Thus, data indicated major role of quinone(s) in the observed antimutagenicity of spinach (Table 4).

Among vegetables selected, spinach quinone extract displayed highest antimutagenicity in Rif^R assay (Table 4). This was also evaluated in TK6 gene mutation assay against EMS and 5-azacytidine (5-AZ) induced mutagenesis. The induced mutation in human lymphoblast cell line at gene ($tk^{+/-}$) loci was found to be negligible in control cells, whereas in the case of EMS and 5-AZ treated cells the relative number of mutants increased by ~21and 24-fold, respectively (Figure 12 A). At 0.5 mg/mL concentration aqueous, methanolic, total soluble phenolic, and quinone extracts reduced the mutagenic effect of EMS by 27, 16, 13, and 42%, respectively (Figure 12A). Similarly, these extracts reduced the mutagenic effect of 5-AZ by 26, 30, 11, and 45%, respectively (Figure 12B).

The yield of phenolics ranged between 0.5 mg/g (in iceberg lettuce) to 5 mg/g dry wt (in broccoli). In case of quinone, yield varied between 1.5 mg/g (in lettuce) to 3.4 mg/g dry wt (in broccoli). Correlation coefficient (R^2) of antimutagenicity vs. phenolics yield was found to be (-) 0.15 and that of quinone was 0.23. The lower correlation value between yield and activity indicated that antimutagenic activity of vegetables could be depending upon the structural types of phenolics or quinones present instead of their yield.

3.1.2. Spinach quinones resolved in 4 major bands in TLC

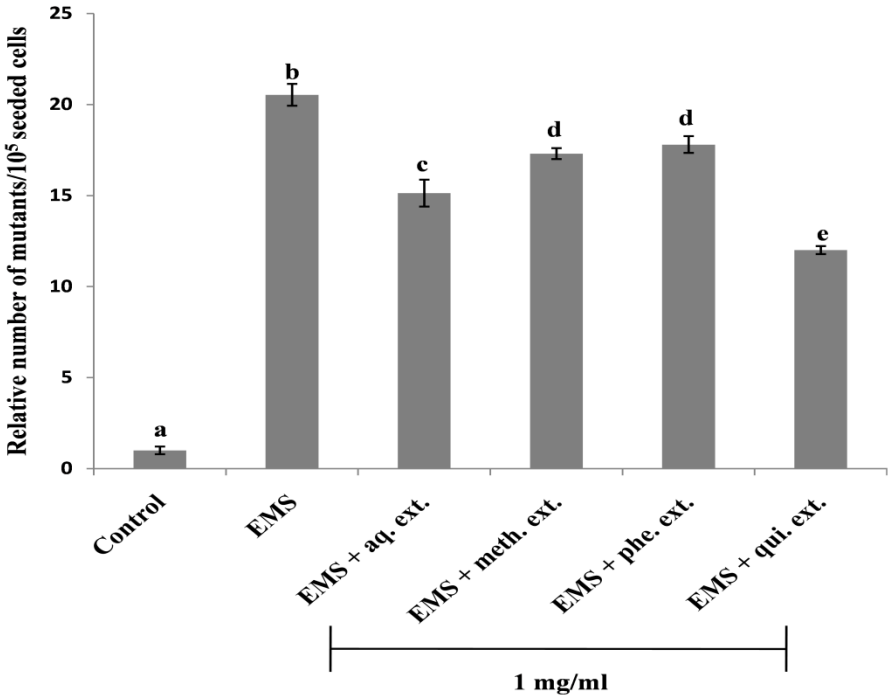
The quinone extract of spinach which displayed highest antimutagenicity was resolved using TLC. Various combinations of solvents were tested for the better resolution of quinones. Finally, optimized developing solvent consisting of petroleum ether, chloroform, and acetone (5:1:0.106) was found to resolve most of the naphthoquinone standards at 254 nm such as 1,4-naphthoquinone (R_f, 0.337), juglone (R_f, 0.358), menadione (R_f, 0.379), plumbagin (R_f, 0.453), lepachol (R_f, 0.347), and phylloquinone (R_f, 0.789), as well as one of the spinach quinones (C1; R_f, 0.789) (Figure 13A). However, the R_f of other spinach quinones (C2; 0.758 and C3; 0.495 at 254 nm) and (C4; 0.315at 366 nm) was not found to match with any standard naphthoquinones (Figure 13A and B). The content of quinone band (C1) was found to be 64 ± 7 μg/g (dry weight), which was quite close to the reported phylloquinone content from frozen whole leaf of spinach (i.e., ~3.7 μg/g of fresh weight) [75]. Among all these spinach quinones, the C4 displayed fluorescence at 366 nm, and its yield was found to be 396± 14 μg/g of dry weight.

Table 4. Antimutagenicity (%) of solvent extracts from naphthoquinone rich vegetables and yield of total soluble phenolics and quinones.

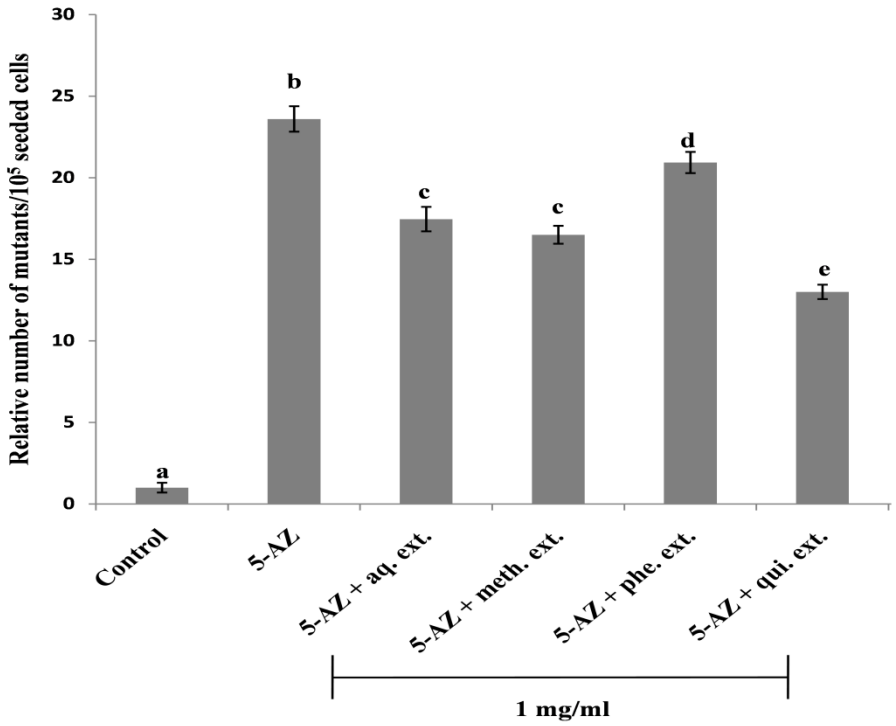
Vegetables	Plant part	Antimutagenicity (%) using Rif^R assay*				Yield (mg/g dry weight)	
		Aqueous extract	Methanol extract	Extract rich in total soluble phenolics	Extract rich in quinones	Extract rich in total soluble phenolics	Extract rich in quinones
Spinach	Leaf	$66\pm3^{a,x}$	$61\pm6^{a,x}$	$35\pm4^{b,x}$	$72\pm6^{c,x}$	$3.0\pm1.1^{a,x}$	$2.5\pm0.6^{a,x}$
Lettuce	Leaf	$63\pm3^{a,x}$	$65\pm5^{a,x}$	$78\pm4^{b,y}$	$2\pm1^{c,y}$	$1.5\pm0.9^{a,x}$	$2.4\pm0.9^{b,x}$
Iceberg lettuce	Leaf	$62\pm4^{a,x}$	$48\pm3^{b,y}$	$65\pm3^{a,z}$	$21\pm3^{c,z}$	$0.5\pm0.2^{a,y}$	$2.6\pm0.8^{b,x}$
Cabbage	Leaf	$61\pm3^{a,x}$	$41\pm4^{b,y}$	$68\pm5^{a,z}$	$5\pm3^{c,y}$	$2.0\pm0.7^{a,x}$	$1.5\pm0.5^{a,y}$
Broccoli	Inflorescence	$58\pm5^{a,x}$	$78\pm5^{b,z}$	$70\pm4^{b,z}$	$11\pm3^{c,w}$	$5.0\pm0.8^{a,z}$	$3.4\pm1.0^{b,x}$
French bean	Fruit	$73\pm6^{a,y}$	$88\pm6^{b,w}$	$63\pm6^{c,z}$	$1\pm1^{d,y}$	$1.0\pm0.4^{a,y}$	$1.8\pm0.7^{a,x}$

* Extracts concentration: 2 mg/ml; Spontaneous Rif^R mutation frequency = $1/10^8$; EMS (133 mM) induced mutation frequency = $2700/10^8$ cells.

Note: Different letters in columns (w-z) and in rows (a-c) indicate significant differences among means ($p \leq 0.05$).

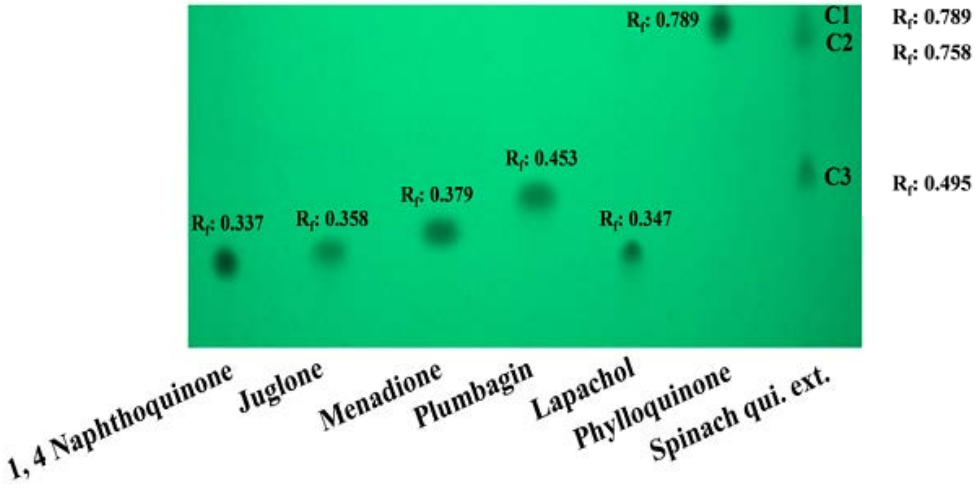


(A)

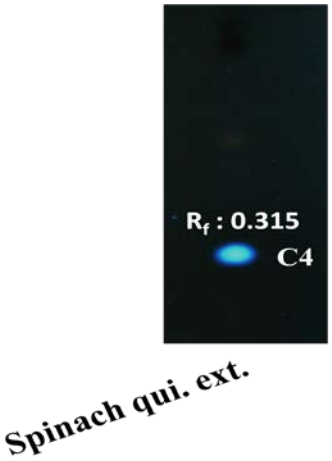


(B)

Figure 12. Antimutagenicity of spinach extracts by human lymphoblast TK6 gene ($tk^{+/-}$) mutation assay against (A) EMS and (B) 5-AZ.



(A)



(B)

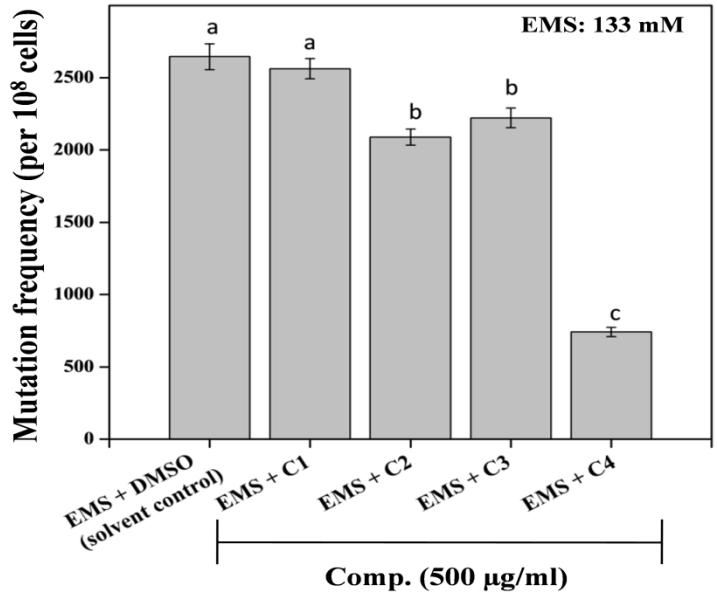
Figure 13. TLC profile of standard naphthoquinones and spinach quinone extract (A) 254 nm and (B) 366 nm.

3.1.3. TLC band (R_f: 0.315; termed as C4) displayed maximum antimutagenicity

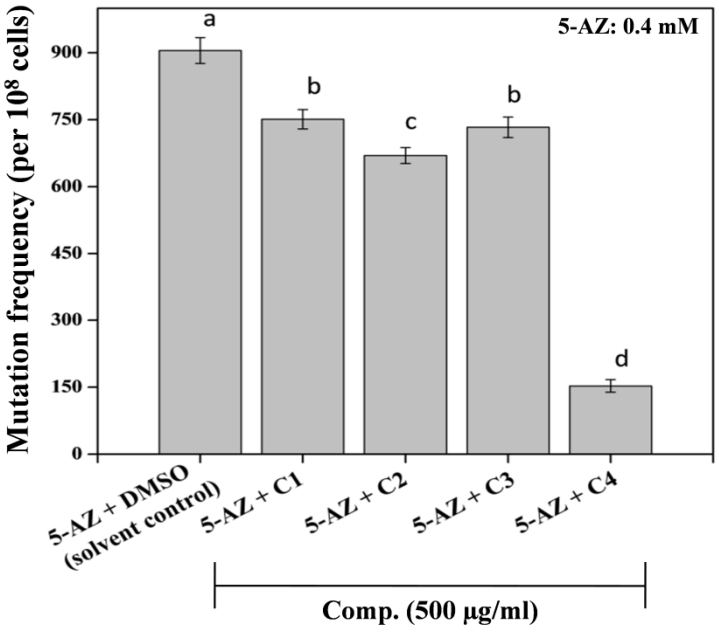
The antimutagenicity of these TLC eluted spinach quinones was also analyzed in various assay systems. In the Rif^R assay, C4 compound (at the optimized concentration of 0.5 mg/mL= 500 µg/mL) exhibited the highest reduction of mutation frequency, i.e., by 72 and 83% against EMS and 5-AZ induced mutagenicity, respectively. However, reduction in mutation frequency was just 13 and17% by C1, 21 and 26% by C2, and 16 and 19% by C3, against EMS and 5-AZ, respectively (Figure 14A and B).

The EMS induced mutation in human lymphoblast cell line at gene loci ($tk^{+/-}$) was found to be reduced to 28, 47, 30, and 56% by C1, C2, C3, and C4 compounds (each at 0.5 mg/mL concentration), respectively (Figure 15A). Similarly, 5-AZ induced mutation was reduced by 23, 34, 32, and 58%, respectively (Figure 15B). At further higher concentration (1 mg/mL) the C4 compound reduced the mutagenic effect ofEMS and 5-AZ both by ~75% (Figure 15 A and B). This was also reflected in reduced cellular aggregation in ($tk^{-/-}$) cells exposed to mutagens (EMS or 5-AZ) in presence of C4 in comparison to those exposed to these mutagens alone (Figure 15C). The C4 compound was not found to display any cytotoxicity in TK6 cells at the concentration used (1 mg/mL).

The Ames test measures reversion of auxotrophic his^- mutants of *S. typhimurium* [67]. The C4 compound (0.5 mg/mL) was also found to reduce EMS and 5-AZ induced his^- reversion in *S. typhimurium* TA100 and TA102 (Figure 16). When treated with EMS, the number of revertant/plate of TA100 and TA102 was found to be 1110 and 540, respectively (Figure 16). The C4 compound inhibited these reversions by 50 and 43%, respectively (Figure 16).

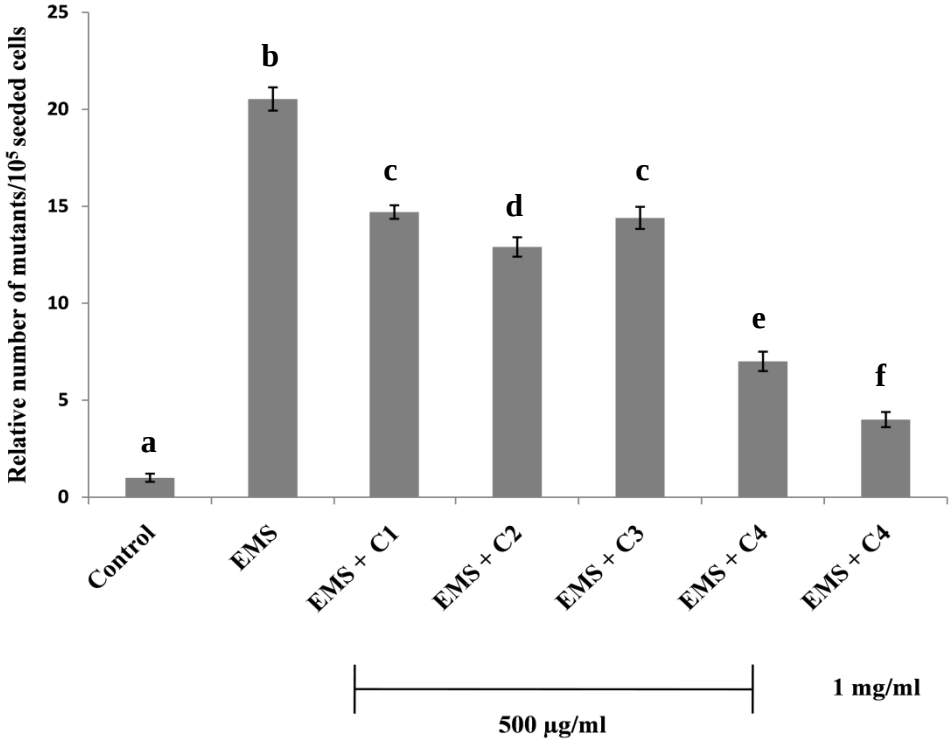


(A)

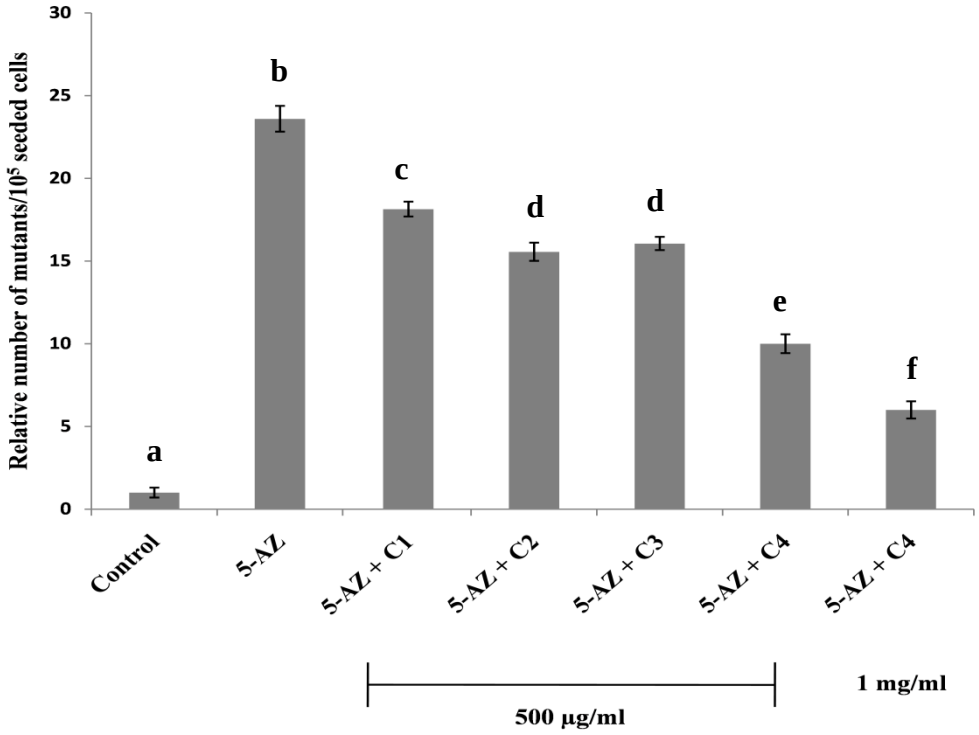


(B)

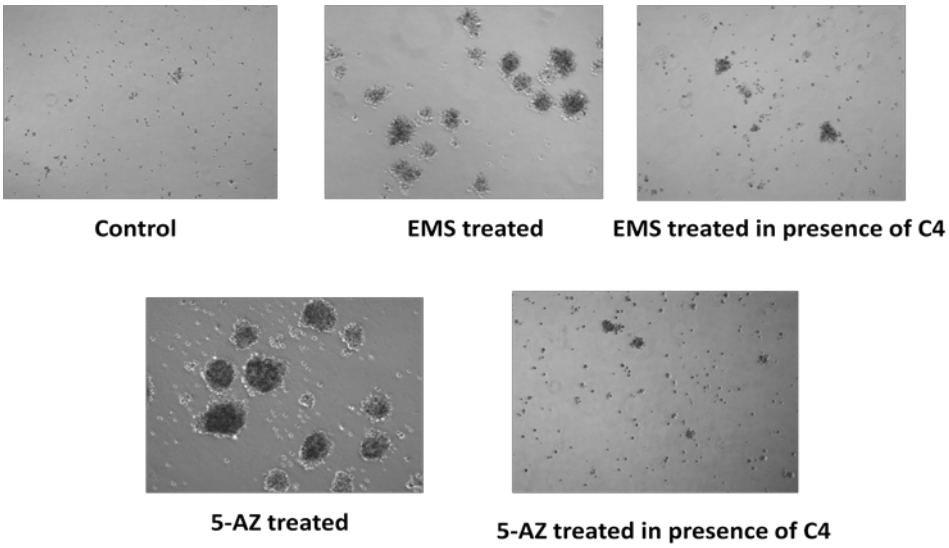
Figure 14. Antimuatagenicity of TLC purified compound by Rif^R assay against (A) EMS and (B) 5-AZ. Different letters (a-d) on the top of the bars show significant differences among means ($p \leq 0.05$).


Relative number of mutants/10^5 seeded cells
25
20
15
10
5
0
a
b
c
d
c
e
f
Control
EMS
EMS + C1
EMS + C2
EMS + C3
EMS + C4
EMS + C4
500 µg/ml
1 mg/ml

(A)


Relative number of mutants/10^5 seeded cells
30
25
20
15
10
5
0
a
b
c
d
d
e
f
Control
5-AZ
5-AZ + C1
5-AZ + C2
5-AZ + C3
5-AZ + C4
5-AZ + C4
500 µg/ml
1 mg/ml

(B)



(C)

Figure 15. Antimuatagenicity of TLC purified compound by human lymphoblast TK6 gene (*tk*$^{+/-}$) mutation assay against (A) EMS, (B) 5-AZ and (C) Mutant (TK$^{-/-}$) cell aggregate in control and treated samples. Different letters (a-f) on the top of the bars show significant differences (in Fig. A and B) among means ($p \leq 0.05$).

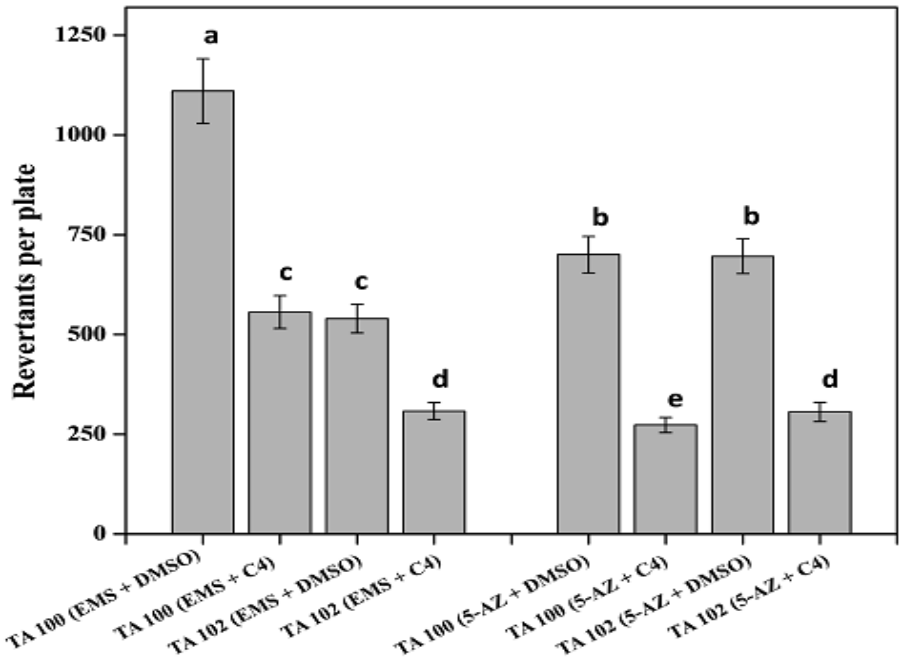


Figure 16. Ames test in *S. typhimurium* TA100 and TA102 strains against EMS (66.5 mM) and 5-AZ (0.4 mM) in presence of highly antimutageinic C4 compound (500 µg/mL). Different letters (a-d) on the top of the bars show significant differences among means ($p \leq 0.05$).

5-AZ treatment of TA100 and TA102 strains resulted in ~700 revertants/plate (Figure 16). Here too C4 significantly inhibited this reversion by 61 and 56%, respectively, in these two strains (Figure 16).

3.1.4. Quinone extract from spinach displayed least antioxidant capacity in comparison to total soluble phenolics (TSP)

Antioxidant activity of quinones extract as well as TLC purified quinones (C1-C4) atthe optimized concentration of 150 µg/ml was found to vary in the range of 16-48% whereas that of total soluble phenolics extract was quite high. Even at much diluted (1: 1000) optimized concentration of 150 ng/ml, phenolics displayed antioxidant activity in the range of 59-78% (Table 5). Thus, in other words quinones possessed very less antioxidant activity compared to phenolics (Table 5). Antioxidant activity of quinones in terms of $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ radical scavenging was equivalent to 11 and 18 µM ascorbic acid, respectively (Table 5).

Table 5. Antioxidant capacity of total soluble phenolics, quionones and TLC purified quinones.

Samples	$ABTS^{\cdot+}$ radical scavenging activity (%)	$DPPH^{\cdot}$ radical scavenging activity (%)
Phenolics (150 ng/ml)	78 ± 8^{x}	59 ± 7^{x}
Quinone (150 µg/ml)	24 ± 3^{y}	18 ± 3^{y}
C1 (150 µg/ml)	23 ± 4^{y}	16 ± 7^{y}
C2 (150 µg/ml)	28 ± 3^{y}	39 ± 6^{z}
C3 (150 µg/ml)	28 ± 4^{y}	48 ± 8^{x}
C4 (150 µg/ml)	36 ± 4^{z}	30 ± 7^{z}
Ascorbic acid equivalent	33±3 (at 11 µM)	38±4 (at 18 µM)

Note: Different letters in columns (x-z) indicate significant differences among means ($p \leq 0.05$).

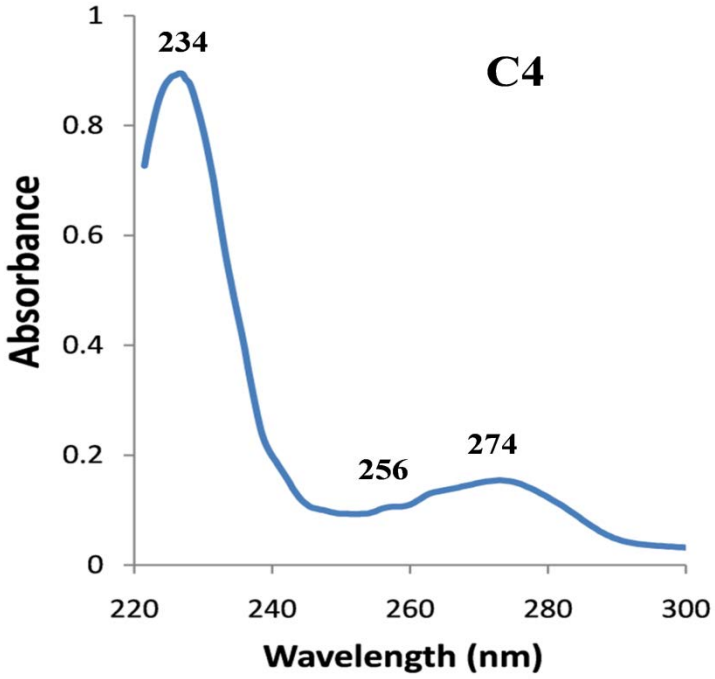
3.1.5. C4 Compound characterized as ethoxy-substituted phylloquinone (ESP) through biochemical, chromatographic and spectral analysis

3.1.5.1. Spectrophotometric analysis

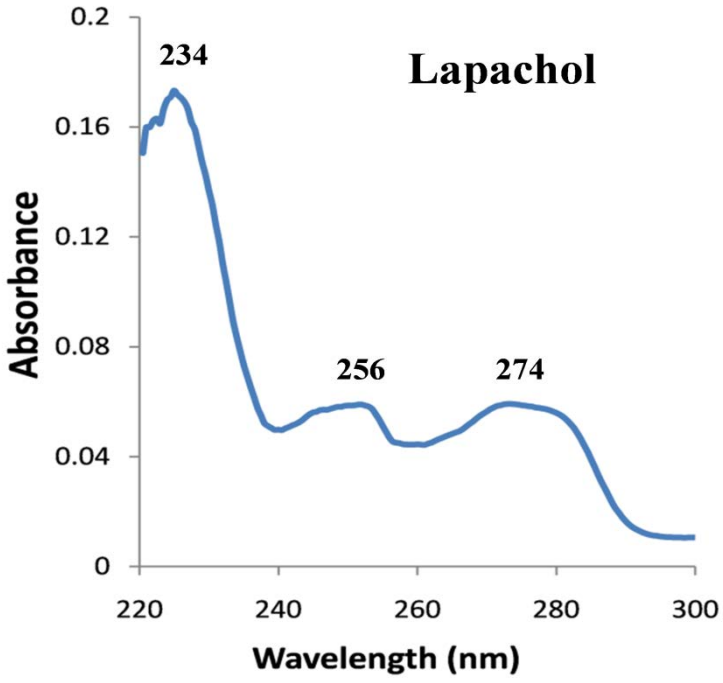
Spectrophtometric analysis of the C4 compound displayed peaks at 234, 256, and 274 nm, which indicated the presence of a benzene nucleus and a quinonoid ring [76, 77]. The peaks at 234 and 274 nm were found to be prominent. The spectrum of C4 was found to be similar to that of lapachol, indicating the presence of related structure at the second position (Figure 17A and B). The alkyl side chain lengths at the third position have been reported for low effects on absorbance spectra (Figure17B and C) [76]. The spectrofluorometric analysis of the C4compound showed excitation and emission wavelengths at 290 and 334 nm, respectively (Figure 17D).

3.1.5.2. HPLC analysis

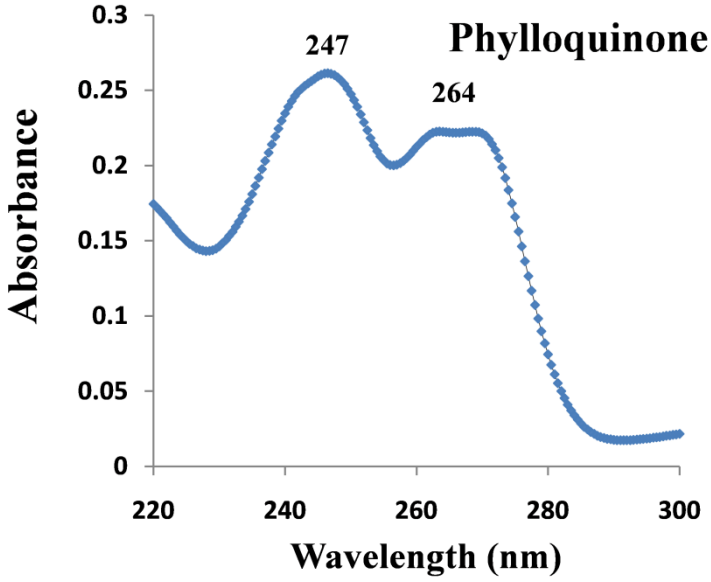
HPLC analysis of the C4 compound showed only one major peak at retention time (t_R) of 15.2 min when analysed at two wavelengths (234 and 274 nm) (Figure 18A and B). Similarly, only one major peak of same t_R was detected even at 280 nm, the wavelength that is primarily used to detect phenolics (Figure 18C). Furthermore, at 335 nm (wavelength used to detect flavonoid), no peak was observed (Figure 18D). Thus, the HPLC profile at various wavelengths confirmed the high purity level of the C4 compound. The standard phylloquinone peak was observed at t_R of 14.6 min at 267 nm, which matched with the C1 band (Figure 18E).


C4
234
256
274
Absorbance
Wavelength (nm)
1
0.8
0.6
0.4
0.2
0
220
240
260
280
300

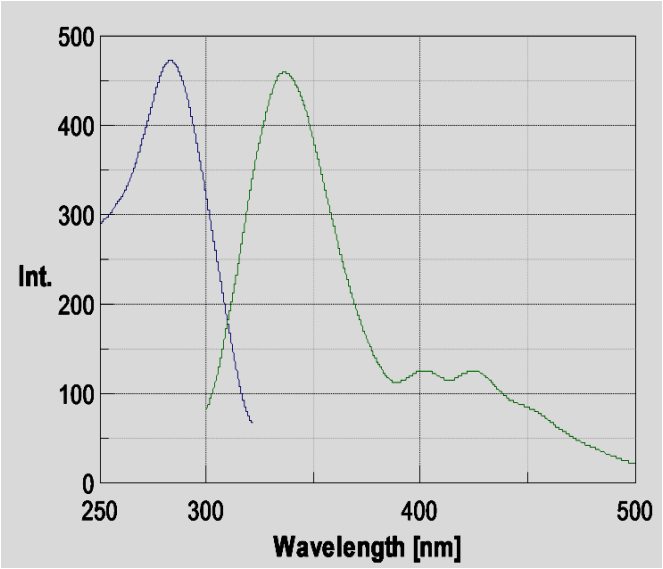
(i)


Lapachol
234
256
274
Absorbance
Wavelength (nm)
0.2
0.16
0.12
0.08
0.04
0
220
240
260
280
300

(ii)

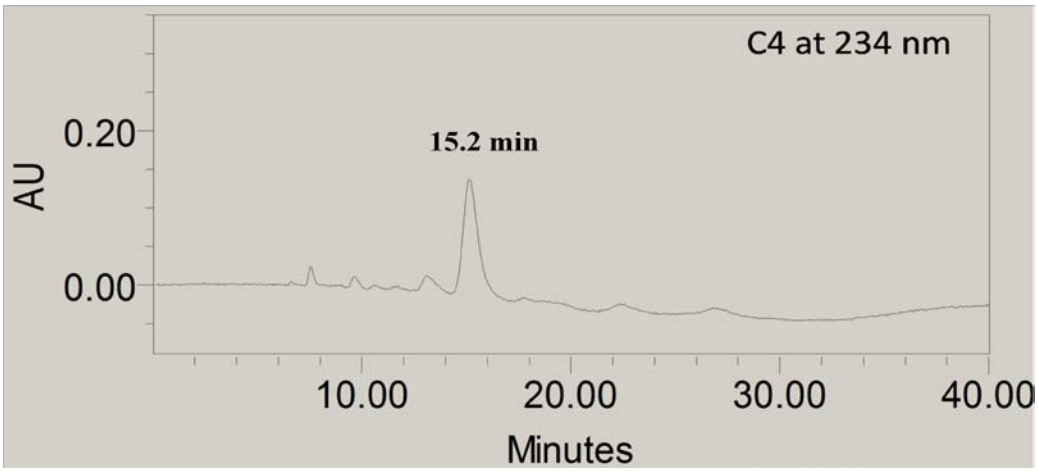


(iii)

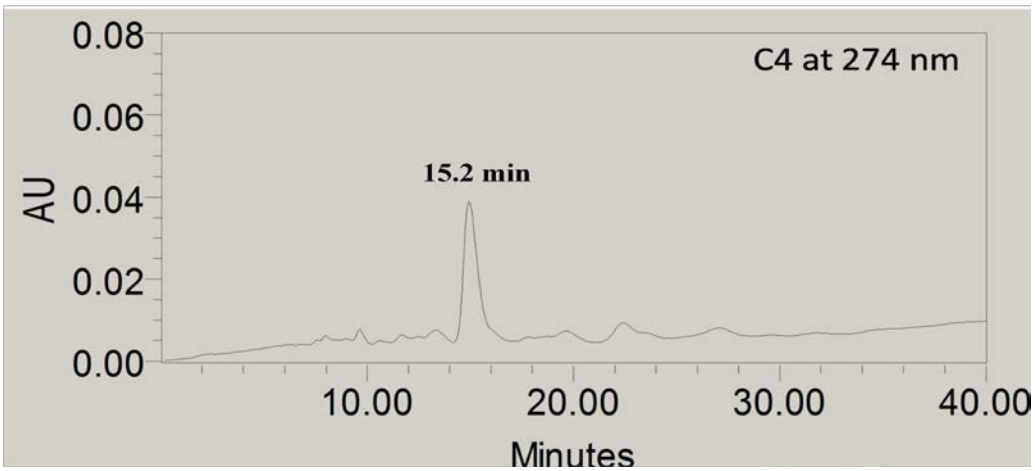


(B)

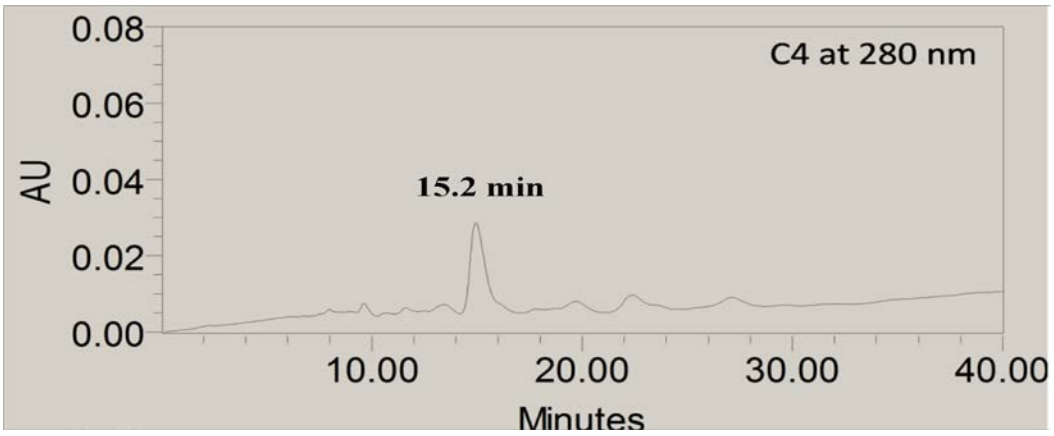
Figure 17. (A) Spectrophotomeric analysis: (i) C4 compound; (ii & iii) standard naphthoquinones: (ii) lapachol and (iii) phylloquinone; and (B) Spectrofluorometric analysis of C4 compound.


C4 at 234 nm
15.2 min
AU
0.20
0.00
10.00
20.00
30.00
40.00
Minutes

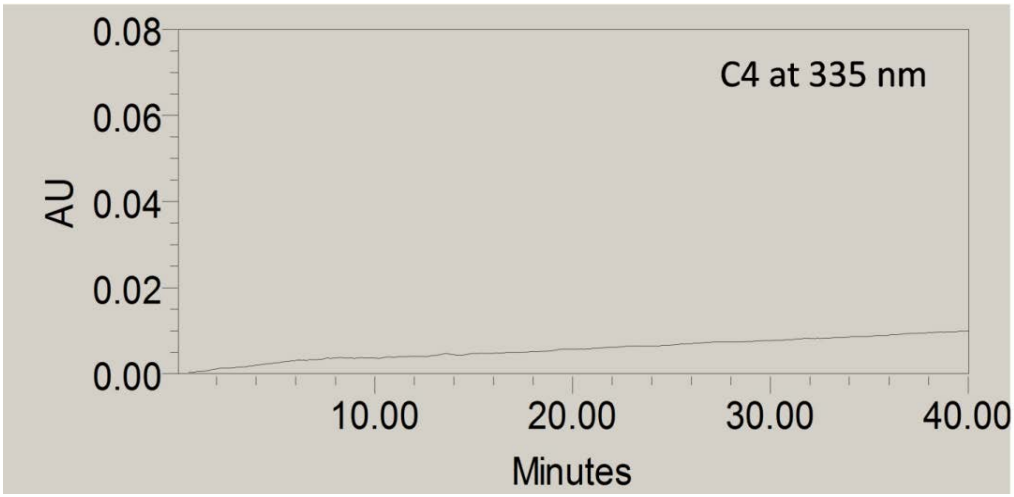
(A)


C4 at 274 nm
15.2 min
AU
0.08
0.06
0.04
0.02
0.00
10.00
20.00
30.00
40.00
Minutes

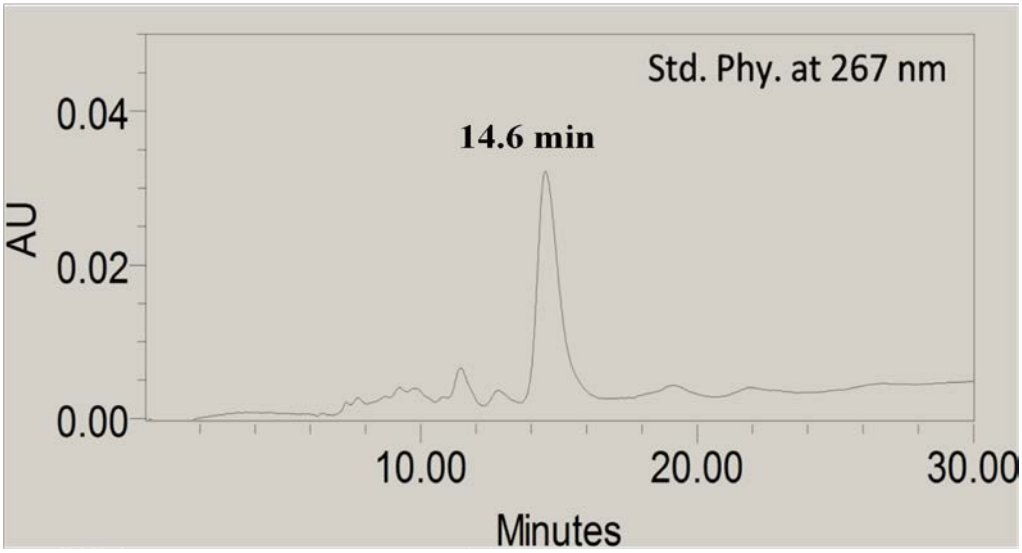
(B)


C4 at 280 nm
15.2 min
AU
0.08
0.06
0.04
0.02
0.00
10.00
20.00
30.00
40.00
Minutes

(C)



(D)

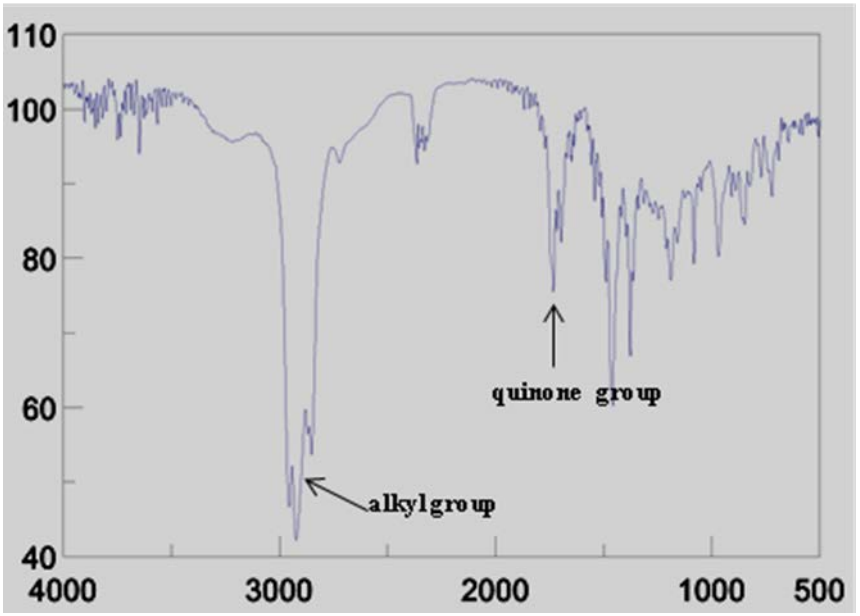


(E)

Figure 18. HPLC analysis of the C4 compound at various wavelengths and comparison with standard phylloquinone.

3.1.5.3. FTIR Analysis

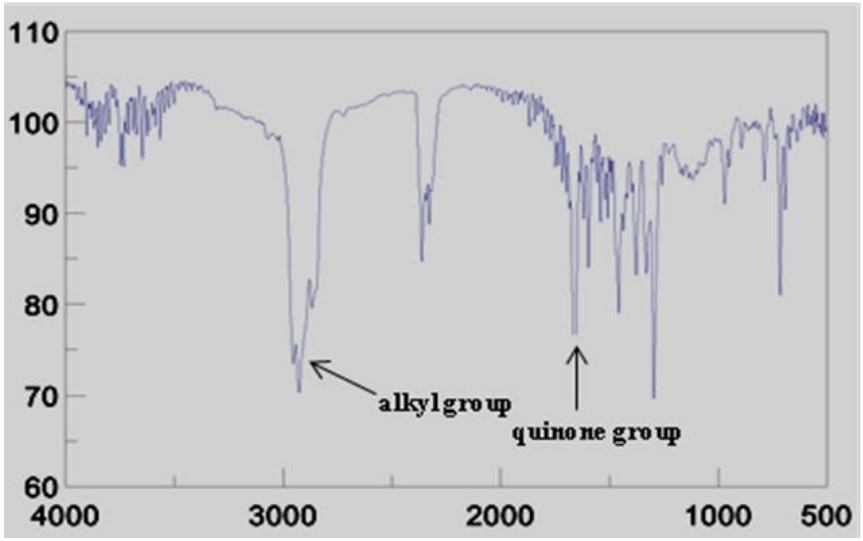
FTIR peaks of the C4 compound observed at 2954, 2923, and 2851 cm^{-1} as well as 1376 cm^{-1} indicated the presence of an alkyl chain similar to phylloquinone (Figure 19A and B). A broad hydroxyl peak at 3073 cm^{-1} was present in juglone but not in C4, indicating the lack of this group (Figure 19A and C). The bioactive compound (C4) displayed quinonic peaks in the range of 1610-1705 cm^{-1} as observed in the case of both standard naphthoquinones (Figure 19). FTIR spectroscopic study performed earlier with various quinones also corroborated these results [78].



% T

Wavenumber [cm^{-1}]

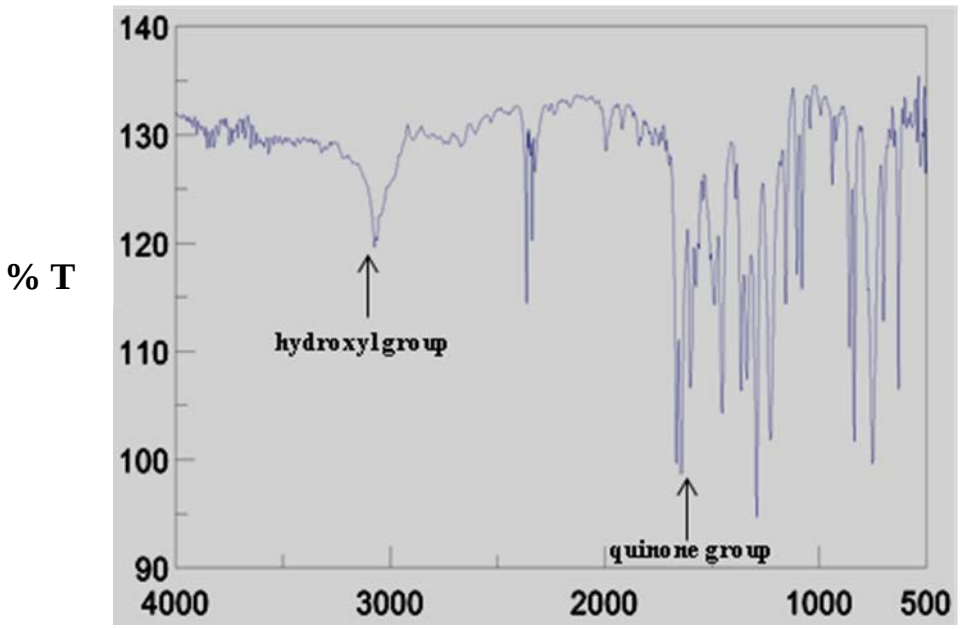
(A)



% T

Wavenumber [cm^{-1}]

(B)



(C)

Figure 19. FTIR analysis of (A) C4 compound; (B) phylloquinone and (C) juglone.

3.1.5.4. Analysis of the hydroxyl group substitution

In the ferric chloride test, spinach quinone C4 was not found to develop any colour, similar to 1,4-naphthoquinone, phylloquinone, and menadione standards devoid of hydroxyl group (Figure 20). However, the hydroxyl group containing quinones such as juglone, plumbagin, and lapachol showed colour development (Figure 20). Thus, the ferric chloride spray on the TLC plate led to the development of a purple colour spot on the uniform background only in the case of hydroxylated quinones [70]. This result also confirmed the finding of FTIR, where no hydroxyl group in C4 was indicated.

3.1.5.5. Analysis of 2, 3 position for presence or absence of functional group

The assay provided information for the presence or absence of functional group at second or third position of quinone compounds. It was found that an increase in substitution at the second or third position of quinone reduced its reaction with 1,3-dimethylbarbituric acid (DMB), probably due to decreased positive charge on these positions of the quinone moiety (Figure 21) [71]. In the C4 compound the reaction was almost negligible, similar to phylloquinone and lapachol standards where both the second and third positions are substituted, indicating substitutions at both of these positions in the C4 compound (Figure 21).

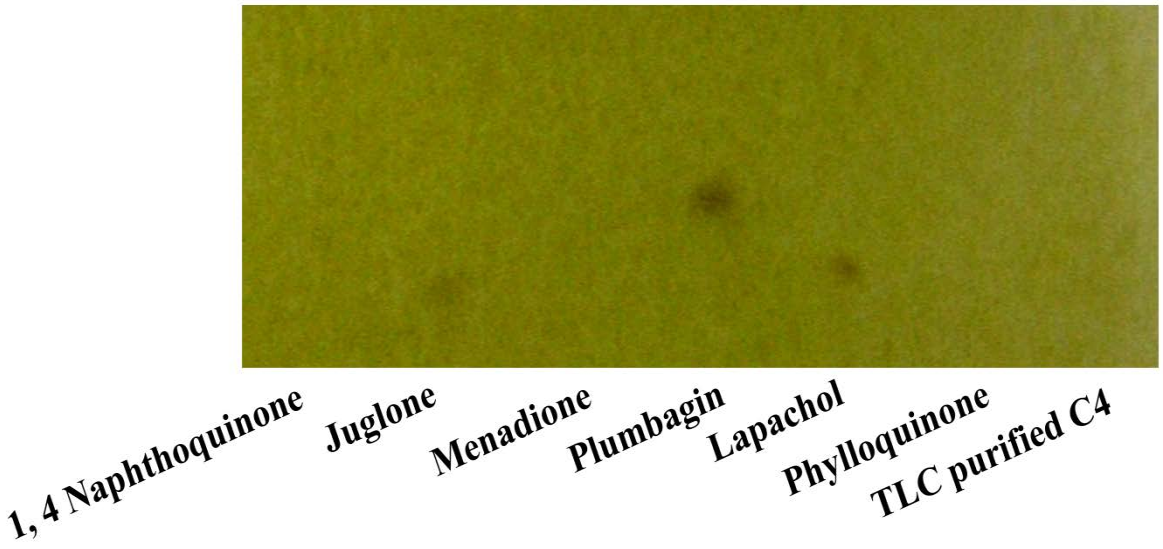


Figure 20. Analysis for the hydroxyl group in C4 compound along with standard naphthoquinones using ferric chloride test.

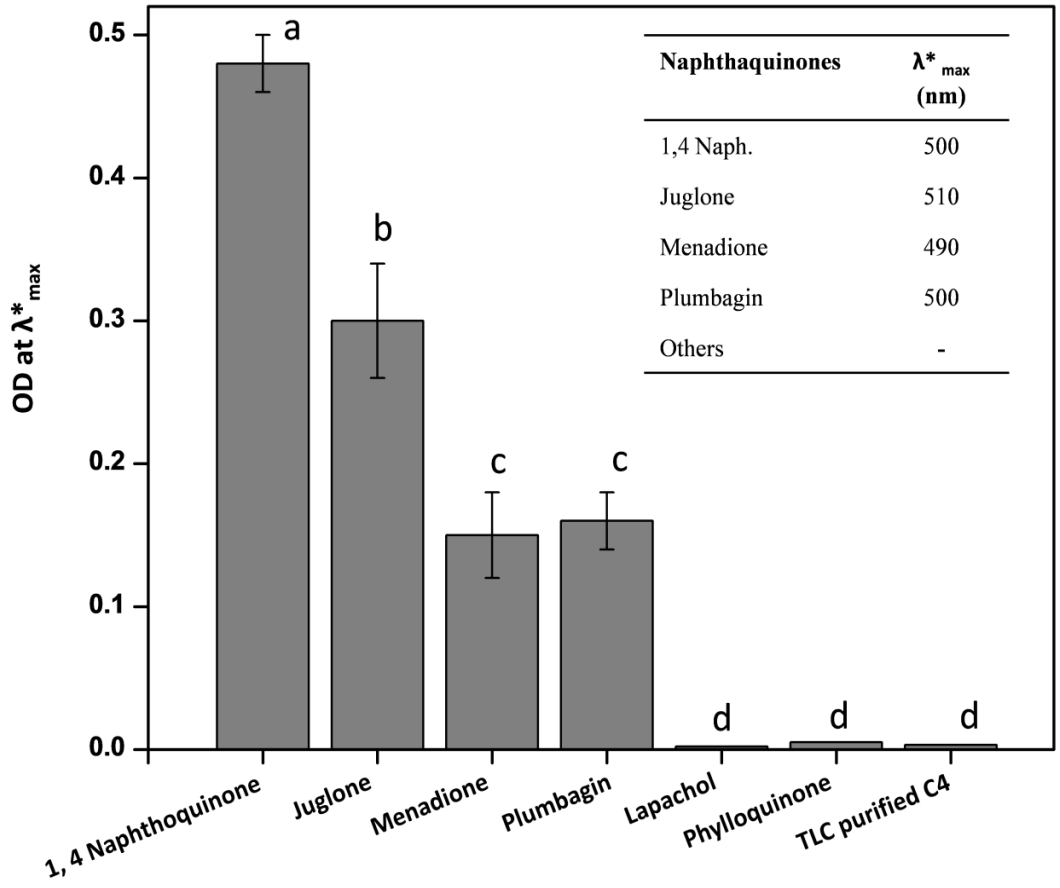
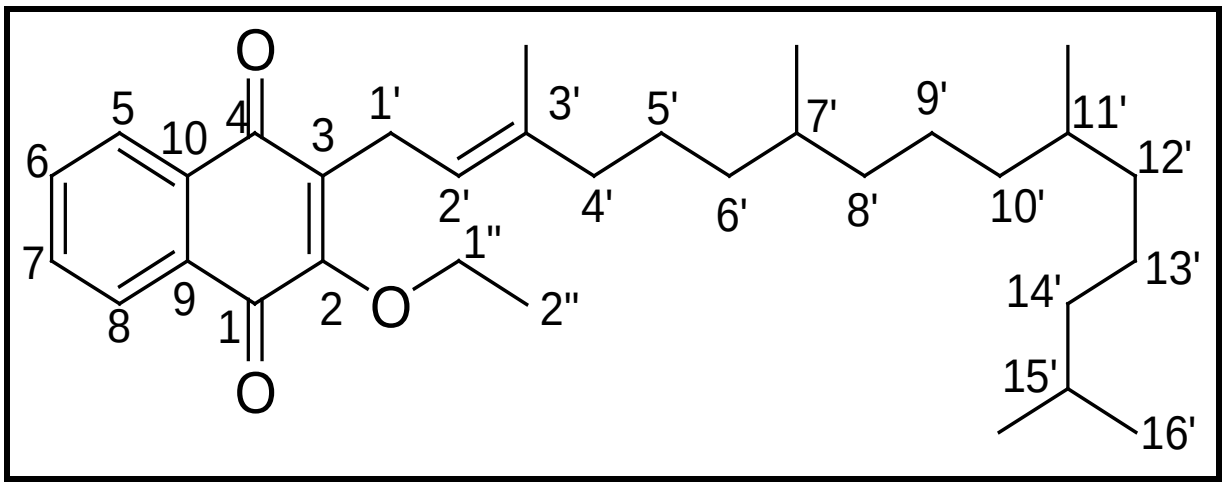


Figure 21. Test for 2, 3 position for presence or absence of functional group of C4 compound using the 1, 3-dimethylbarbituric acid (DMB) assay. Different letters (a-d) on the top of the bars show significant differences among means ($p \leq 0.05$).

3.1.5.6. NMR analysis

^{1}H and ^{13}C NMR spectra of C4 and its possible structure are shown in Table 6. The NMR data having four aromatic proton signals (two δ_H 7.70, dd, J = 6.0, 2.21 and two δ_H 7.54, multiplet) indicated a disubstituted benzene ring, and two carbon signals (δ_C 182.9 and 175.5) confirmed the presence of naphthoquinone moiety as reported earlier [79]. Four methyl groups at 3′,7′,11′, and 15′ positions were ascertained by both ^{1}H and ^{13}C NMR spectra [79]. The ^{13}C NMR spectrum of the compound showed 32 signals. The presence of the ethoxy group at the C-2 position was also indicated by the spectral data (Table 6). Thus, the NMR result suggested the C4 compound to be a naphthoquinone (phylloquinone derivative) (Table 6) (Figure 22) and assigned as:



2-Ethoxy-3-(3,7,11,15-tetramethylhexadec-2-ethyl)naphthaquinone-1,4-dione

(in short, Ethoxy-Substituted Phylloquinone, ESP)

Figure 22. Structure of C4 compound based upon NMR analysis

Table 6. ^{13}C and ^{1}H NMR data of the most antimutagenic C4 compound.

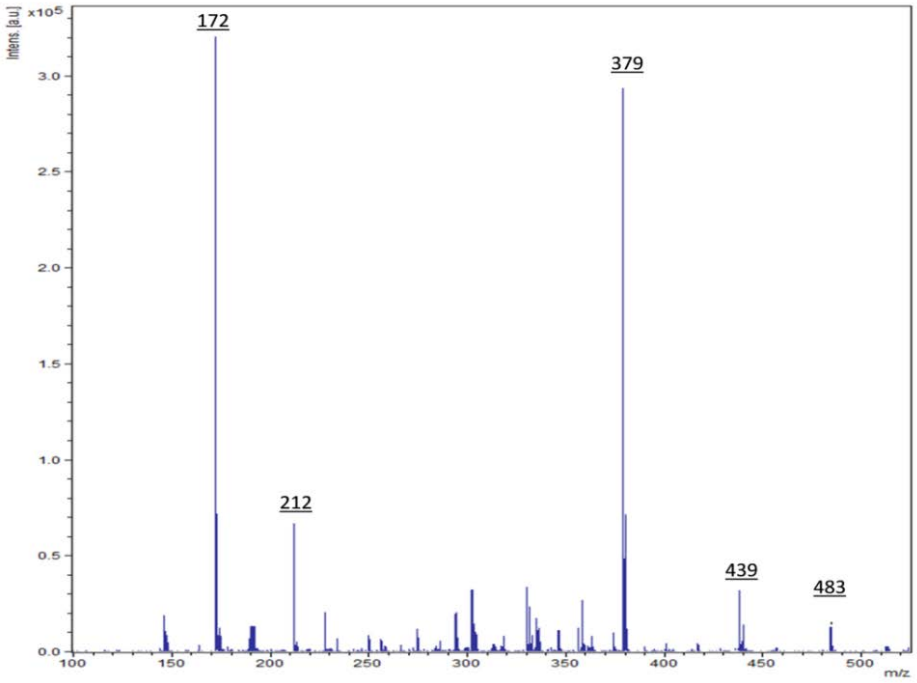
Carbon Position	Chemical Shift (δ_C)	Proton Position	Chemical Shift (δ_H)	No. of protons	Peak Multiplicity
1	182.9	5	7.70	1	dd (*J*=6.0,2,1)
2	121.9	6	7.54	1	m
3	158.6	7	7.54	1	m
4	175.5	8	7.70	1	dd (*J*=6.0,2,1)
5,8	129.6	1′	2.23	2	m
6,7	124.4.	2′	5.22	1	t
9,10	134.8	3′-methyl	1.68	3	s
1′	22.5	4′	2.05	2	t
2′	124.2	5′	1.33	2	m
3′	140.1	6′, 8′, 10′, 12′, 14′	1.29	2 protons each	m
3′-methyl	17.6	7′	1.69	1	m
4′	39.5	9′	1.31	2	m
5′	25.9	11′	1.59	1	m
6′	38.8	13′	1.30	2	m
7′	34.7	7′ and 11′ methyl	1.05	3 protons each	both d (*J*= 6.6)
7′-methyl	21.6	15′	1.92	1	m
8′	38.1	16′	0.90	3	d (*J* =6.6)
9′	25.5	15′- methyl	0.90	3	d
10′	38.5	1′′	4.00	2	q
11′	33.1	2′′	1.21	3	t
11′-methyl	22.0				
12′	38.4				

13′	25.2
14′	39.4
15′	29.8
15′-methyl	23.7
16′	23.7
1′′	68.3
2′′	19.8

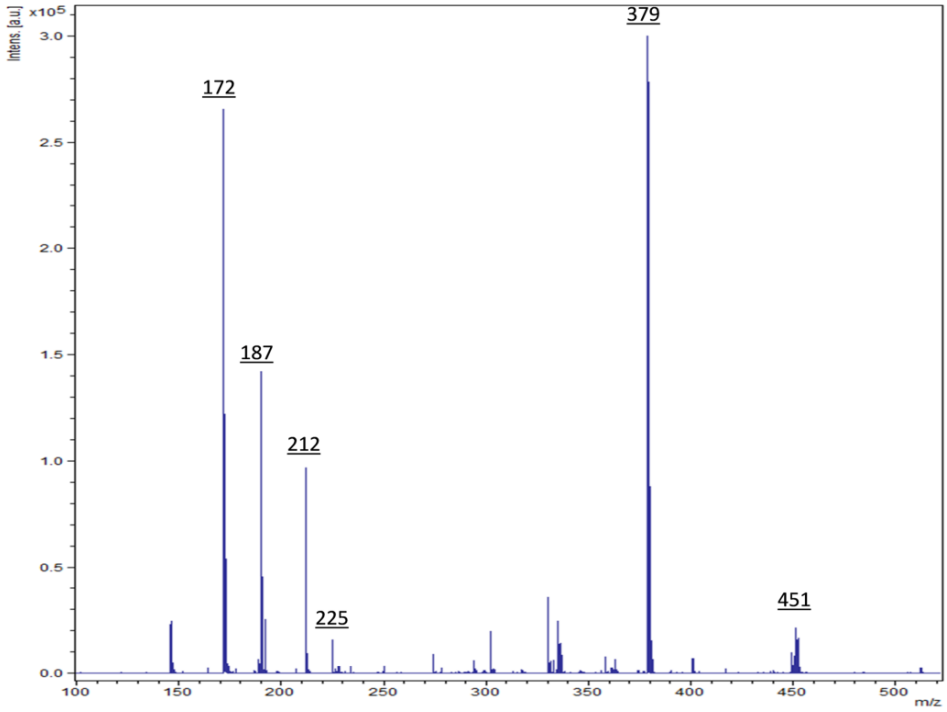
3.1.5.7. MALDI-TOF MS analysis

The protonated molecular ion of C4 compound (ESP) was detected at m/z 483 (Figure 22A) (Table 7). Its MS/MS fragmentation, m/z 439 resulted from the loss of [CH_3CH_2O-] moiety (45 amu), m/z 379 from the loss of [CH_3CH_2O-] (45 amu) and [-$CH_2CH(CH_3)_2$] (57 amu), m/z 212 from the loss of [CH_3CH_2O-] (45 amu) and[-$CH_2CH_2CH_2CH(CH_3)]_3CH_3$] (225 amu), and m/z 172from the loss of [CH_3CH_2O-] (45 amu) and –C=C(CH_3)[$CH_2CH_2CH_2CH(CH_3)]_3CH_3$] (264 amu) (Figure 22) (Table 7). Thus, the fragmentation pattern validated the above proposed structure of C4 compound (ESP).

The phylloquinone standard protonated molecular ion was detected at m/z 451 (Figure 22B) (Table 7) [80]. Its MS/MS fragmentation, m/z 225 resulted from the loss of [-$CH_2CH_2CH_2CH$-(CH_3)]$_3CH_3$] (225 amu) (Figure 22B) (Table 7) [80]. Other fragments such as 379, 212, and 172 were produced due to the loss of [CH3-] and ions indicated above other than[CH_3CH_2O-] (Figure 22A and B) (Table 7).



(A)



(B)

Figure 23. MALDI-TOF MS analysis of (A) C4 compound (ESP) and (B) phylloquinone.

Table 7. The fragmented masses produced during MALDI-TOF MS analysis and confirmation of ESP structure.

C4		Phylloquinone	
m/z	**Fragmentation**	**(m/z)**	**Fragmentation**
483	Molecular mass	451	Molecular mass
439	Loss of [CH_3CH_2O-] (45 amu)	225	Loss of [-$CH_2CH_2CH_2CH(CH_3)]_3CH_3$] (225 amu)
379	Loss of [CH_3CH_2O-] (45 amu) [$CH_2CH(CH_3)_2$] (57 amu)	379	Loss of CH_3- (15 amu) [$CH_2CH(CH_3)_2$] (57 amu)
212	Loss of [CH_3CH_2O-] (45 amu) [-$CH_2CH_2CH_2CH(CH_3)]_3CH_3$] (225 amu)	212	Loss of CH_3- (15 amu) [-$CH_2CH_2CH_2CH(CH_3)]_3CH_3$] (225 amu)
172	Loss of [CH_3CH_2O-] (45 amu) -C=C(CH_3)[$CH_2CH_2CH_2CH(CH_3)]_3CH_3$] (264 amu)	172	Loss of CH_3- (15 amu) -C= (CH_3)[$CH_2CH_2CH_2CH(CH_3)]_3CH_3$] (264 amu)

3.2. Comparative bioactivity evaluation of ESP with other naphthoquinones and understanding the underlying mechanism

3.2.1. Comparative antimutagenicity

Antimutagenicity of different naphthoquinones was compared in *E. coli* MG1655 based Rif^R assay. Phylloquinone displayed significantly less antimutagenicity (~12%) as compared to the ethoxy-substituted phylloquinone (ESP), which displayed ~75% antimutagenic activity, at the same concentration (1 mM) (Figure 23). Based upon current findings it seems that the functional groups at the C-2 position might be playing the important role in higher antimutagenicity of ESP with respect to phylloquinone.

Structure-function relationship with respect to antimutagenicity was also observed in other naphthoquinones, plumbagin, juglone, and menadione at equimolar concentration (0.1 mM) (Figure 23). At higher concentration these naphthoquinones were found to affect the viability of *E. coli* cells. Antimutagenicity displayed by plumbagin (which contains -OH and $-CH_3$ groups at C-5 and C-2, respectively) and juglone (that contains only -OH group at C-5) was 69 and 64%, respectively, indicated importance of hydroxyl (-OH) group in the backbone structure. On the other hand less (~24%) antimutagenicity displayed by menadione (which contain only $-CH_3$ group at C-5) indicated limited role of $-CH_3$ group to the antimugenicity (Figure 23). These results have also indicated that the presence of substitution such as ($-OCH_2CH_3$ / -OH) with naphthoquinone backbone structure enhances its antimutagenic potential.

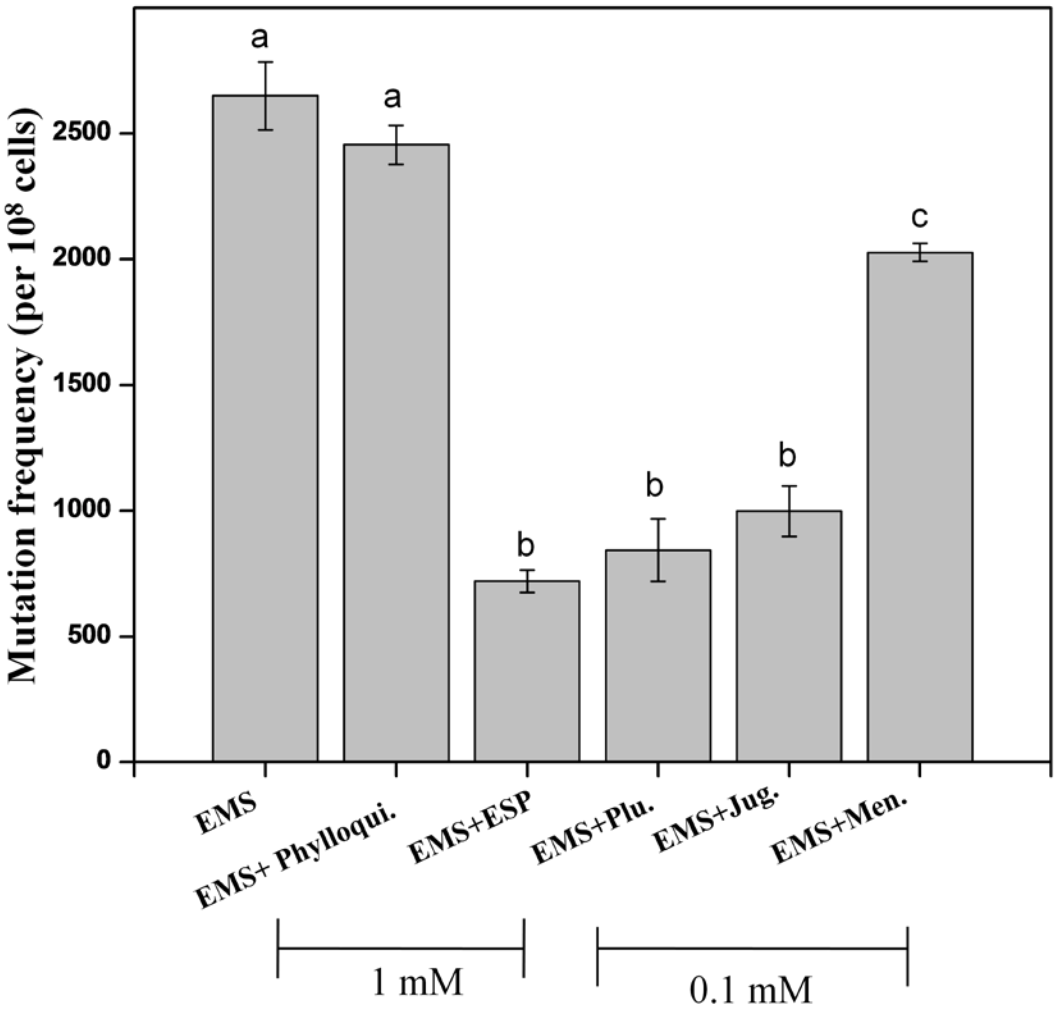


Figure 24. Antimutagenicity of spinach bioactive ESP as compared to standard naphthoquinones in Rif^R assay. Different letters (a-c) on the top of the bars show significant differences among means ($p \leq 0.05$).

Note: At higher (0.2 mM) concentrations, plumbagin, juglone and menadione affected the viability of *E. coli*.

3.2.2. Comparative antioxidant capacity

Naphthoquinones such as ESP, phylloquinone, plumbagin, juglone, and menadione displayed variable antioxidant capacity in terms of $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ radical scavenging activities when assayed at optimized equimolar concentration of 300 µM concentration. $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ radical scavengingactivities ranged between 5-25 and 6-34%, respectively (Table 8). The correlation of antioxidant activity with the antimutagenicity was quite low (R^2 = 0.19), which ruled out the major role of radical scavenging in the observed antimutagenicity.

Table 8. Antioxidant capacity of ESP and standard naphthoquinones.

Compounds	$ABTS^{\cdot+}$ radical scavenging activity (%)	$DPPH^{\cdot}$ radical scavenging activity (%)
ESP (300 µM)	34 ± 6^{x}	25 ± 5^{x}
Phylloquinone(300 µM)	16 ± 4^{y}	12 ± 4^{y}
Plumbagin(300 µM)	7 ± 4^{z}	5 ± 3^{z}
Juglone(300 µM)	6 ± 2^{z}	5 ± 3^{z}
Menadione(300 µM)	9 ± 5^{z}	7 ± 2^{z}
Ascorbic acid equivalent	33±3 (11 µM)	38±4 (18 µM)

Note: Different letters in columns (x-z) indicate significant differences among means ($p \leq 0.05$).

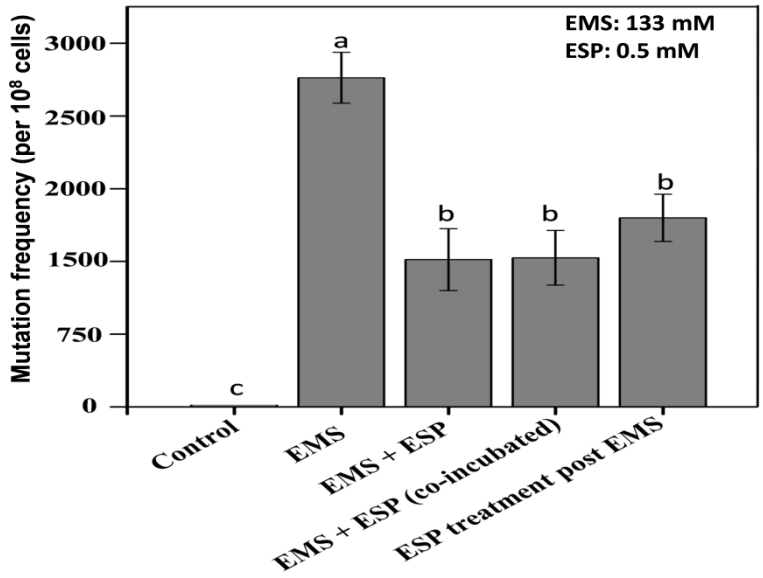
3.2.3. Possible mechanism of antimutagenicity of ESP

3.2.3.1. Antimutagenicity of ESP against EMS under different conditions

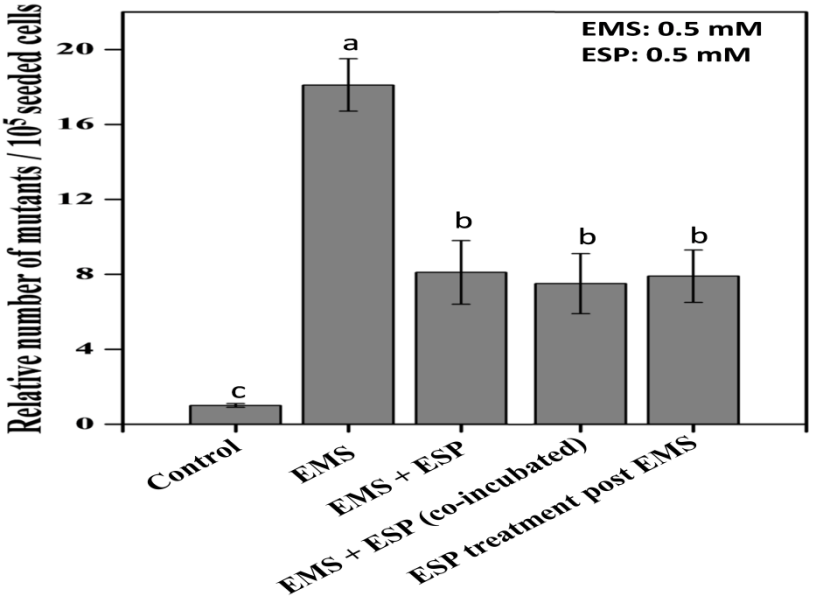
Mutation frequency in presence of EMS in *E. coli* MG1655 was observed to be $2750/10^8$ cells. In Rif^R assay, when EMS (the optimized concentration of 133 mM) and ESP (the optimized concentration of 0.5 mM) were added simultaneously, reduction in mutation frequency (antimutagenicity) was found to be ~46% (Figure 24A). No significant change in antimutagenicity was observed when EMS and ESP were co-incubated for 2h prior to the adding in the culture (Figure 24A). This indicated no direct interaction between EMS and ESP. Interestingly, when cells were treated with EMS followed by ESP, ~38% antimutagenicity was observed which could be related to induction of general protective mechanism due to ESP treatment (Figure 24A). Similar results were obtained with TK6 human lymphoblast cell line at the optimized concentration of 0.5 mM for both EMS and ESP where, antimutagenicity in above conditions, i.e., simultaneous, co-incubated and post treatment was ~56, 61, and 58%, respectively (Figure 24B).

3.2.3.2. Assessment of direct physical interaction between EMS and ESP

EMS (8 mM) and ESP (8 mM) was co-incubated and analyzed by TLC, no change in fluorescence or R_f was observed at 366 nm (Figure 25A). Besides, no other spot(s) were observed at 366 as well as 254 nm (Figure 25A and B). This result corroborated the above observations concluding no direct interaction between mutagen and ESP compound which otherwise would have led to modification or complex formation.

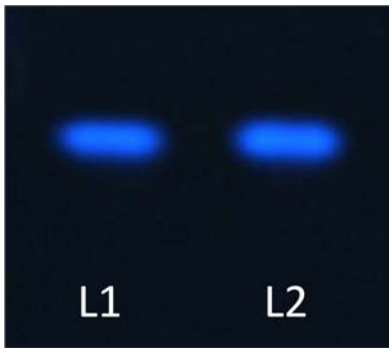


(A)



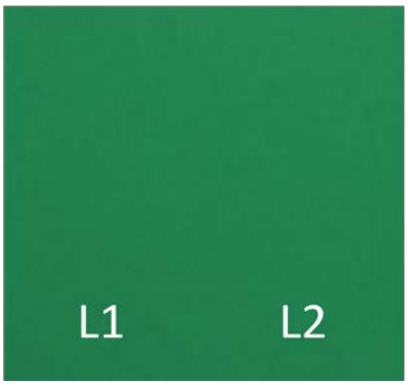
(B)

Figure 25. Antimutagenicity of ESP: (A) Rif^{R} and (B) TK6 gene (*tk*$^{+/-}$) mutation assays under various treatment conditions, i.e., EMS+ESP: simultaneously added to the cells; EMS+ESP (co-incubated): added to the cells after 4 h co-incubation outside; ESP treatment post EMS: cells treated with EMS for 4h and then ESP added. Different letters (a-c) on the top of the bars show significant differences among means (p≤0.05).



366 nm

(A)



254 nm

(B)

Figure 26. TLC analysis of ESP at (A) 366 and (B) 254 nm; L1: after incubation in the solvent, and L2: after co-incubation with EMS.

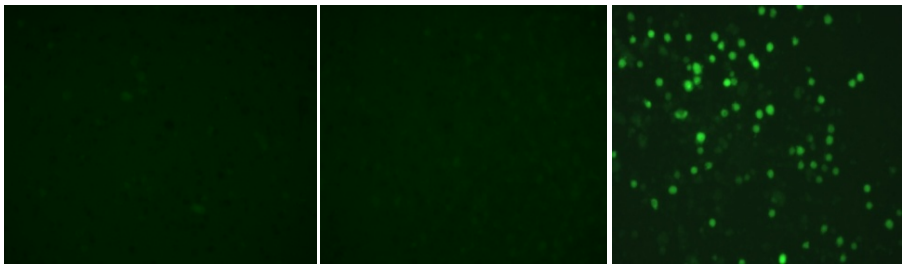
3.2.3.3. Role of antioxidant activity in antimutagenicity of ESP

EMS treatment was not found to induce any oxidative stress in TK6 cells at the optimized concentration (0.5 mM) used for antimutagenic study (Figure 26A). However, in case of *E. coli*, EMS (133 mM) treatment used for antimutagenic study was found to induce ~2 fold oxidative stress as compared to untreated control cells which was not reduced significantly in the presence of ESP (Figure 26B and C). In hydrogen peroxide treated cells significant (~23 fold) oxidative stress was induced as compared to the control cells (Figure 26B and C). The findings thus indicated that whatever antioxidant activity is possessed by ESP that does not contribute to its antimutagenicity against EMS.

3.2.3.4. Interaction of ESP with DNA

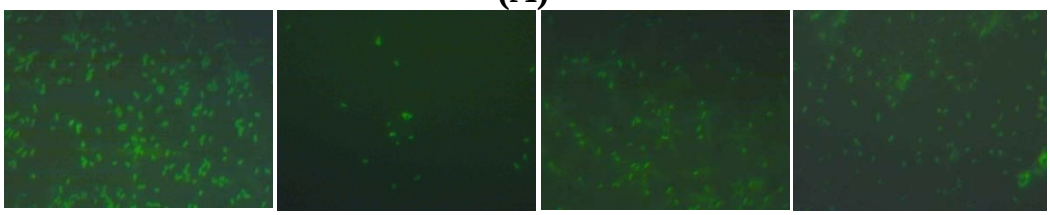
3.2.3.4.1. Spectrophotometric analysis

As expected CT DNA absorbance maxima (λ_{max}) was observed at 260 nm (Figure 27). Increase in ESP (10-60 µM) concentration enhanced the absorbance value of CT DNA (Figure 27). Such hyperchromic effect is a spectral feature that depicts non-covalent interaction, particularly in groove binding between compounds and CT DNA which leads to change in its secondary structure [81]. Intercalation of small molecules into the DNA helix results in bathochromic (red) shift as well as hypochromic effect [82]. Absorbance of ESP alone was found to be negligible even at the highest concentration used.



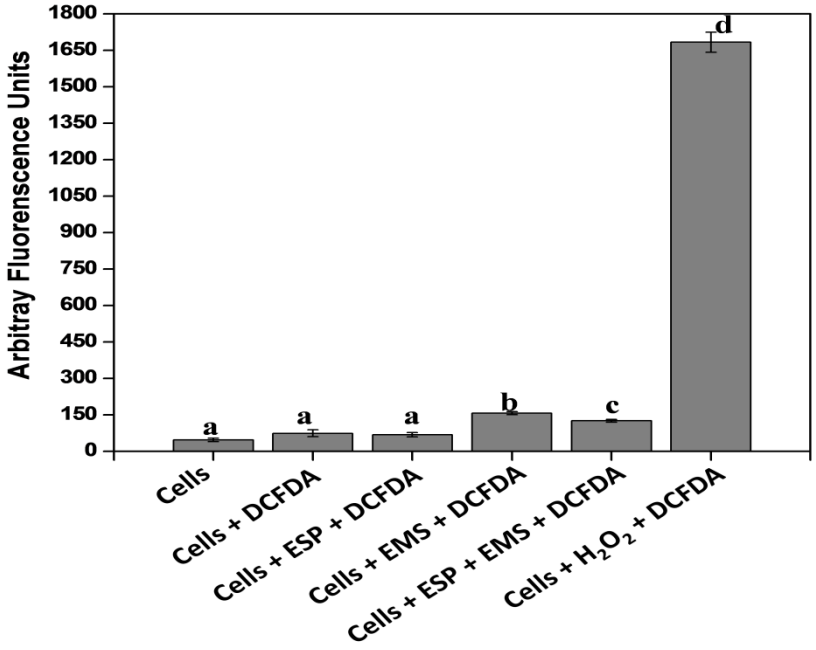
a b c

(A)



a b c d

(B)



(C)

Figure 27. Microscopic analysis of DCFDA stained (A) TK 6 cells: a) Control, b) EMS (0.5 mM) treated, c) H_2O_2 (50 µM) treated; (B) *E. coli* MG1655 cells: a) Control, b) EMS (133 mM) treated, c) ESP (0.5 mM) + EMS (133 mM) treated, d) H_2O_2 (50 µM) treated; (C) Histogram depicting level of ROS in *E. coli* cells measured through fluorometric analysis (λ_{Ex}: 488 and λ_{Em}: 525 nm). Different letters (a-d) on the top of the bars show significant differences among means ($p \leq 0.05$).

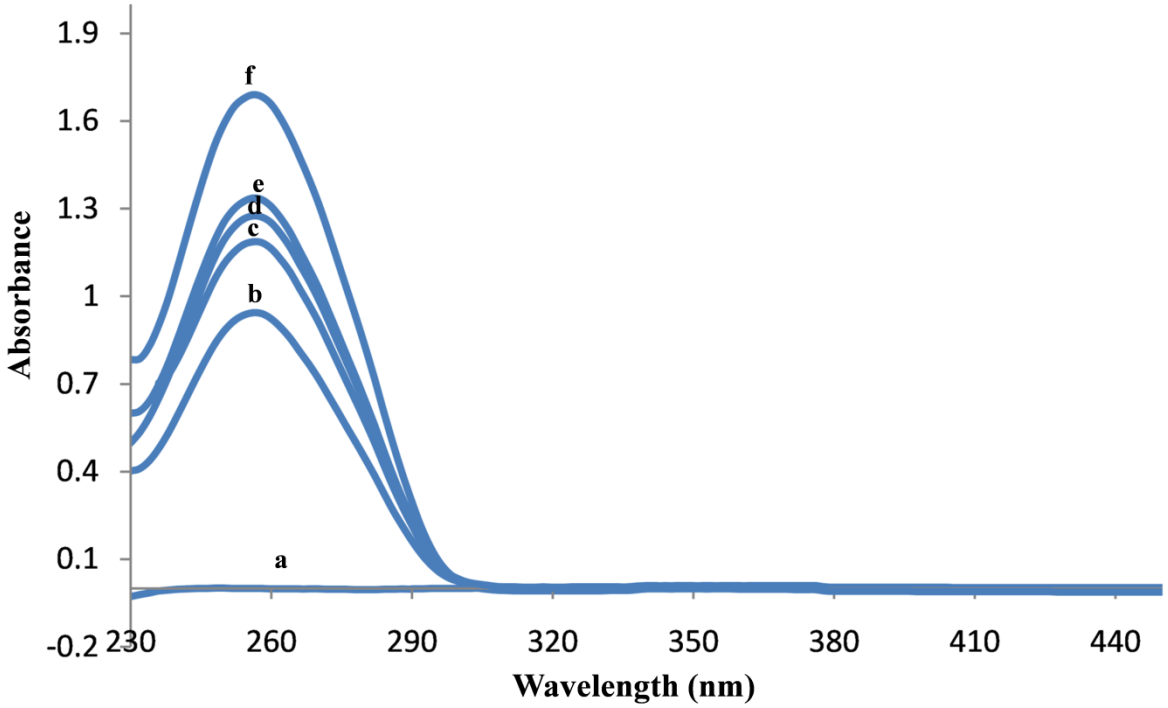


Figure 28. Spectrophotometric analysis of Calf Thymus (CT) DNA treated with ESP at different concentrations: a) ESP (60 µM), b) CT DNA (120 µM), c) CT DNA (120 µM) + ESP (10 µM), d) CT DNA (120 µM) + ESP (20 µM), e) CT DNA (120 µM) + ESP (40 µM), f) CT DNA (120 µM) + ESP (60 µM).

3.2.3.4.2. Circular dichroism (CD) spectropolarimetry

The CD pattern observed for CT DNA provides further and definitive confirmation of the probable mode of CT DNA binding with ESP. The CD spectrum of CT DNA consists of a positive band at 275 nm due to base stacking and a negative band at 245 nm due to helicity and it is also characteristic of DNA in a right-handed B form [83]. These CD signals of DNA are considered to be highly sensitive toward interaction of small molecules with DNA and corresponding changes in its structure [84]. The CT DNA displayed typical spectrum of B-form with no induction of new band and the increase in concentration of ESP was not found to affect this feature (Figure 28) [85]. However, there was decrease in the elipticity at 245 and 275 nm at enhanced concentrations of compound in groove binding (Figure 28) [72, 86].

3.2.3.4.3. DAPI (4', 6-Diamidino-2-phenylindole dihydrochloride) binding assay

Similarly, DAPI fluorescence increases significantly upon its binding to minor groove of DNA at 450 nm (λ_{Ex} 338 nm) (Figure 29). A significant decrease in this fluorescence was observed with the increase in the concentration (10-40 μM) of ESP (Figure 29). This suggested that ESP possibly bind to the minor groove of the DNA (Figure 29).

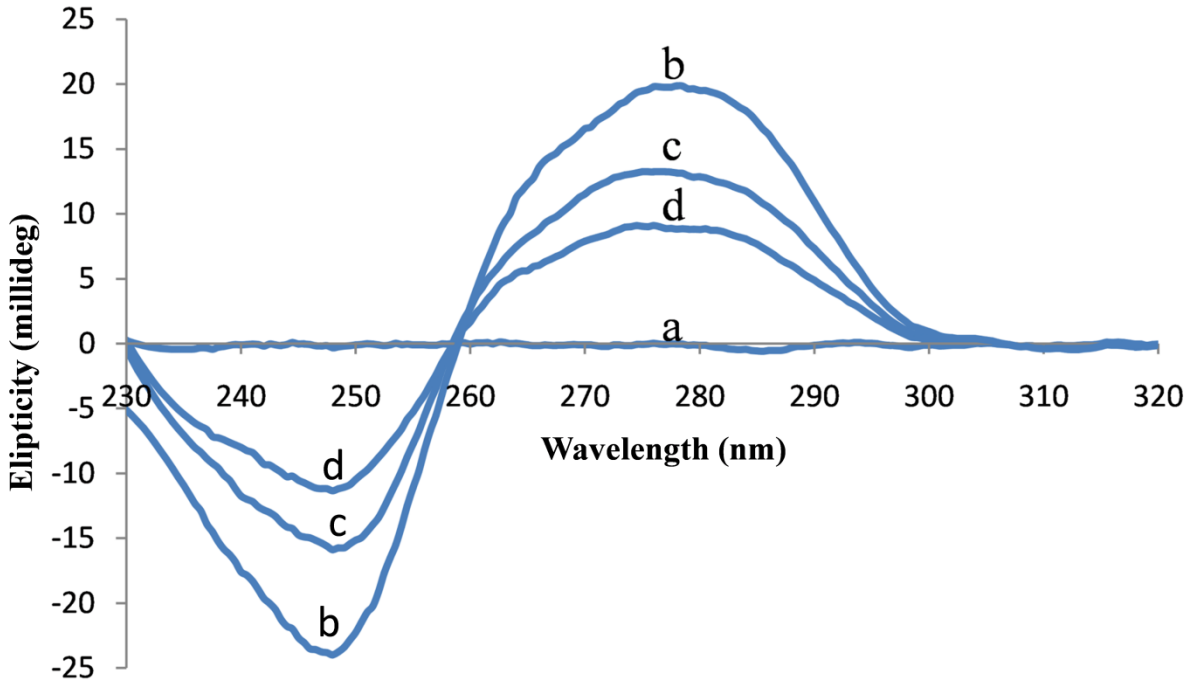


Figure 29. Circular dichroism spectropolarimetric analysis of CT DNA treated with ESP at different concentrations: a) ESP (60 µM), b) CT DNA (50 µM), c) CT DNA (50 µM) + ESP (40 µM), d) CT DNA (50 µM) + ESP (60 µM).

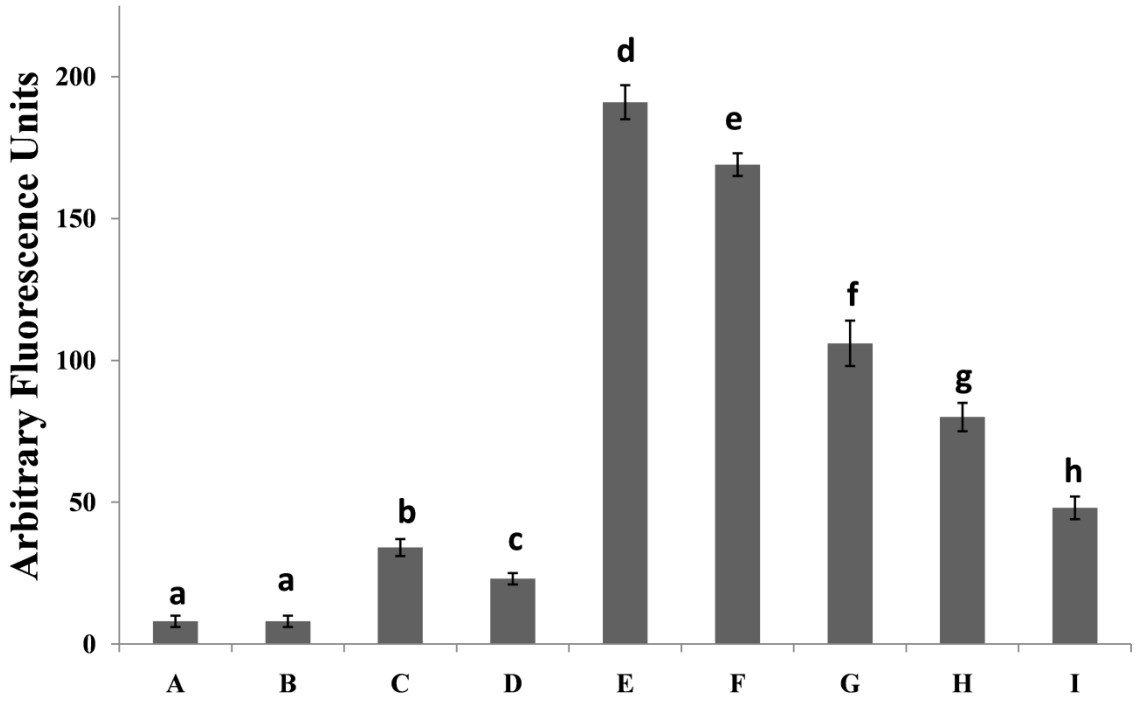


Figure 30. DAPI binding assay (CT DNA treated with ESP at different concentrations): A) Phosphate buffer, B) CT DNA (120 µM), C) DAPI (15 µM), D) ESP (40 µM), E) CT DNA (120 µM) + DAPI (15 µM), F) CT DNA (120 µM) + DAPI (15 µM) + ESP (40 µM), G) CT DNA (120 µM) + ESP (10 µM) + DAPI (15 µM), H) CT DNA (120 µM) + ESP (20 µM) + DAPI (15 µM), I) CT DNA (120 µM) + ESP (40 µM) + DAPI (15 µM). Different letters (a-h) on the top of the bars show significant differences among means ($p \leq 0.05$).

3.2.3.4.4. Ethidium bromide (EtBr) binding assay

EtBr is an intercalator to the DNA. Binding to DNA increases its fluorescence drastically at 610 nm (λ_{Ex} 480 nm) (Figure 30). Presence of ESP was not found to affect this fluorescence of EtBr at (10-40 µM) concentrations (Figure 30). This indicated that ESP was not intercalating to the CT DNA.

3.2.3.4.5. Methyl green (MG) binding assay

MG is major groove binder of DNA. Its absorbencies are at 254, 310, 420, and 630 nm, however among these its absorbance is highest and specific at 630 nm and most appropriate for DNA interaction study [87, 88]. MG absorbance was highest in the buffer under free condition (Figure 31). Its binding to CT DNA reduced the absorbance value (Figure 31). The presence of ESP (10-40 µM) was not found to significantly affect the absorbance value (Figure 31). This indicated that ESP does not bind the major groove of CT DNA and therefore ESP which binds minor groove can not interfere with the binding of MG to the CT DNA major groove.

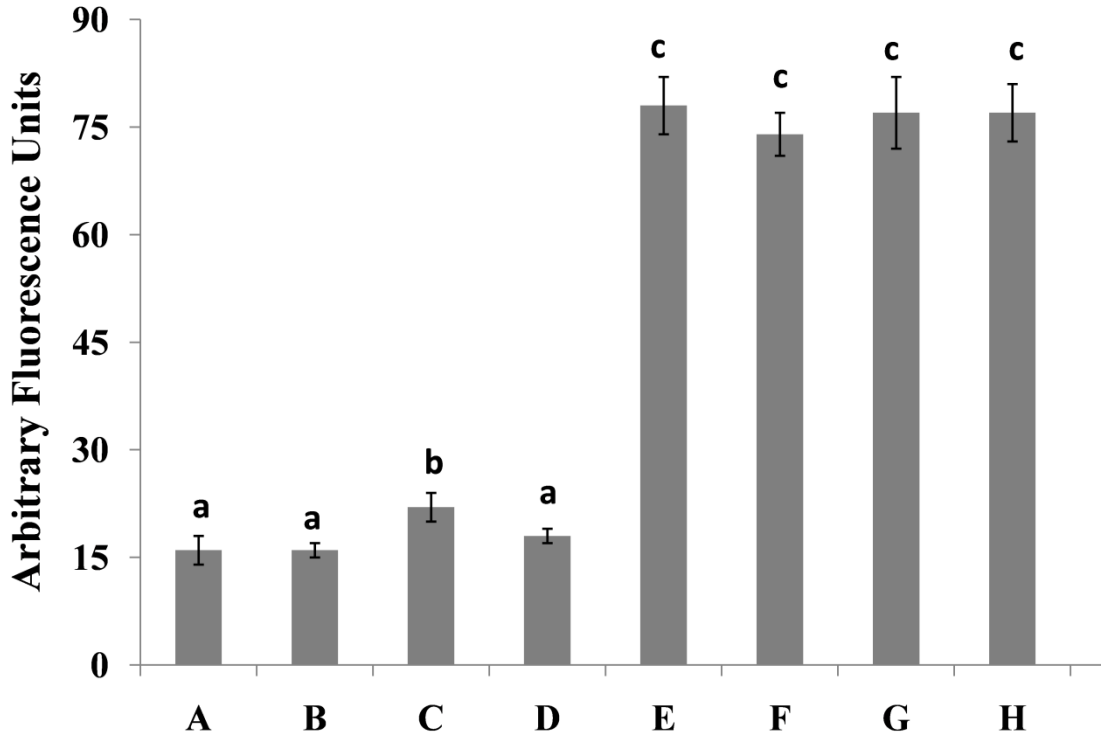


Figure 31. Ethidium bromide (EtBr) binding assay (CT DNA treated with ESP at different concentrations): A) Phosphate buffer, B) CT DNA (120 µM), C) EtBr (10 µM), D) ESP (40 µM), E) CT DNA (120 µM) + EtBr (10 µM), F) CT DNA (120 µM) + ESP (10 µM) + EtBr (10 µM), G) CT DNA (120 µM) + ESP (20 µM) + EtBr (10 µM), H) CT DNA (120 µM) + ESP (40 µM) + EtBr (10 µM). Different letters (a-d) on the top of the bars show significant differences among means ($p \leq 0.05$).

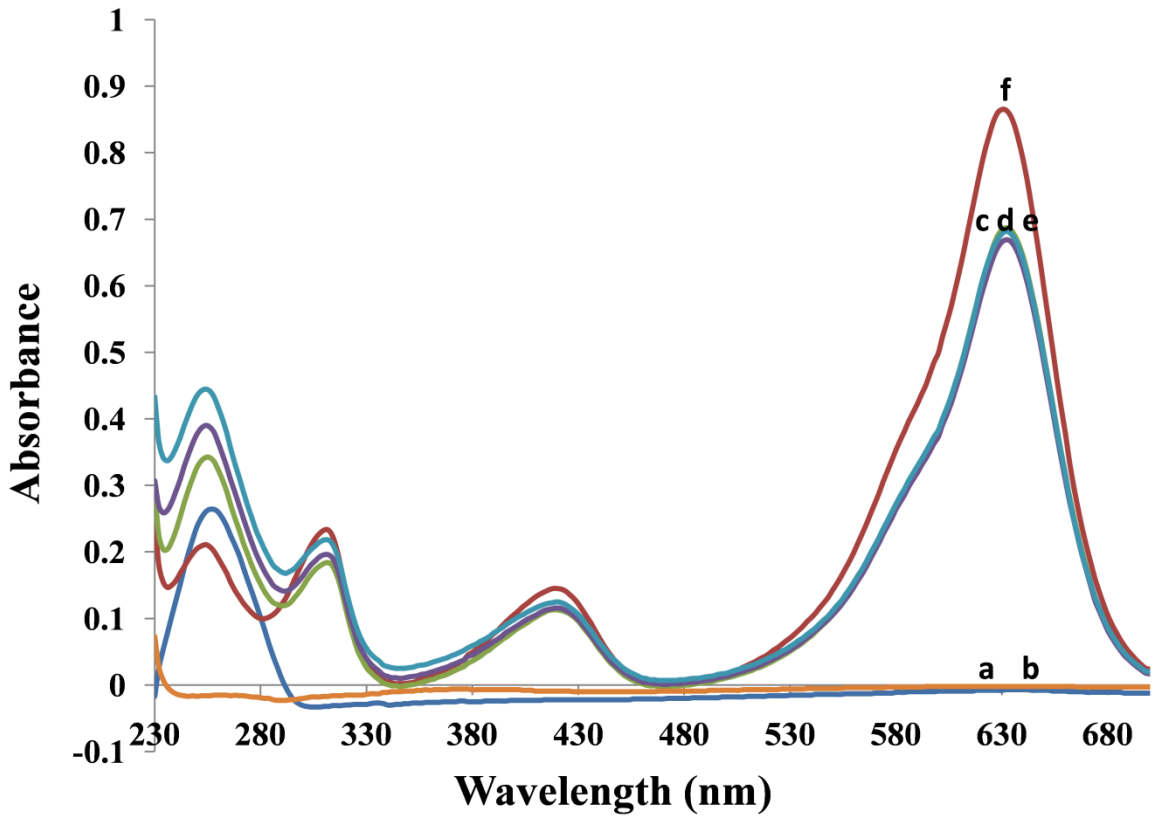


Figure 32. Methyl green (MG) binding assay (CT DNA treated with ESP at different concentrations): a) ESP (40 µM), b) CT DNA (120 µM), c) CT DNA (120 µM) + MG (20 µM), d) CT DNA (120 µM) + ESP (20 µM) + MG (20 µM), e) CT DNA (120 µM) + ESP (40 µM) + MG (20 µM), f) MG (20 µM).

3.2.3.5. 2-D analysis, MALDI-TOF MS/MS and antimutagenicity analysis in *E. coli* gene knockout strains

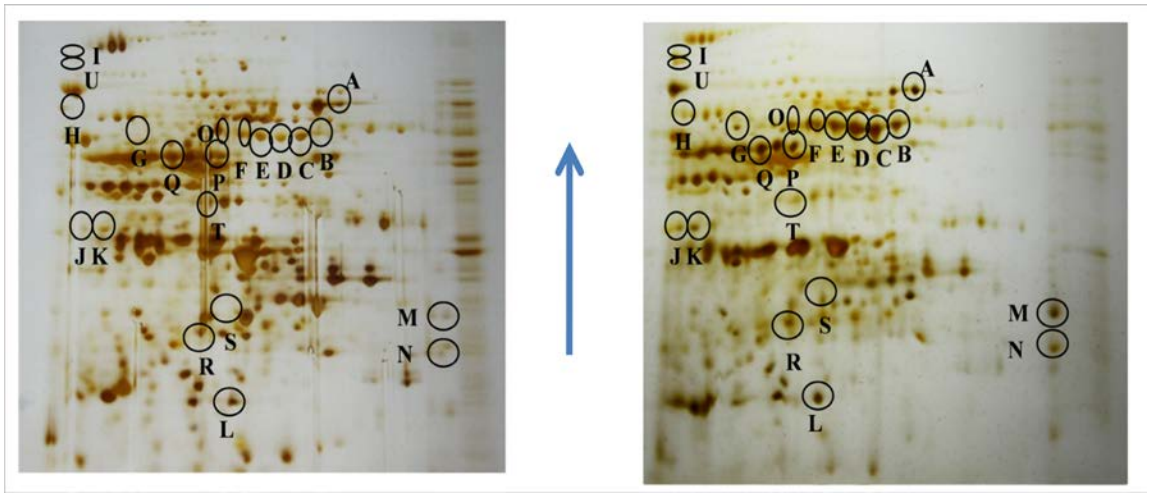
Around 20 proteins were prominently up-regulated in ESP and EMS treated *E. coli* cells as compared to EMS alone treated cells (Figure 32, Table 9). Besides, around 32 proteins were also found to be prominently down-regulated in ESP and EMS treated cells as compared to EMS alone treated cells (Figure 33, Table 9). Differentially expressed proteins those showing more than 4 fold change (increase/ decrease) and some proteins which prominently expressed only in the presence of EMS+ESP treated cells, were selected for identification using MALDI-TOF MS/MS (Table 10). The expression profile of these proteins in EMS and ESP+EMS were also compared with control (untreated) cells (Figure 34) (Table10).

Among the up-regulated proteins, based upon their reported functionality and the fold change in ESP+EMS treated cells as compared to EMS, proteins like TnaA (16.3 fold), DgcP (7.3 fold), SelA (prominently expressed only in the presence of EMS+ESP) and RpoH (8.5 fold) could be among the important proteins contributing to the observed antimutagenicity due to ESP (Table 10). Interestingly these genes were also found to be significantly up-regulated in ESP+EMS treated cells compared to control cells (Table 10).

Thus, to confirm the role of these up-regulated proteins in antimutagenicity, their knockout strains were used for antimutagenicity analysis. Interestingly, mutation frequency of these selected knock out strains were found to be higher (~2970-3250/10^8 cells) than the wild type strain (2700/10^8 cells) indicated their importance in preventing mutation (Figure 35). Antimutagenicity of ESP was ~74% in *E. coli*

MG1655 (wild type) strain whereas in *E. coli MG1655 ΔdgcP* (F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, ΔyeaP790::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514), *E. coli MG1655 ΔtnaA* (genotype: F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, rph-1, ΔtnaA739::kan, Δ(rhaD-rhaB)568, hsdR514), and *E. coli MG1655 ΔselA* (genotype: F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, ΔselA775::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514) this activity was found to be ~48, 27, and 69%, respectively (Figure 35). Thus, it seems that *tnaA* and *dgcP* gene functions were found to be the much more required for the antimutagenicity of ESP. The *tnaA* gene encodes for tryptophanase enzyme which convert L-tryptophan to indole. The indole is known to inhibit the cell division of *E. coli* [89]. The *dgcP* gene encodes for diguanylate cyclase which synthesizes cyclic-di-GMP (c-di-GMP) via condensation of 2 GTP molecules. The c-di-GMP is a messenger molecule and can prevent cell division by septal invagination [90]. The *rpoH* being essential gene, its knockout study could not be performed.

Some important down-regulated proteins were found to be *dps* (DNA protection during starvation protein), *iptE* (LPS-assembly lipoprotein) and *rpsQ* (30S ribosomal protein S17) in ESP+EMS treated cells as compared to EMS alone treated cells (Table 10).



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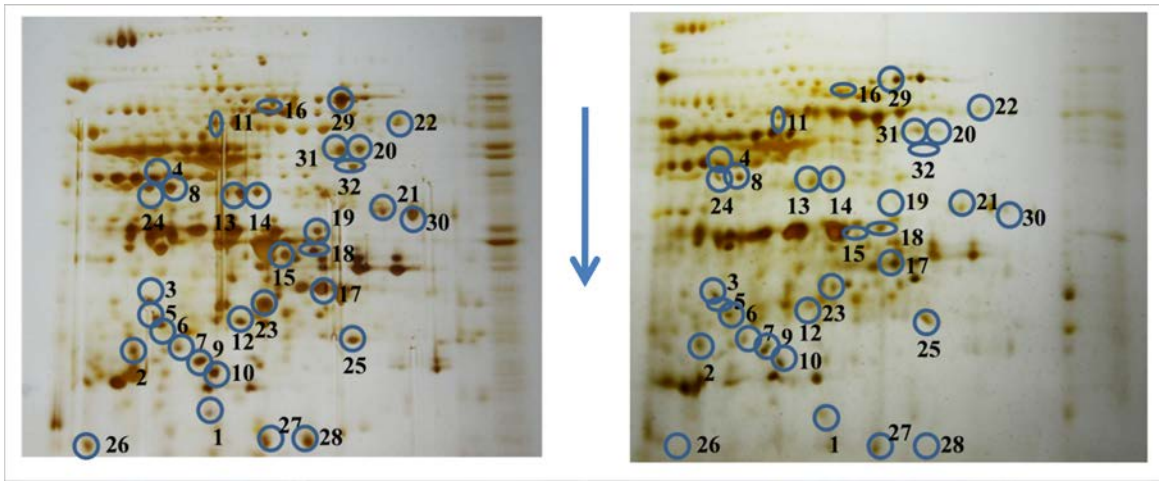

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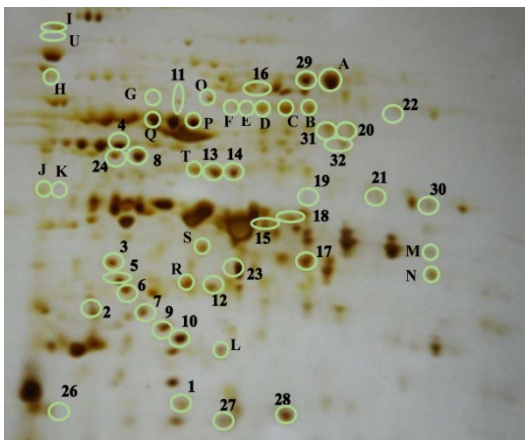


(E)

Figure 33. Two dimensional gel protein profiles showing: up-regulated protein in (B) ESP+EMS treated cells as compared to (A) EMS alone treated cells; down-regulated proteins in (D) ESP+EMS treated cells as compared to (C) EMS alone treated cells; (E) untreated control.

Table 9. Fold change in expression of prominent proteins in 2-D gel upon ESP+EMS treatment as compared to EMS.

Fold change	Proteins up-regulated	Protein down-regulated
≤ 2.5	7	16
2.6 – 4	0	6
4.1 – 9	6	1
> 9	3	5
Exclusive protein (observed only in ESP + EMS treated cells)	4	4

Table 10. Fold change (> 4) in expression of proteins in 2-D gel upon ESP+EMS treatment as compared to EMS and their identity based on MALDI-TOF-MS/MS.

Protein code	Fold change in EMS vs. control ↑(up) ↓(down)	Fold change in (ESP+ EMS) vs. control ↑(up) ↓(down)	Fold change in (ESP+EMS) vs. EMS ↑(up) ↓(down)	Identity	Function
A	7.1↓	3.2↓	4.5↑	OppA	Periplasmic oligopeptide-binding protein
B	3.7↓	9.6↑	16.3↑	TnaA	Tryptophanase; synthesizes indole from L-tryptophan
C	1.6↓	5.5↑	4.2↑	YbcV	Uncharacterized protein
D	1.7↑	14.3↑	8.5↑	RpoH	RNA polymerase sigma factor
E	3.5↑	19.8↑	7.3↑	DgcP	Synthesis of c-di-GMP
F	7.5↑	12.3↑	4.4↑	RutA	Pyrimidine monooxygenase
G	-	-	EP	SelA	L-seryl-tRNA (Sec) selenium transferase (Selenocysteine synthase)
M	1.2↑	18.1↑	17.3↑	StfR	Prophage side tail fiber protein
N	2.2↓	13.4↑	15.1↑	Cca	Multifunctional CCA protein
Protein code	**Fold change in EMS vs. control ↑(up) ↓(down)**	**Fold change in ESP+EMS vs. control ↑(up) ↓(down)**	**Fold change in ESP+EMS vs. EMS**	**Identity**	**Function**
12	3.1↑	8.3↓	14.3↓	MglB	D-galactose-binding periplasmic protein
15	3.5↑	12.8↓	17.5↓	TdcE	Catalyzes the cleavage of 2-ketobutyrate to propionyl-CoA and formate.
20	18.1↑	6.8↓	25.7↓	IptE	LPS-assembly lipoprotein
23	9.8↑	17.1↓	31.4↓	OmpA	Outer membrane protein A
26	EP	-	-	ParM	Plasmid segregation protein
28	4.3↑	30.8↓	36.7↓	Dps	DNA protection during starvation protein
29	3.1↑	15.1↓	20.4↓	RpsQ	30S ribosomal protein S17
30	15.7↑	9.2↓	27.8↓	CarB	Carbamoyl-phosphate synthase large chain
31	4.2↑	3.2↓	7.9↓	NfrA	Bacteriophage adsorption protein A
32	5.6↑	-	-	UgpQ	Glycerophosphodiester phosphodiesterase

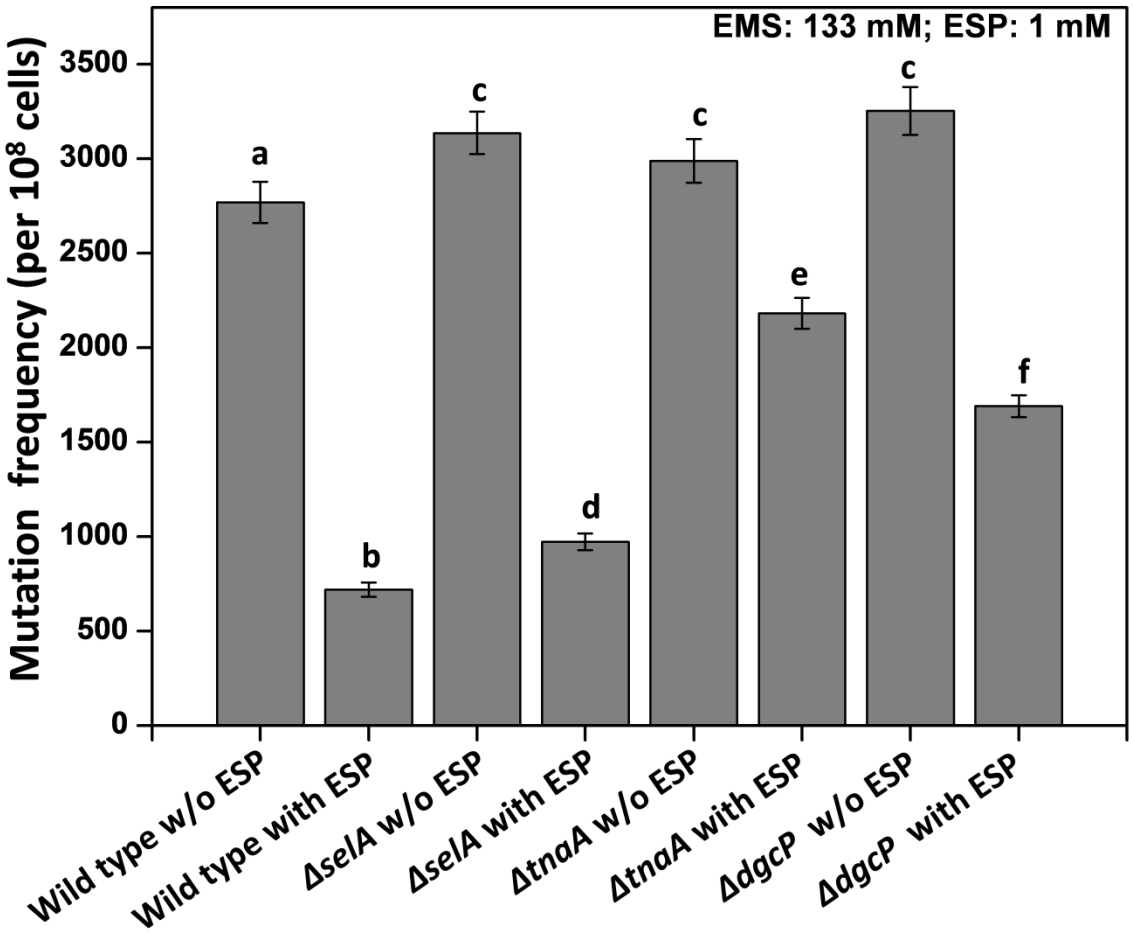


Figure 34. Antimutagenicity (Rif^R assay) of ESP in wild type (*E. coli* MG1655) and knockout strains upon EMS treatment. Different letters (a-f) on the top of the bars show significant differences among means ($p \leq 0.05$).

Chapter 4:

Discussion

Mutations are one of the prime causes for many chronic diseases including cancer [46]. The mutagenic agents such as chemicals directly or post activation, damages the DNA (Figure 35) [25]. Besides, radiation and oxidants too damages the DNA leading to mutations [49] (Figure 35).

In the current study, naphthoquinone rich foods, spinach, lettuce, iceberg lettuce, cabbage, broccoli and French bean were analyzed for antimutagenicity using aqueous, methanolic, total soluble phenolic and quinonic extracts.Variations were observed in antimutagenicity of extracts as also reported earlier in the cultivar of rose and apple [46, 48]. This could be due the presence of bioactive(s) in different concentration due to their differential hydrophilicity/ hydrophobicity [52]. The yield was found to have weak correlation with antimutagenicity again indicated the significance of phytoconstituent in this activity. Among the naphthoquinone rich foods, only spinach quinone extract displayed highest antimutagenicity as compared to its phenolic extract. Thus, from the quinone extract of spinach antimutagenic fluorescent compound was characterized as an ethoxy-substituted phylloquinone (ESP). Another naphthoquinone derivative (2-methyl-1,4,4a,8a-tetrahydro-endo-1,4-methanonaphthalene-5,8-dione) has been reported earlier from nutmeg (*Myristica fragrance*) to be fluorescent [91].

This novel compound possessed -OCH_2CH_3 instead of -CH_3 in case of phylloquinone at the 2^{nd} position. Ethoxy-substituted anthocyanin, i.e., peonidin was reported earlier to contribute to higher antimutagenicity [46]. In other study, 13 flavonoids and related compounds were reported from spinach, which displayed antimutagenicity against dietary carcinogen 2-amino-3-methylimidazo [4,5-f]-quinoline in *S. typhimurium* TA98 [92]. However, only 5,6,3′,4′-tetrahydroxy-7-methoxyflavonol 3-O-disaccharide

was reported as a potent antimutagen. In the current study, too, among different TLC purified quinones, ESP displayed maximum antimutagenicity.

Antimutagenicity of ESP was compared with standard naphthoquinones. High antimutagenicity in ESP, plumbagin and juglone, but comparatively less in phylloquinone and menadione indicated that -OH / $-OCH_2CH_3$ substitutions contributed to the antimutagenicity of naphthoquinones. In another study, K vitamins such as phylloquinone (K1), menadione (K3), and 1,4-diacetoxy-2-methylnaphthalene (K4) were analyzed for antimutagenicity against mutagens such as heterocyclic amine (HCA) where phylloquinone was found to be the least antimutagenic [93]. Naturally occurring quinones such as biflorin and plumbagin have been reported earlier for antimutagenicity against various mutagens such as hydrogen peroxide, 2-nitrofluorene, 3-nitrofluoranthene and l-nitropyrene [94, 95]. In general, most quinones displayed poor antioxidant activity but high antimutagenicity indicating the lack of correlation between these two important bioactivities.

The mutagen induced DNA damages are controlled by antimutagens especially from diet by various mechanisms such as mutagen inactivation/ transformation, antioxidant activity, regulatory responses and DNA repair (Figure 35) [36]. Antimutagens can be classified as desmutagens, which inhibits interaction between mutagen and DNA; and bio-antimutagens, which prevent mutagenesis after DNA damage by the mutagen mostly through induction of DNA repair [96]. The characterized bioactive compound ESP was also analyzed for the mechanism of antimutagenicity against ethyl methanesulfonate (EMS). The simultaneous treatment or co-incubation of ESP and EMS resulted in similar level of antimutagenicity. This indicated that the compound ESP does not directly inactivate the mutagen (EMS) through either complex formation

or modification. TLC analysis of co-incubated ESP and EMS reconfirmed this finding. Several compounds have been reported which directly inactivate mutagen. Co-incubation of phenolic extract from Fresh bean and aflatoxin B1 was reported to significantly reduce the mutagenicity which indicated possibility of chemical complex formation [44]. Similarly, gallic acid displayed antimutagenicity possibly by scavenging electrophilic mutagens [97]. In the post-treatment condition too, ESP displayed significant reduction in mutagenicity which indicated this compound is acting as bio-antimutagen. The antimutagenicity of ESP is very significant in preventive and treatment models such as human lymphoblast cell line.

Role ESP in reducing the oxidative stress was also ruled out. Also, induction of oxidative stress by EMS was quite low. Similarly, in other studies too, different cultivars of apple, honey, and various vegetables, the extract or purified bioactives did not display good correlation between the antioxidant and antimutagenic activities (40, 47, 48]. Besides, antioxidant related enzymes like *sodC* (superoxide dismutase), *xthA* (oxidative repair) etc were up-regulated in menadione which did not display significant antimutagenicity [98]. However, certain compounds like thiols (captopril, cysteine, and glutathione) and plumbagin have both antioxidant and antimutagenic activities but mechanism of antimutagenicity was not found to be primarily due to antioxidant enzymes [98, 99].

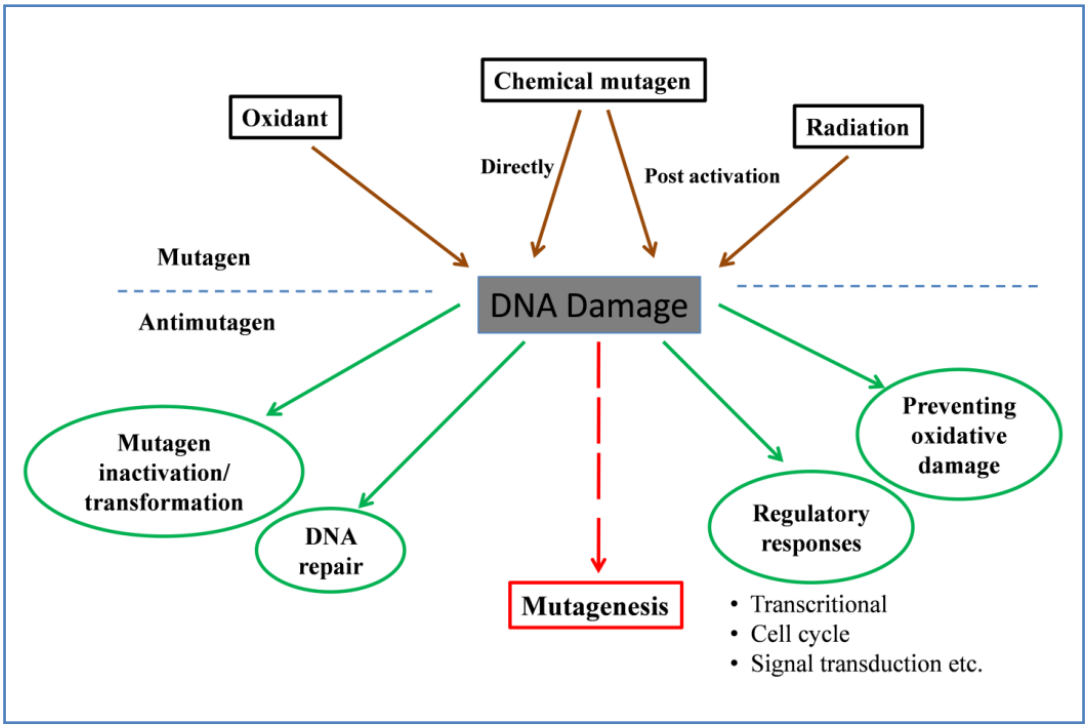


Figure 35. Possible mechanism of mutagenesis and antimutagenesis.

During mechanistic study, ESP was found to bind to the minor groove of DNA which could be one of the mechanisms for its antimutagenicity as minor groove is often recognized by transcription factors or other cellular DNA targeted proteins and such interaction may lead to change in gene expression profile [100, 101, 102, 103]. The minor groove binding is preferred over intercalative binding for therapeutics such as cancer due its lower toxicity and non-mutagenic effects to normal cells [104]. However, some intercalators such as quercetin and troxerutin have also been reported to be non- toxic to normal cells [73, 105].

During proteomic analysis, genes like *tnaA*, *dgcP*, *selA*, and *rpoH* were up-regulated in ESP and EMS co-treated cells as compared to EMS alone treated cells. However, TnaA protein was down regulated but up-regulation in DgcP and RpoH proteins (< 4 fold) was observed in EMS treated cells as compared to control cells. Alkylation damage to DNA has been reported to change the expression of genes related to replication, repair, and product that block cell division as an adaptive response [106].

Further, antimutagenicity analysis in *E. coli* gene knockouts indicated role of *tnaA* and *dgcP* in observed antimutagenicity. The gene *tnaA* encodes for tryptophanase which is involved in biosynthesis of indole. It is considered important for inhibiting division by an ionophore-based mechanism and allowing repair and maintenance of cells during the period of starvation [107]. Indole is a proton ionophore that make the cytoplasmic membrane permeable to hydrogen ions leading to the reduction in electrochemical potential (ECP) across the cytoplasmic membrane.This is known to inhibit cell division in *E. coli* by preventing MinCD oscillation as well as formation of the FtsZ ring which is a prerequisite for division [89, 108] (Figure 36). Indole has been also

reported to increase the survival of cells under stress conditions like antibiotic, high temperature etc [109].

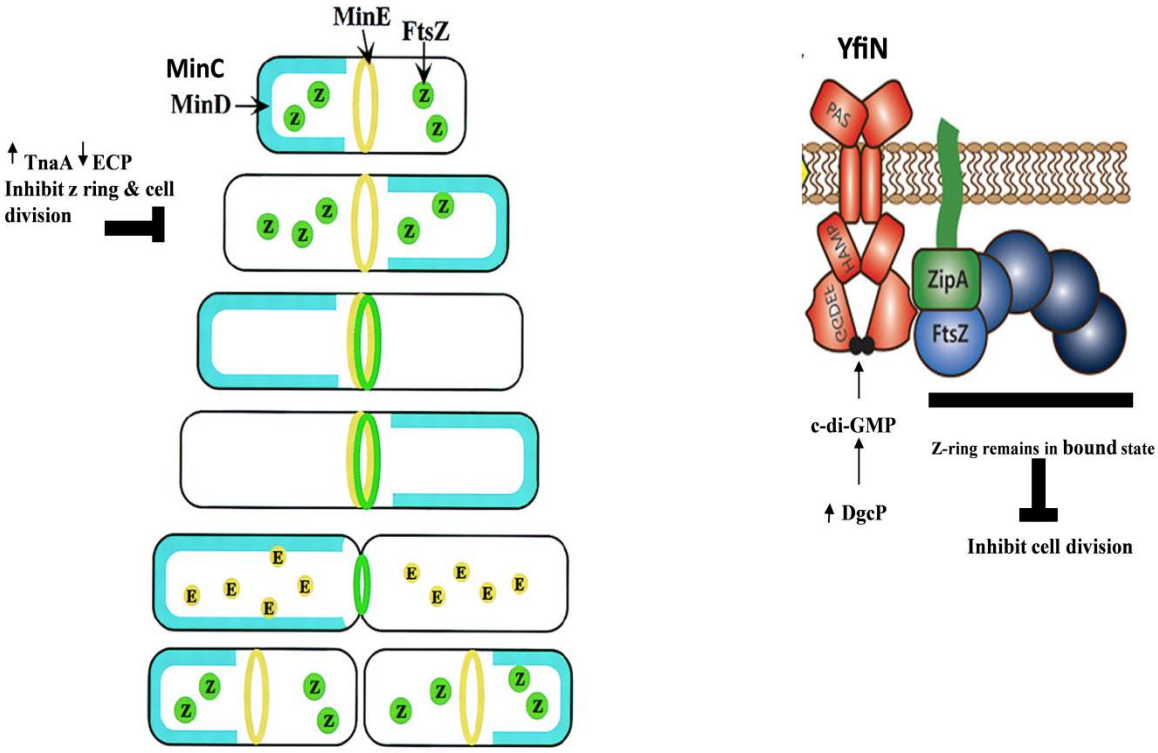
The *dgcP* gene is involved in c-di-GMP formation which is a messenger molecule which can bind with another diguanylate cyclase (YfiN) under stress and exposes its binding sites for FtsZ and ZipA and thus retains the Z ring at the mid cell but stalls cell division by preventing initiation of septal invagination (Figure 36) [90, 110]. FtsZ assembly is the major target of cell division checkpoints during stresses, including DNA damage, defective cell wall synthesis and nutrient starvation [111].

The up-regulation of these genes may retard the cell growth and provide additional time period for proof reading leading to DNA repair. Cell cycle arrest in response to DNA damage is common in eukaryotic and prokaryotic cells [112, 113]. Thus, the proposed mechanism of antimutagenicity may hold true for other mutagens with different mechanism. However, the extent of repair and thus antimutagenicity may vary based upon mutagen. The inhibitors of tryptophanase enzyme like S-phenylbenzoquinone-L-tryptophan, alpha-amino-2-(9,10-anthraquinone)-propanoic acid, L-tryptophane-ethylester, and N-acetyl-L-tryptophan may act as inhibitors of antimutagenicity of ESP [114]. Similarly, inhibitors of diguanylate cyclase such as N-(4-anilinophenyl) benzamide and N-{[(2-phenylethyl) amino] carbonothioyl} benzamide may act as inhibitors of antimutagenicity of ESP [115].

The *rpoH* gene encodes for RNA polymerase sigma factor that initiate the transcription of heat shock genes [116]. Interestingly, this gene has been shown to be link to cell division events [117]. However, its role in antimutagenecity could not be ascertained due to non-existence of its knockout strain.

Due to cell division inhibition, genes like *dps* (protect DNA during starvation), *iptE* (assembly of lipopolysaccharide at the surface of the outer membrane) and *rpsQ* (30S ribosomal protein S17) have been found to be down-regulated in ESP and EMS co-treated *E. coli* cells as compared to EMS alone treated cell. Mechanisms of antimutagenicity differ based upon structure of compound. In our other study, naphthoquinones, plumbagin and juglone were reported to display antimutagenicity by up-regulation of genes like *speE* (spermidine synthase) and *ybaK* (tRNA editing) genes known to be involved in preventing mistranslation (Kumar et al 2013).

Thus, overall expression profile indicated inhibition of cell division as major mechanism of antimutagenicity by ESP. The possible mechanism of antimutagenicity by ESP has been displayed by a model (Figure 37).



A **B**

Figure 36. Role in cell division inhibition of A) TnaA and B) DgcP proteins in bacteria.

Note: Structures taken from Google images (https://mbio.asm.org; http://www.pnas.org). These are required for the basic understanding of the problem addressed in the current study.

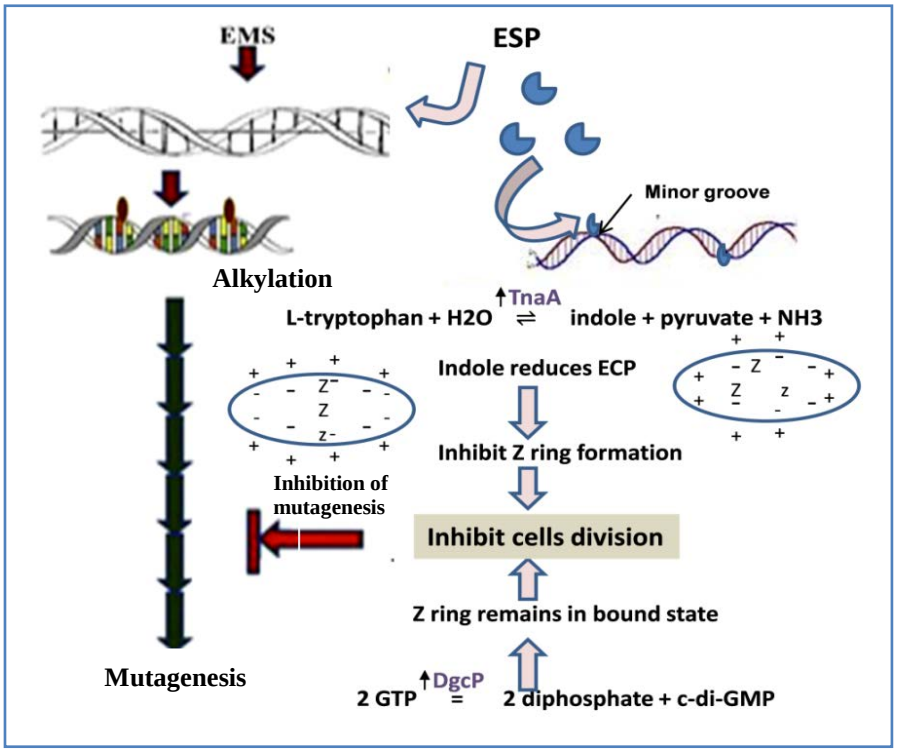


Figure 37. Model explaining mechanism of antimutagenicity by ESP. ESP interacts with minor groove of DNA leading to up-regulation of genes like *tnaA* and *dgcP* which plays important role in cell division inhibition. Retardation of cell division provides additional time for the repair of DNA damage and mutagenesis.

Chapter 5:

Summary and Conclusions

Naphthoquinone rich vegetables such as spinach, lettuce, iceberg lettuce, cabbage, broccoli and French bean were analyzed for antimutagenicity using solvent extracts. Naphthoquinone extract from spinach displayed high antimutagenic activity as compared to other naphthoquinone rich vegetables. The extract was resolved to four different compounds in TLC. A fluorescent compound (C4) displayed highest antimutagenicity and found to have high purity level when analyzed using HPLC. The compound was characterized using biochemical, chromatography and spectral analysis as an 2-ethoxy-3-(3,7,11,15-tetramethyl hexadec-2-ethyl) naphthaquinone-1,4-dione or in short ethoxy-substituted phylloquinone (ESP).

ESP was not found to interact directly to the mutagen. Induction of oxidative stress or its reduction was not found to be the major contributing factor to the observed antimutagenicity. A strong DNA binding activity was observed at minor groove which could be one of the mechanisms for its antimutagenicity as it is often recognized by transcription factors or other cellular DNA targeted proteins and such interaction may lead to change in gene expression profile. The 2-D proteomics profile, mass spectrometry and *E. coli* gene knockout studies confirmed the role of *tnaA* and *dgcP* genes reported to inhibit cell division in observed antimutagenicity of ESP. Retardation of cell division provides additional time for the repair of DNA damage and mutagenesis.

ESP displayed significantly high antimutagenicity as compared to phylloquinone. Here, ethoxy ($-OCH_2CH_3$) was found to be important for antimutagenicity. In case of other structural analogues of naphthoquinones (plumbagin, menadione and Juglone), plumbagin and juglone (having -OH group in their structure) displayed antimutagenicity. This thus indicated that functional groups like $-OCH_2CH_3$/ -OH are

important for antimutagenicity of naphthoquinones. In general, antioxidant capacity of naphthoquinones was found to be quite low. Findings of this study provided fundamental information pertaining to functional and nutraceutical potential of dietary ingredients.

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Purification and Characterization of the Principal Antimutagenic Bioactive as Ethoxy-Substituted Phylloquinone from Spinach (*Spinacea oleracea* L.) Based on Evaluation in Models Including Human Lymphoblast $TK^{+/-}$ Cells

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Supporting Information

ABSTRACT: During in vitro analysis, spinach (*Spinacea oleracea* L.) leaf extracts displayed varying antimutagenicity when analyzed in models including human lymphoblast ($TK^{+/-}$) cell line (thymidine kinase gene mutation assay) and *Escherichia coli* MG1655 (rifampicin resistance assay) against chemically (ethyl methanesulfonate and 5-azacytidine) induced mutagenicity. Highest antimutagenicity was displayed by the quinone extract. The principal bioactive compound exhibited fluorescence in TLC at 366 nm (termed C4) resolved at R_f 0.32 and t_R 15.2 min in TLC and HPLC, respectively. On the TLC plate, three spots (C1−C3), observed at 254 nm, displayed comparatively lesser antimutagenicity. Furthermore, biochemical and spectroscopic analyses using MALDI-TOF MS and NMR indicated the nature of the potent compound (C4) as an ethoxy-substituted phylloquinone derivative [2-ethoxy-3-((*E*)-3,7,11,15-tetramethylhexadec-2-enyl)naphthalene-1,4-dione]. The C4 compound did not display any cytotoxicity and hence possesses significant nutraceutical-based intervention possibility to combat the onset of mutation-associated disease(s).

KEYWORDS: *prophylactic, thymidine kinase assay, rifampicin resistance assay, nuclear magnetic resonance, quinone*

INTRODUCTION

Quite often living organisms are exposed to various genotoxic agents from industrial sources, biocides (insecticides, herbicides, and pesticides), and natural biotic (viruses, toxigenic microbes including fungi) or abiotic (radiation) sources.[1] This may result in increased DNA damage and subsequent mutations that possibly are translated and manifested in chronic diseases such as atherosclerosis, cardiovascular diseases (CVD), and neoplastic inductions.[2,4] Dietary intervention has been postulated to minimize the onset of such diseases mostly due to the occurrence of natural antimutagens.[5−10] As per the World Health Organization (WHO), cancer prevention is closely linked to diet, and around one-third of all cancer deaths are preventable.[1]

Spinach (*Spinacia oleracea*; family Amaranthaceae) is an annual herb, native to central and southwestern Asia. It has the ability to grow throughout the year in diverse locations. Its leaves are consumed worldwide in different food preparations including as vegetables and as an ingredient of salads. It is reported to be one of the most nutritionally adequate foods and can provide ≥20% (100 g fresh weight basis) of the recommended dietary intake (RDI) of minerals (magnesium, manganese, and iron), phylloquinone (vitamin K_1), ascorbic acid (vitamin C), α-tocopherol (vitamin E), carotene (β-carotene and lutein), and folate (vitamin B_9).[11−13] Earlier studies have reviewed the possible benefits of spinach in different health concerns such as eye disorders, viral infections, oxidative stress, and iron deficiency and also in the context of diabetes, cancer, and hepatotoxicity.[14,15] Although spinach has also been illustrated to have antimutagenic activity in some earlier papers, its bioactive phytochemicals have not been isolated and properly characterized.[16−18]

In the current study, lyophilized powder of spinach was solvent extracted and screened for antimutagenic activity using models such as human lymphoblast $TK^{+/-}$ cells [thymidine kinase ($tk^{+/-}$) assay], *Escherichia coli* MG1655 cells [rifampicin resistance (Rif^R) assay], and the Ames test against known chemical mutagens (ethyl methanesulfonate and 5-azacytidine). Mutagenic agents are known to work across living systems irrespective of their organizational complexity, that is, prokaryotic (bacteria) or eukaryotic cells.[19] Later, the extract displaying maximum antimutagenic activity was purified and characterized using various standard analytical techniques.

MATERIALS AND METHODS

Collection of Spinach Plant. Most commonly grown spinach (*S. oleracea* var. Semisavoy) leaves were procured from the agricultural field of Nashik, Maharashtra, India, within a few hours of harvest. The plant material was thoroughly washed three times with tap water to remove any debris and then washed with double-distilled water. The cleaned material was shed dried to remove residual water and weighed using a fine balance before and after lyophilization (Alpha 2-4 freeze-dryer, Martin Christ, Osterode, Germany) to determine its dry weight (g%).

Preparation of Spinach Extracts. *Aqueous and Methanolic Extraction.* One gram of the lyophilized spinach powder was homogenized in 20 mL of Milli-Q water or methanol using a Polytron

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Antimutagenic and antioxidant properties of plumbagin and other naphthoquinones

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ABSTRACT

The structure–function relationships of the naphthoquinone phytochemicals, plumbagin, juglone, and menadione, have been studied with regard to antimutagenic and antioxidant activities. Antimutagenicity of these compounds was assessed by the Ames test and RNA polymerase B (*rpoB*)-based rifampicin resistance assay. Antioxidant potential was evaluated by radical scavenging assays and reducing power measurement. Protection of cells and DNA against gamma radiation-induced oxidative damage was assayed by survival analysis and gel electrophoresis profiling, respectively. On the 1,4-naphthoquinone nucleus, plumbagin possesses 5-hydroxyl and 2-methyl functional groups, whereas juglone has only the 5-hydroxyl and menadione only the 2-methyl group. Plumbagin showed strong antimutagenic (against ultraviolet and ethyl methanesulfonate) and antioxidant activities, whereas juglone displayed only strong antimutagenic, and menadione only strong antioxidant activities. Thus, these two functional groups (5-OH/2-CH_3) play important roles in the differential bioactivity of naphthoquinones. *Escherichia coli*, microarray analysis showed upregulation of the genes *rep* (replication/repair), *ybaK* (tRNA editing), *speE* (spermidine synthesis), and *yjfC* (glutathionyl spermidine synthesis) by plumbagin or juglone, and *sodC* (superoxide dismutase), *xthA* (oxidative repair), *hycB* (electron carrier between hydrogenase 3 and fumarate dehydrogenase), and *ligA* (formation of phosphodiester bond in DNA) by plumbagin or menadione. Studies with *E. coli* single-gene knockouts showed that *ybaK and speE*, reported to prevent mistranslation, are likely to be involved in the antimutagenicity displayed by juglone, and *sodC* to be involved in the antioxidant activity of menadione.

1. Introduction

Mutations are an important cause of the initiation and progression of many diseases, including atherosclerosis, heart diseases, and cancer. Phytochemicals that reduce mutagenesis may offer preventive potential. Naphthoquinones, such as ubiquinone, plastoquinone, and K vitamins, may have multiple health promoting effects [1]. However, further insight into their mechanisms of action is needed. In this study, three structurally similar naphthoquinones – plumbagin, menadione, and juglone (Fig. 1) – were examined. Plumbagin (5-hydroxyl-2-methyl-1,4-naphthoquinone), a yellow compound isolated from the genus *Plumbago* [2], has anti-inflammatory, anticarcinogenic, immunosuppressive, and anti-atherosclerotic activities [3–5]. Juglone (5-hydroxyl-1,4-naphthoquinone), known in the food industry as natural brown, is isolated from the black walnut (*Juglans nigra*) and other plants of the Juglandaceae family. It has been used as an herbicide, a dye for cloth and inks, and a colouring agent for foods and cosmetics [6]. Menadione (2-methyl-1,4-naphthoquinone), also known as vitamin K_3, is a synthetic compound. The antimutagenic properties of these naphthoquinones against ultraviolet (UV) and ethyl methanesulfonate (EMS) mutagenesis were evaluated by the Ames test and rifampicin resistance assay. The Ames test measures reversion of auxotrophic his$^-$ mutants of *Salmonella typhimurium* and the rifampicin resistance assay measures acquisition of rifampicin resistance by *Escherichia coli* cells due to mutations in the *rpoB* gene, which encodes the β subunit of RNA polymerase [7,8].

Antioxidant activity was determined by quantifying the scavenging activities of free radicals such as DPPH, hydroxyl, and superoxide, and by measuring reducing power. Plasmid DNA protection from gamma radiation-induced oxidative damage was assayed using agarose gel electrophoresis, and its function by measuring transformation efficiency. Protection of *E. coli* cells against oxidative damage upon gamma radiation was also assayed by survival analysis using fluorescence activated cell sorting (FACS),

Abbreviations: Rif^R, rifampicin resistant; his, histidine; PL, plumbagin; JU, juglone; ME, menadione; AP, antimutagenic potential; SR, spontaneous reversion; SPC, standard plate count; aRNA, amplified RNA; cfu, colony-forming units; WT, wild type.

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Review article

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Fruits and Vegetables as Dietary Sources of Antimutagens

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Abstract

Mutation is the process leading to heritable changes in the genetic material of an organism and caused mainly by the external factors, including chemical and physical agents, or can also occur spontaneously due to errors in DNA replication, repair, and recombination. Agents contributing to the mutagenic burden in the environment could be from industrial sources, wide spectrum applications of biocides in the agriculture, and other contaminants. As many of these mutagenic chemicals can induce severe disorders in humans including cancer and a large spectrum of inherited diseases, it is important to detect such mutagenic agents precisely and rapidly, and also look for an approach to combat them. Natural occurring dietary antimutagens primarily from health protective foods such as fruits and vegetables could provide a mechanism to counteract the deleterious effect of these mutagens. The World Health Organization (WHO) indicates that one-third of all cancer deaths are preventable and that diet is closely linked to cancer prevention. These health protective phytochemicals particularly antimutagenic ones could provide an effective solution to these concerns. The current review deals with understanding of the mutagenic events, methods of its analysis and a brief compilation of the existing scientific findings related to the dietary sources having potential to counteract the effects of the mutagenic exposures from different sources. The review would provide an opportunity to look into the science, think about the possible future perspectives and mechanism to translate the outcome of the scientific research for benefits of the mankind.

Keywords

Mutagens, Vegetables, Fruits, Antimutagenicity.

Introduction

Mutation is the process leading to heritable changes in the genetic material of an organism. The mutagenesis is primarily caused by the chemical and physical agents called as mutagens. Additionally, mutations can also occur spontaneously due to errors in DNA replication, repair, and recombination. Some mutagenic events can affect only one or a few nucleotides within a gene and hence called as point mutations. These base pair substitutions i.e. the replacement of one base pair

STRUCTURE BASED VARIATION IN THE ANTIMUTAGENIC/ ANTIOXIDANT PROPERTIES OF DIFFERENT NAPHTHOQUINONES

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Abstract

The structure-function relationships of the naphthoquinone phytochemicals, plumbagin (5-hydroxyl-2-methyl-1,4-naphthoquinone), juglone (5-hydroxyl-1,4-naphthoquinone), and menadione (2-methyl-1,4-naphthoquinone), have been studied. Plumbagin showed strong antimutagenic and antioxidant activities, whereas juglone displayed only strong antimutagenic, and menadione only strong antioxidant activities. Thus, functional groups (5-OH/2-CH_3) seem to play important roles in the differential bioactivity of naphthoquinones. Escherichia coli microarray analysis as well as studies with its single-gene knockouts showed that *ybaK* (tRNA editing) and *speE* (spermidine synthesis), reported to prevent mistranslation, are likely to be involved in the antimutagenicity displayed by juglone, and sodC (superoxide dismutase) to be involved in the antioxidant activity of menadione.

Introduction

Mutation is an important cause of the initiation and progression of many diseases, including atherosclerosis, heart diseases, and cancer. Hence, phytochemicals that reduce mutagenesis may be considered to have health protective potential. Naphthoquinones, such as ubiquinone, plastoquinone, and K vitamins, have already been reported for multiple health promoting effects. However, still in many cases their mechanism of action is not clear. Hence, to address this issue, three structural naphthoquinones analogues - plumbagin, menadione, and juglone were examined for antimutagenic and antioxidant potential. Plumbagin (PL), a yellow compound isolated from the genus Plumbago, has been reported to have anti-inflammatory, anticarcinogenic, immunosuppressive, and anti-atherosclerotic activities [1]. Juglone (JU), known in the food industry as natural brown, is isolated from the black walnut *(Juglans nigra)* and other plants of the Juglandaceae family. It has been used as an herbicide, a dye for cloth and inks, and a colouring agent for foods and cosmetics. Menadione (2-methyl-1,4-naphthoquinone) (ME) is a synthetic compound also known as vitamin K3. On the 1,4-naphthoquinone nucleus, plumbagin possesses 5-hydroxyl and 2-methyl functional groups, whereas juglone has only the 5-hydroxyl and menadione only the 2-methyl group. To further understand the molecular mechanisms contributing to these activities, microarray transcriptome analyses were performed using *E. coli* cells grown in the presence of each of these naphthoquinones, separately. The relevance of certain upregulated genes was tested using *E. coli* knockout mutants.

Material and Methods

Analysis of antimutagenicity

The antimutagenic properties of these naphthoquinones were evaluated by the Ames test and

Structure Based Variation in the Antimutagenic/Antioxidant Properties of Different Naphthoquinones

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Naphthoquinones such as ubiquinone, plastoquinone and K vitamins have several physiological roles in the biological system. The interest in this group of compounds is due to their broad-range of health promoting effects. A variety of naphthoquinone derivatives are reported in vegetables such as spinach, lettuce, broccoli, mustard/turnip green as well as herbals such as walnut, chitrak, garden balsam. In the current study, structure-function relationship in conferring antimutagenic and antioxidant properties to different naphthoquinones has been investigated. For this naphthoquinones, plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone) and its structural analogs, juglone (5-hydroxy-1,4-naphthoquinone) and menadione (2-methyl-1,4-naphthoquinone) were used. Plumbagin displayed broad spectrum antimutagenicity against both ultraviolet and ethyl methanesulfonate and also showed strong antioxidant properties. Surprisingly, its analogue, juglone displayed strong antimutagenic but weak antioxidant property. On the other hand, another analog, menadione displayed strong antioxidant but weak antimutagenic property. Therefore, the two functional groups appear to be important for differential bioactivity of naphthoquinones. Microarray analysis performed with *E. coli* cells grown separately in presence of these compounds showed that genes *rep* (replication/repair), *ybaK* (tRNA editing), *speE* (spermidine synthesis), and *yjfC* (glutathionyl spermidine synthesis) were upregulated in the presence of juglone, whereas, *sodC* (superoxide dismutase), *xthA* (oxidative repair), *hycB* (electron carrier between hydrogenase 3 and fumarate dehydrogenase), and *ligA* (formation of phosthodiester bond in DNA) were upregulated in the presence of menadione. All of these genes were found to be upregulated in presence of plumbagin. Studies with *E. coli* single gene knockouts showed that genes *ybaK and speE*, reported to prevent mistranslation, were likely to be involved in antimutagenicity of juglone and *sodC* was involved in antioxidant activity of menadione. Thus, these studies showed that minor structural variations in a phytochemical can drastically affect its bioactivity as evidence in case of plumbagin and its structural analogues.

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Antimutagenicity of spinach naphthoquinone extract using *Escherichia coli* RNA polymerase B (*rpoB*) based Rif^S to Rif^R (rifampicin sensitive to resistant) forward mutation assay

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Mutations are an important cause of the initiation and progression of many diseases, including atherosclerosis, heart diseases, and cancer. Phytochemicals that reduce mutagenesis may offer preventive potential to these diseases. There are limited reports on antimutagenicity of naphthoquinone extracted from its rich sources such as spinach, lettuce, iceberg lettuce, cabbage, broccoli, and French bean. Thus, antimutagenic activity of naphthoquinone extracts from these plants were analyzed using rifampicin resistance assay which measures acquisition of rifampicin resistance by *Escherichia coli* cells due to mutations in the *rpoB* gene, which encodes the β subunit of RNA polymerase. The antimutagenicity ranged between 1-72% and was found to be highest with naphthoquinone extract from spinach. The naphthoquinone extract from spinach which displayed high antimutagenicity was analyzed for its bioactive compound. HPLC analysis of the naphthoquinone extract revealed a major peak at R_t: 7.3 min. During TLC analysis, a fluorescent compound at R_f: 0.93 was observed. The TLC eluted compound displayed ~58 and 77% antimutagenicity at 1 and 2 mg/ml against ethyl methanesolfonate (EMS) induced mutagenesis, respectively. Biochemical tests for quinone, Fourier transform infrared (FTIR) spectroscopy, and proton nuclear magnetic resonance (NMR) spectroscopy indicated the possible structure of the bioactive compound from spinach as ethoxy substituted phylloquinone derivative.

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