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Antioxidative and pro-oxidative property of *Matricaria chamomilla* L. flower for the variants of deoxyribose degradation

Suchita Srivastava*, Ritesh Kumar and Suaib Luqman

Academy of Scientific and Innovative Research (AcSIR),
CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow-226015, Uttar Pradesh, India
**CSIR-Institute of Microbial Technology, Chandigarh-160036, Punjab, India*

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Abstract

In traditional system of medicine, *Matricaria chamomilla* L. is an important plant and has been used as herbal tea for its antioxidative and ROS scavenging property. The free radicals are one of the fundamental basics for any biochemical processes and are essential moieties governing the aerobic metabolism. The presence of free radicals implicates various diseases and disorders including cancer, diabetes mellitus, atherosclerosis, cardiovascular diseases, cataract, rheumatism, and other autoimmune diseases, *etc*. The antioxidants from natural products have gained utmost importance in treatment of these disastrous diseases and disorders. In our present investigation, we have analyzed the interaction of *M. chamomilla* flower extracts with the auto-oxidation of ascorbate and also determined the auto-oxidative property of the *M. chamomilla* extracts by modifying the deoxyribose degradation assay. Our study also envisaged a comparative concentration-dependent analysis of the aqueous and alcoholic extracts of *M. chamomilla* for the modified deoxyribose degradation assay. The assay system provides insight into the specific antioxidative and pro-oxidative properties of the extracts. In addition, an extensive characterization of the redox properties of the extracts with iron is possible, if iron ions are added in the free form or complexed with EDTA. Furthermore, DNA nicking assay was performed as an expedient method to elucidate DNA damage, through conversion of supercoiled DNA to a nicked DNA, for variants of Fenton reagents. The ethanolic extract of *M. chamomilla* flowers proved to be pro-oxidative in a wider range of milieus than the aqueous extract in both set of experiments. At lower investigated concentration (10-50 µg/ml), both the ethanolic and aqueous extracts protected the DNA damage from hydroxyl radicals formed during the Fenton reaction. Whilst, the ethanolic extract on their higher concentrations (100-250µg/ml), augmented the DNA damage by enhancing the hydroxyl radical formation. The nicking of the supercoiled DNA in the presence of higher concentration of extracts, result in release of topological constraints, leading to the formation of circularized and linear plasmid DNA. This effect of the ethanolic extract could be due to the presence of flavonoids and their glycosides.

Key words : *Matricaria chamomilla* L. antioxidants, 2-Deoxy-D ribose, DNA nicking, ROS, RNS, hydroxyl radical scavenging

1. Introduction

Living organism produces energy, essential for life processes by harnessing oxidation-reduction reaction mechanisms (Valko *et al*., 2007). Among redox reactions, formation of free radical occurs as a part of homeostasis where an electron detaches from a molecule and then reattaches instantly. Failure of free radicals to reattach to the redox molecule or an antioxidant can lead to detrimental effect to the human body, thereby, causing various diseases and disorder (Aust *et al*., 1993; Stohs, 1995; Arouma, 1998). During the evolutionary course of biological processes, multicellular organisms develop numerous enzymatic and non-enzymatic antioxidant ramparts to armor the toxic effects of free radicals encompassing reactive oxygen species (ROS), reactive nitrogen species (RNS), superoxide, hydrogen peroxide, hydroxyl, peroxyl and alkoxyl radicals (Blokhina *et al*., 2003; Acker *et al*., 2006; Bedard and Krause, 2007). Nonetheless, when these cellular antioxidant defences are feeble to completely inactivate the free radicals, there is a cellular loss of antioxidant defenses and a plight of oxidative stress, ensuing oxidative damage to the cell (Halliwell and Gutteridge, 1989). Usually none of the initiators $O_2^{\cdot-}$/ H_2O_2 are strong oxidizing agents and are unable to interact directly with any intracellular target other than iron or iron-contained molecules. Presence of iron in the redox reactions of the biological systems leads to the formation of extremely reactive intermediates through Fenton Chemistry (Galaris *et al*., 2008). Although other metals like copper, catalyzes the synthesis of free radicals even more effectively than iron, due to its wider availability in biological systems as the main catalyst in the living cells (Mello-Filho and Meneghini, 1991; Barbouti *et al*., 2001).

Author for correspondence: Dr. Suaib Luqman
Academy of Scientific and Innovative Research (AcSIR), CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow-226015, Uttar Pradesh India
E-mail: s.luqman@cimap.res.in
Tel.: +91-9450904995; +91-522-2718635
Fax.: +91-522-2716141

The repercussion of the oxidative stress has already been observed and reported as the damage of macromolecules such as nucleic acids, lipids, and proteins due to their chemical reactivity, changes in the membrane potential causing mitochondrial permeability transition and sinking the cellular antioxidants (Cadenas and Davies, 2000; Davies, 2000; Marnett *et al*., 2003). Thus, severely compromising cell health and viability, leading to the development of multiple acute and chronic human ailments, such as atherosclerosis, neurodegeneration, malaria, influenza, down's syndrome, hepatitis, cardiovascular disorders, rheumatoid arthritis, sickle cell disease, diabetes, obesity, ageing and cancer and eventually leading to cell death (Wallace, 1999; Reiter *et al*., 2002; Halliwell and Gutteridge, 2007; Cohen and Tong, 2010; Galasko and Montine, 2010; Pandey *et al*., 2010; Ziech *et al*., 2010). Currently, owing to the traditional therapeutic approach to these catastrophic conditions, use of antioxidant supplements, especially those from natural sources with privileged bioavailability and superior protective efficacy than synthetic antioxidants have been reported (Berger, 2005; Fusco *et al*., 2007; Herrera *et al*., 2009).

Chamomile (*M. chamomilla* L., Asteraceae), one of the most traditionally used medicinal herb often referred to as 'star among medicinal species', has long been recognized in the Ayurvedic and Unani systems of medicine (Mughal *et al*., 1999). Two species of chamomile are generally used in traditional herbalism, *M. chamomilla (Chamomilla recutita*; German chamomile; Hungarian chamomile) and *Chamaemelum nobile* (Roman chamomile). Nearly every part of this plant, including root, bark, gum, leaf, fruit, flowers, seed, and seed oil have been used for various ailments in the indigenous medicine (Odebiyi and Sofowora, 1999). Chamomile traditionally used as an anti-inflammatory, antispasmodic, mild astringent and healing medicine, has been very helpful as an external agent for rapid healing of ulcers and burns without infection, as well as persistent skin problems such as eczema and psoriasis (Pazyar *et al*., 2014; Cvetanovic *et al*., 2015; Mehmood *et al*., 2015). Modern research ascertained the plant for its antibacterial, antifungal, antiviral, analgesic, antioxidant, antipyretic and carminative properties (Nwosu and Okafor, 1995; Ruckmani *et al*., 1998; Guevara *et al*., 1999; Siddhuraju and Becker, 2003; Bajpai *et al*., 2005; Fahey, 2005; Rao *et al*., 2008; Selvakumar and Natarajan, 2008; Mahajan *et al*., 2009; Saadabi and Abu, 2011; Muangnoi *et al*., 2012). The essential oils of chamomile extracted from dried and fresh flowers are used extensively in cosmetics and aromatherapy (Srivastava *et al*., 2010). The chamomile owes its therapeutic activity to different groups of effective substances, which make up the complex effect of the drug. The largest group of its medically important compounds is the essential oils extracted from the floral buds of the plant, primarily composed of terpenoids such as chamazulene, -bisabolol, bisabolol oxides, bisabolon oxide A, farnesene, spathulenol and the cis/ trans-en-in-dicycloethers. The plant contains a high amount of flavone glycosides (apigenin 7-glycoside and its 6'-acetylated derivative); flavonols (luteolin glucosides, quercetin glycosides, and isohamnetin); mucilage polysaccharides; choline; and coumarins (umbelliferone and its methyl ether, herniarin); which again impart this plant their medicinal attributes (McKay and Blumberg, 2006; Farooq *et al*., 2012). Adding to the therapeutic attributes of *M. chamomilla*, we investigated the preventive effect of alcoholic and aqueous extract of flower on site specific and non-site specific degradation of deoxyribose sugar as a measure of hydroxyl radical formation substantiated by DNA nicking assay.

2. Materials and Methods

2.1 Collection of plant material and extract preparation

The flowers of *M. chamomilla* L. were collected from the research farm of CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow, India. The plant was identified and authenticated by the taxonomist of the Institute and a voucher specimen was deposited to the herbarium (9075) for future reference. The collected flowers of chamomile were shade dried for 7 days and extracted at least three times with distilled water and/or absolute ethanol (Luqman and Kumar, 2012a). The prepared extracts (ethanol 3.711% and aqueous 3.47% w/w) were filtered through Whatman No.1 filter paper and then concentrated on a rotary evaporator (Buchi, Flavil, Switzerland) at 45ºC, dried and kept at 4ºC till used for the assay. The extract was dissolved in DMSO (100 mg/ml) and further diluted with distilled water to obtain the working concentrations.

2.2 Reagents, chemicals and biochemicals used in hydroxyl radical scavenging (HRS) assay

Potassium dihydrogen phosphate, Disodium hydrogen phosphate, Thiobarbituric acid (TBA), Trichloroacetic acid (TCA), Dimethyl sulfoxide (DMSO), Deoxyribose, Ascorbic acid, Ethylenediamine tetra acetic acid (EDTA), Ferric Chloride ($FeCl_3$) and Hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich Chemical Co., USA.

2.3 Reaction cocktail for deoxyribose degradation assay

Phosphate buffer (20 mM, pH 7.4): 272.2 mg potassium dihydrogen phosphate and 356 mg disodium hydrogen phosphate dissolved in 100 ml distilled water and pH was adjusted to 7.4. Four different types of reaction cocktails were prepared in phosphate buffer for HRS assay as described previously (Luqman *et al*., 2008; Luqman and Kumar, 2011; 2012b):

A. 3.75 mM deoxyribose (50 mg), 1 mM H_2O_2 (10 µL), 100 µM $FeCl_3$ (1.6 mg), 100 µM ascorbic acid (1.761 mg) dissolved in potassium phosphate buffer. It lacks EDTA in solution.

B. 3.75 mM deoxyribose (50 mg), 1 mM H_2O_2 (10 µL), 100 µM $FeCl_3$ (1.6 mg), 100 µM EDTA (3.7 mg), 100 µM ascorbic acid (1.761 mg) dissolved in potassium phosphate buffer.

C. 3.75 mM deoxyribose (50 mg), 1 mM H_2O_2 (10 µL), 100 µM $FeCl_3$ (1.6 mg) dissolved in potassium phosphate buffer. It lacks both EDTA and ascorbic acid in solution.

D. 3.75 mM deoxyribose (50 mg), 1 mM H_2O_2 (10 µL), 100 µM $FeCl_3$ (1.6 mg), 100 µM EDTA (3.7 mg) dissolved in potassium phosphate buffer. It lacks ascorbic acid in solution.

Thiobarbituric acid (1%) and trichloroacetic acid (2%) solutions were also prepared in double distilled water.

2.4 DNA nicking assay

DNA nicking assay was performed using genomic DNA by the method of Lee *et al*. (2002) with minor modifications. Briefly, pBR322 plasmid DNA (500 ng) in 20 µl of PBS (pH 7.4) was incubated with 50 and 100 µg of *M. chamomilla* flower extract at room temperature for 15 min. After incubation, the samples were treated with 10 µl of four variants of reaction cocktail for Fenton reagents:

Reaction cocktail A : 100 mM H_2O_2, 80 µM $FeCl_3$ and 50 µM ascorbic acid.

Reaction cocktail B : 100 mM H_2O_2, 80 µM $FeCl_3$, 100 µM EDTA and 50 µM ascorbic acid.

Reaction cocktail C : 100 mM H_2O_2 and 80 µM $FeCl_3$.

Reaction cocktail D : 100 mM H_2O_2, 80 µM $FeCl_3$ and 100 µM EDTA.

The samples were then incubated for 45 min. at 37°C temperature in a controlled incubator and analyzed for DNA nicking on 1.0% agarose gel using ethidium bromide dye.

3. Results and Discussion

The concentration-dependent hydroxyl radical scavenging activity of aqueous and alcoholic extracts of *M. chamomilla* flowers was tested by performing *in vitro* deoxyribose degradation assay and observations are presented in Figures (1-4).

$OH^{\cdot}$, a highly reactive oxygen-centered radical, holds an exclusive position among ROS because of its utmost reactivity and oxidative potential towards the physiologically stable compounds. In the midst of only 10^{-9}s of half-life in cells, it begets another radical and attacks all proteins, DNA, PUFA in membranes (Sies, 1993; Stohs, 1995). The redox state of the cell is fundamentally linked to a transition metal redox couple such as iron, copper, chromium, manganese and is maintained inside strict physiological limits. In a biological system, the concentration of free intracellular iron is tightly regulated by existing antioxidative systems; however, *in vivo*, under stress conditions, an excess of superoxide releases 'free iron' from iron-containing molecules such as (4Fe-4S) cluster (Liochev and Fridovich, 1994). The released Fe^{2+} can participate in the Fenton reaction, generating highly reactive hydroxyl radical ($Fe^{2+} + H_2O_2$' $\rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$). Thus, under stress conditions, $O_2^{\bullet-}$ acts as an oxidant of (4Fe-4S) cluster-containing enzymes and facilitates $OH^{\bullet}$ production from H_2O_2 by making Fe^{2+} available for the Fenton reaction (Leonard *et al*., 2004; Valko *et al*., 2005). The superoxide radical participates in the Haber-Weiss reaction ($O_2\bullet$" $+H_2O_2$' $\rightarrow O_2 + \bullet OH + OH^{-}$) which combines a Fenton reaction and the reduction of Fe^{3+} by superoxide, yielding Fe^{2+} and oxygen ($Fe^{3+} + O_2\bullet$" $\rightarrow Fe^{2+} + O_2$) (Liochev and Fridovich, 2002).

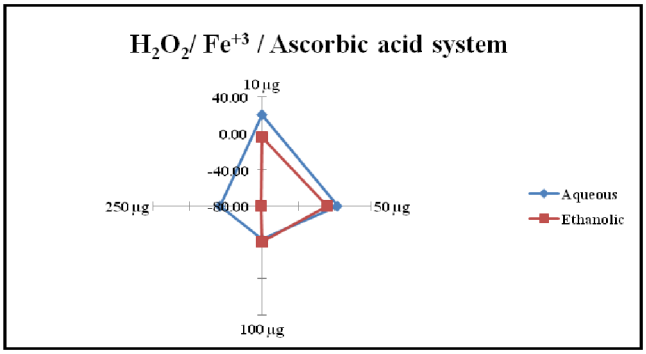
Deoxyribose degradation assay allows the exposure of interactions of the test samples (extracts and / or compounds) with the hydroxyl radicals by following the Fenton Chemistry (Gutteridge, 1984; Aruoma *et al*., 1987; Halliwell *et al*., 1987). The detection molecule deoxyribose is degraded by hydroxyl radical in Fenton reaction with a rate constant of 3.1 x 10^9 $M^{-1}s^{-1}$ and leads to the formation of malonaldehyde, which can be detected spectrophotometrically by the creation of pink chromogen on reaction with 2-thiobarbituric acid. The pro-oxidant and antioxidant effect of plant extracts are due to the balance of two activities: free radical-scavenging activity and reducing power on iron ions, which may drive the Fenton reaction *via* reduction of iron ions (Ling *et al*., 2010). In our present experimental analysis, we prepared four different reaction cocktails to study the effect of aqueous and ethanolic extract in HRS assay system with original and modified Fenton reactions.

3.1 H_2O_2/Fe^{+++}/ascorbic acid reaction cocktail

In this system, the iron ions are complexed with 2-deoxy-D-ribose generating hydroxyl radical in close proximity to 2-deoxy-D-ribose molecule, heading to its degradation. Ascorbic acid initializes the Fenton reaction by reducing the Fe^{+++} and recyclizes it into the system (Aruoma, 1994). The molecules having autooxidation properties compete with the 2-deoxy-D-ribose for Fe^{+++} ions decreasing its degradation that is caused by the iron catalyzed hydroxyl radical attack. In this system, the flavonoid group of molecules present in form of glycosides in the extract chelates the non-bounded Fe^{+++}.

Our results are in concomitant to the hypothesis that the aqueous extract of the chamomile flowers consists mainly of the components rich in flavones, flavonols, glycosides, mucilage polysaccharides, ascorbate and minerals like Mn which on lower concentrations of extract scavenge the hydroxyl radical generated within the Fenton reaction, but augmentation of the concentration increases the small molecular weight phenolics such as quercetin, gallic acid and their glycosides due to their autooxidation in presence of Fe^{+++} aid to the synthesis of hydroxyl radical, conducive of its scavenging activity (Li and Trush, 1994; Hsu *et al*., 2008; Babich *et al*., 2011). This is probably due to the different redox properties of flavonoids-iron complex (Figure 1). As reported earlier, the attachment of methyl, methoxy and glycosides moieties to the polyphenolic flavones and flavonoids decreases its antioxidant property; a result comparable to our observations. The presence of free hydroxyl groups of non-glycosidic flavonols and flavones, coumarins, tannins, terpene alcohols and oxides in the alcoholic extract of chamomile flower shows the similar behavior with the enhancement of hydroxyl radical formation (Figure 1).

Figure 1 : Concentration-dependent hydroxyl radical scavenging effect of extracts of *M. chamomilla* L. in the presence of ascorbic acid



Percent scavenging values are Mean ± SE of three independent experiments in duplicates at each concentration.

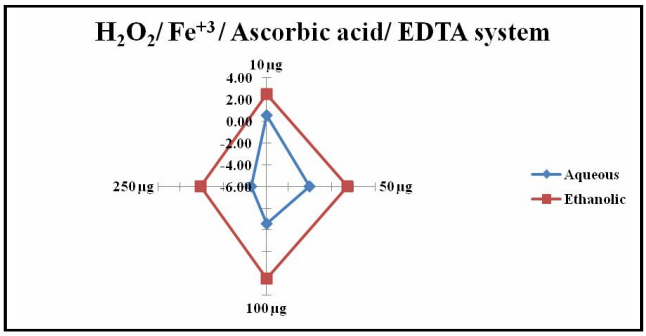
3.2 H_2O_2/Fe^{+++}/ascorbic acid/ EDTA reaction cocktail

In this system, the specific site reaction of Fenton's chemistry in presence of ascorbate has been explored. When there is presence of EDTA, it forms a complex with Fe^{+++} which circumvents the complex formation with the tested substances, 2-deoxy-D-ribose (Gutteridge, 1984) or ascorbic acid (Buettner, 1988), but does not prevent the participation of the iron in the Fenton reaction. According to Gutteridge (1984), redox active scavengers inhibit efficiently 2-deoxy-D-ribose degradation by hydroxyl radicals that were formed in the solution; the iron ions were complexed by EDTA.

The alcoholic extract proved to be better than aqueous as the free hydroxyl moieties of the flavonoids and terpenes are able to check the pro-oxidative nature of ascorbate at lower concentrations. But

with an enhancement in the concentration, again the pro-oxidative nature of the molecules present in the alcoholic extract was observed (Figure 2).

Figure 2: Concentration-dependent hydroxyl radical scavenging effect of extracts of *M. chamomilla* L. in the presence of both EDTA and ascorbic acid

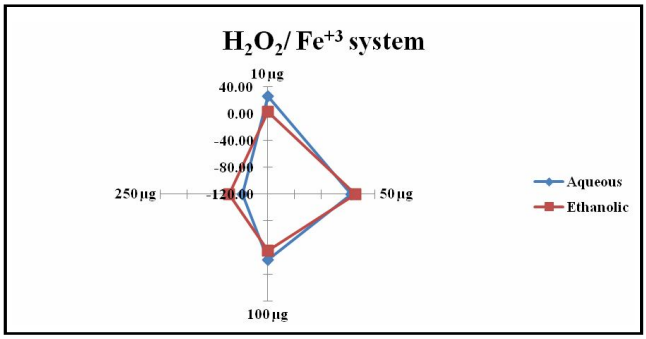


Percent scavenging values are Mean ± SE of three independent experiments in duplicates at each concentration.

3.3 H_2O_2/ Fe^{+++} reaction cocktail

This system explores if the compound is able to substitute the function of ascorbic acid and start the generation of hydroxyl radical through Fenton reaction with the reduction of Fe^{+++}. In the absence of ascorbic acid and EDTA, the components of aqueous extracts were initially able to scavenge the miniature amount of hydroxyl/ superoxide radicals formed by the reduction of iron by H_2O_2. But later, the flavonoid glycosides present in the aqueous extract replaces the activity of ascorbate and start with a speeding activity of hydroxyl radical generation through Fenton chemistry (Chobot, 2010). Thus, a pro-oxidant activity of extracts was observed in concentration-dependent comportment, but the extract proves to be better than alcoholic one with free hydroxyl groups on flavonoid moieties (Figure 3). Apart from occupying free OH groups necessary for hydrogen abstraction and radical scavenging, any sugar substituent is capable of (i) diminishing coplanarity of the B-ring relative to the rest of the flavonoid, and/or (ii) lending hydrophilicity and altering access to lipid peroxyl and alkoxyl radicals during propagation of lipid peroxidation in membranes (Heim *et al*., 2002).

Figure 3: Concentration-dependent hydroxyl radical scavenging effect of extracts of *M. chamomilla* L. in the absence of both EDTA and ascorbic acid

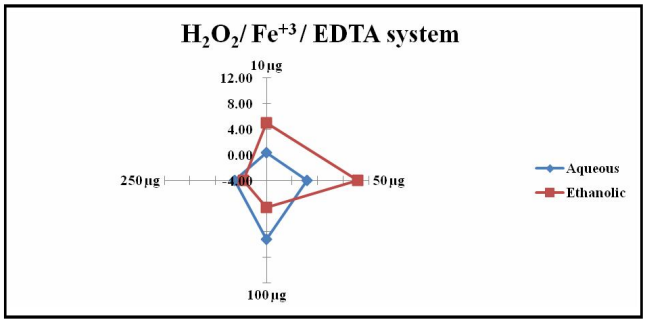


Percent scavenging values are Mean ± SE of three independent experiments in duplicates at each concentration.

3.4 H_2O_2/ Fe^{+++}/ EDTA reaction cocktail

The alcoholic extract in this reaction cocktail variant showed better hydroxyl scavenging property at a concentration of 10 and 50 µg/ mL, but with the increase in the concentration, a strong pro-oxidative effect was observed, in contrary to the aqueous extract which showed rise in hydroxyl radical scavenging activity in a concentration-dependent manner (10-100 µg), while the concentration of 250 µg/ml showed a weak pro-oxidative effect (Figure 4).

Figure 4: Concentration-dependent hydroxyl radical scavenging effect of extracts of *M. chamomilla* L. in the presence of EDTA

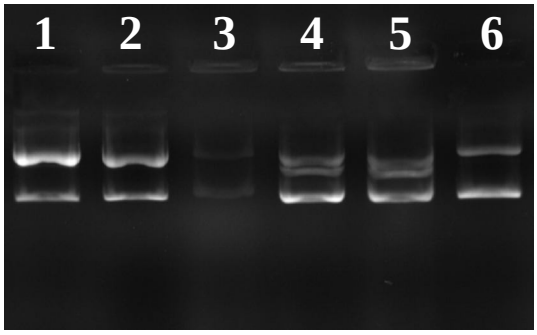


Percent scavenging values are Mean ± SE of three independent experiments in duplicates at each concentration.

3.5 DNA nicking assay

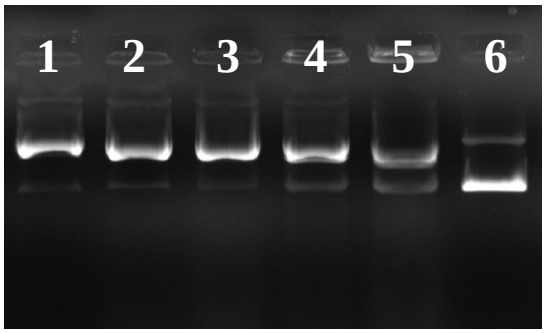
In DNA nicking assay, the same 4 reaction cocktails were checked for the nicking of DNA at concentrations 50 and 100 µg/ ml and our results depict that at lower concentration of the extracts, the DNA nicking was preserved by the extracts, depicting the antioxidative property the extracts, whereas at higher concentration of the extracts, the nicking of DNA supercoiling into relaxed linear form is observed, showing the pro-oxidative effect of the extracts (Figure 5). The ethanolic extract of *M. chamomilla* degraded the DNA content completely, whereas the aqueous extracts show the pro-oxidative effect, by linearizing the supercoil form. The extracts contributed to hydroxyl radical formation in the absence of ascorbate at higher concentrations. The presence of EDTA, in the reaction cocktail leads to the specificity of DNA degradation by the extracts. Our observations were concomitant to the results pragmatic in the case of deoxyribose degradation assay.

Figure 5 : Effect of extracts of *M. chamomilla* L. on DNA nicking among the variants of deoxyribose degradation assay reaction cocktails



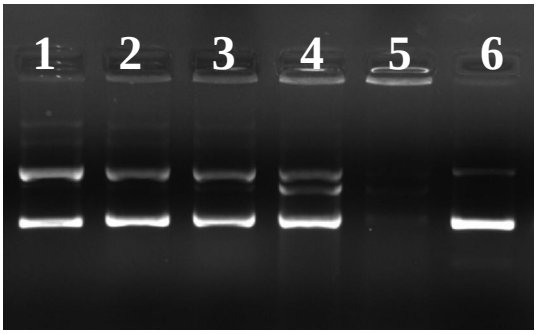
A

Reaction cocktail : H_2O_2 + Fe^{+3} + Ascorbic acid



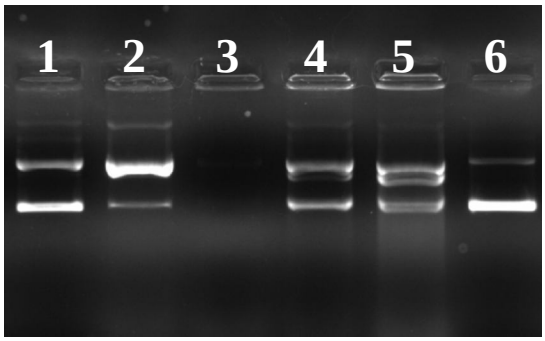
B

Reaction cocktail : H_2O_2 + Fe^{+3} + Ascorbic acid + EDTA



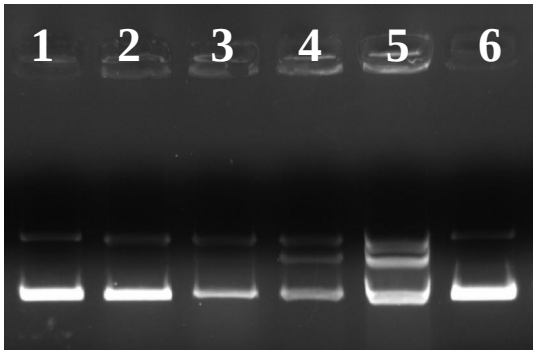
C

Reaction cocktail : H_2O_2 + Fe^{+3}



D

Reaction cocktail : H_2O_2 + Fe^{+3} + EDTA



E

Reaction cocktail : H_2O_2

Lane 1 : pBR322 DNA+ Reaction cocktail;

Lane 2 : pBR322 DNA+ Reaction cocktail + Ethanolic extract of *M. chamomilla* flower (50 µg);

Lane 3 : pBR322 DNA+ Reaction cocktail + Ethanolic extract of *M. chamomilla* flower (100 µg);

Lane 4 : pBR322 DNA+ Reaction cocktail + Aqueous extract of *M. chamomilla* flower (50 µg);

Lane 5 : pBR322 DNA+ Reaction cocktail + Aqueous extract of *M. chamomilla* flower (100 µg);

Lane 6 : pBR322 Control DNA Reaction cocktail A: H_2O_2 + Fe^{+3} + Ascorbic acid; Reaction cocktail B: H_2O_2 + Fe^{+3} + Ascorbic acid + EDTA; Reaction cocktail C: H_2O_2 + Fe^{+3}; Reaction cocktail D: H_2O_2 + Fe^{+3} + EDTA; Reaction cocktail E: H_2O_2

4. Conclusion

Alcoholic and aqueous extracts of *M. chamomilla* were tested for hydroxyl radical scavenging activity at different concentrations (10-250 µg/mL), using various variants of deoxyribose degradation assay substantiated by DNA nicking assay. Our results suggest that both the aqueous and alcoholic extracts of *M. chamomilla* flowers show antioxidative property at lower concentration but as the concentration increases the extracts depicts a pro-oxidative effect which may be due to their high concentration of flavonol glycosides in the extract. Our findings also infer that the concentration of polyphenolics and the flavonol glycosides was more in alcoholic extract.

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Conflict of interest

We declare that we have no conflict of interest.

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