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Spectrophotometric Studies On The Interaction Of *Synapis alba* Extract With Salmon milt DNA.

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ABSTRACT

Synapis alba commonly known as White Mustard belongs to the family Brassicaceae. These plants are indigenous to Europe. It is an annual plant and flowering occurs from June to August. The White mustard seed is about 2mm in diameter, globular, testa yellowish, finely pitted, hard, embryo oily. Oxidative DNA damage is an inevitable consequence of cellular metabolism, with a propensity for increased levels following toxic insult. The present work has been carried out to evaluate the extent of oxidative DNA damage repair by *Synapis alba* aqueous extract. UV- spectrophotometric method was used to analyze the extent of DNA protection and DNA repair. The result indicated that Salmon milt DNA on induced oxidative stress can be protected more than 50% when treated with *Synapis alba* aqueous extract. A time based spectrophotometric study was also carried out and it was found that with the increase in the time of reaction, the aqueous extract of *Synapis alba* proved to be very efficient in repairing oxidatively damaged DNA indicating more than 90% of DNA repair. Electrophoresis was carried out to visually prove the DNA protection by *Synapis alba* and also to show that the antioxidant properties of *Synapis alba* were similar and comparable to that of the standard antioxidant taken. The result indicated that Salmon milt DNA on induced oxidative stress can be protected more than 50% when treated with *Synapis alba* aqueous extract. The results of the present work suggest that the seeds of *Synapis alba* possess potent antioxidant property which is a good source of natural antioxidant which can be used for a potent antioxidant drug.

Keywords: Antioxidant, *Synapis alba*, Oxidative DNA Damage, Salmon milt DNA.

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INTRODUCTION

Synapis alba is commonly known as White Mustard. It is sometimes referred to as *Brassica alba* or *B. hirta.* It is an annual plant, with a thinly hirsute stem, 2 to 5 feet high, which belongs to the family Brassicaceae [1]. It is believed that mustard was first cultivated in India around 3000B.C.White mustard seeds are hard round seeds, usually around 1.0 to 1.5 mm in diameter, with a color ranging from beige or yellow to light brown [2]. Mustard seeds generally take three to ten days to germinate if placed under conditions like cold atmosphere and relatively moist soil. Mustard seed is a rich source of oil and protein. The seed has oil as high as 46-48%, and whole seed meal has 43.6% protein [3]. The entire seed is employed in medicine as the seeds have the following medicinal uses: antibacterial, antifungal, appetizer, carminative, diaphoretic, digestive, diuretic, emetic, expectorant and stimulant [4][5][6][7]. It is used in small quantities, internally, as a condiment and mild but efficient excitant of the organs of digestion. It helps to regulate irregular heartbeat, relieve respiratory congestion, cholesterol and blood sugar levels because of its magnesium content [8][9][10[11]. It is also used in the treatment of respiratory infections, arthritic joints, chilblains and skin eruptions etc. The seed has an inhibitory action on the growth of fungus [12].

An antioxidant is a molecule which inhibits the oxidation reaction caused by free radicals. Oxidation is a reaction involving production of free radicals which are produced during various metabolic cellular process which may lead to damage or death to the cell. Free radicals are also called as Reactive Oxygen Species (ROS) or Active Oxygen species (AOS). ROS include free radicals such as hydroxyl radicals, superoxide ions, nitric oxide, alkyl oxide, alkyl carboxylic acid and non-free radicals such as hydrogen peroxide, singlet oxygen and hypochloride radicals [13].

Hyper-production of ROS develops oxidative stress. Oxidative stress causes damage to cell structure and cell function by overly reactive oxygen-containing molecules and chronic excessive inflammation. Oxidative stress seems to play a significant role in many human diseases such as cardiovascular diseases, diabetes, fatty liver, rheumatoid arthritis, epilepsy, arteriosclerosis, Alzheimer disease, Parkinson's disease, tissue injury and cancer [14].

At the molecular level especially in aerobic cells, these ROS are generated as a by-product of normal mitochondrial activity. If not controlled causes damage to cellular macromolecules, especially the DNA. The extent of the DNA damage determines cell fate, cell cycle arrest and DNA repair or the activation of apoptotic pathways. Oxidative damage in DNA is repaired primarily via the base excision repair pathway which appears to be the simplest of the three excision repair pathways. To allow time for DNA repair, the cells activate their cell checkpoint, leading to cell cycle arrest and preventing the replication of the damage and defective DNA [15].

Salmon milt is a waste product of marine industries, it consists of 10% (w/w) DNA which can be readily extracted and purified in the form of lyophilized powder [16]. One of the remarkable significance seen is its resistance to pH and temperature. Our current study intends to find the oxidative stress induced DNA damage reduction by the source - *Synapis alba.*

MATERIALS AND METHODS

Plant Source

Pure yellow mustard seeds obtained from Punjab (India) was used for all extraction experiments.

It was identified and authenticated by experts from Botanical Survey of India, Pune. The dried seeds were stored in airtight containers until further use.

Chemicals

The chemicals used were agarose (AR), ascorbic acid, bromophenol blue (AR), ethanol, ethidium bromide, ferrous sulphate, ferric chloride, gallic acid, potassium ferricyanide, trichloroacetic acid, glycerol and methanol were purchased from Sisco Research Laboratories, Mumbai, India. Molecular biology grade Salmon milt DNA was obtained from Sigma Aldrich.

Preparation of Aqueous Extract

1%, 5%, 10% extracts were prepared by homogenizing the seeds in reverse osmosis water. It was homogenized for half an hour followed by filtration [17][18]. The filtrate was subjected to centrifugation at 8000 rpm for 10 minutes at 4˚C. The extract obtained was stored in airtight containers at 4˚C until use.

Estimation of Total Antioxidant Activity

The total antioxidant content of the *Synapis alba* extract was determined by using Ferric reducing power assay by the method of Hinneburg et al, 2006 with slight modifications [19]. Gallic acid was used as a reference standard for plotting calibration curve. The reaction mixture consists of 1mL of the aqueous extract mixed with 0.5mL of Potassium Ferricyanide (1.0%). The tubes were incubated at 50˚C for 20 minutes in the hot air oven, 0.5ml of 10% Trichloro acetic acid was added to the mixture and centrifuged for 10 minutes at 6000rpm. The pellet was discarded. To the supernatant 0.1ml of water and 0.1ml of Ferric chloride (0.1%) was added. The absorbance of the resulting blue color was measured at 700nm using a colorimeter. The total antioxidant contents were determined from the linear equation of a standard curve prepared with Gallic acid. The content of total antioxidant compounds expressed as mg/g Gallic acid equivalent (GAE) of dry extract.

Detection of DNA Protection by *Synapis alba* Using UV-Spectrophotometer

The level of Salmon milt DNA protection by *Synapis alba* was compared to the standard antioxidant (Ascorbic acid) using DNA Spectroscopy. For the reaction mixtures the standard DNA (0.2mg/ml), Ethidium Bromide (1mg/ml), the standard antioxidant-Ascorbic Acid (100mM) and the standard oxidant-Ferrous Sulphate (10mM) were taken. The absorbance of EtBr from 365 to 380nm was taken with the help of UV Spectrophotometer using distilled water as the reference. Different standard reaction mixtures were prepared as follows and incubated for 15 minutes and the absorbance readings were taken from 365 to 380nm. The first test tube contained DNA plus Ethidium Bromide(EtBr), second test tube contained DNA plus Oxidant plus EtBr, third test tube contained DNA plus Antioxidant plus EtBr, fourth test tube contained DNA plus Antioxidant plus Oxidant plus EtBr and fifth test contained DNA plus Oxidant plus Antioxidant plus EtBr. Different sample reaction mixtures were also prepared as above taking 10, 20, 30, 40, 50µl of 5% aqueous extract.

Detection of DNA Protection by *Synapis alba* Using Agarose Gel Electrophoresis

50ml of 1% agarose was prepared to which 50µl of EtBr was added, the comb was placed and was allowed to solidify in the electrophoresis unit. The level of Salmon milt DNA protection by *Synapis alba* was compared to the standard antioxidant (Ascorbic acid) using agarose gel electrophoresis. For the reaction mixtures the standard DNA (10mg/ml), Ethidium Bromide (1mg/ml), the standard antioxidant-Ascorbic Acid (100mM) and the standard oxidant-Ferrous Sulphate (10mM) were taken. Different reaction mixtures were prepared as follows: DNA, DNA plus Oxidant, DNA plus Antioxidant and DNA plus sample(5%). The reaction was carried out for 15 minutes. It was then loaded into the wells and electrophoresis was carried out at 100mV, 120A, and 1.2 h.m. for 1 hour 15 minutes.

RESULTS AND DICUSSION

Total Antioxidant Content

The total antioxidant content per gram of yellow mustard seeds was found to be 23.92, 30.16, 27.04, 29.12 mg/g respectively for various 1% sample extracts. The 5% sample extracts was found to have maximum antioxidant activity having antioxidant content of 20.8, 24.96, 33.28, 33.28 mg/g respectively. The 10% extracts contained about 15.6, 15.6, 17.68 and 15.6 mg/g respectively. In the *figure 1* the sample extracts -1,2,3,4 correspond to 1% extracts, 5,6,7,8 correspond to 5% extract and 9,10,11,12 correspond to 10% extract respectively.

The antioxidant property of *Synapis alba* may correspond to a protein origin such as glutathione, or even a phenolic origin such as sinapic acid, tannic acid and so on. Similar studies conducted by Yuan H *et al.* showed that mustard seeds which are consumed in considerable amounts by the Japanese people have the longest life expectancy in the world, are known to contain a number of powerful anti-oxidants which enhanced

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the activity of several anti-oxidant enzymes, such as superoxide dismutase (SOD), catalase, and GSH-peroxidase and, moreover, reduced azoxymethane (AOM) -mediated formation of colon adenomas by about 50% [20]. The potential of White mustard as a natural source of the antioxidant alpha-tocopherol has been described by Yusuf MA and Sarin NB [21]. Aqueous extracts of mustard inhibited lipid peroxidation induced by $FeSO_4$ -ascorbate on human erythrocyte membranes was studied by Sujatha R and Srinivas L thus supporting potent antioxidant property of *Synapis alba* [22].

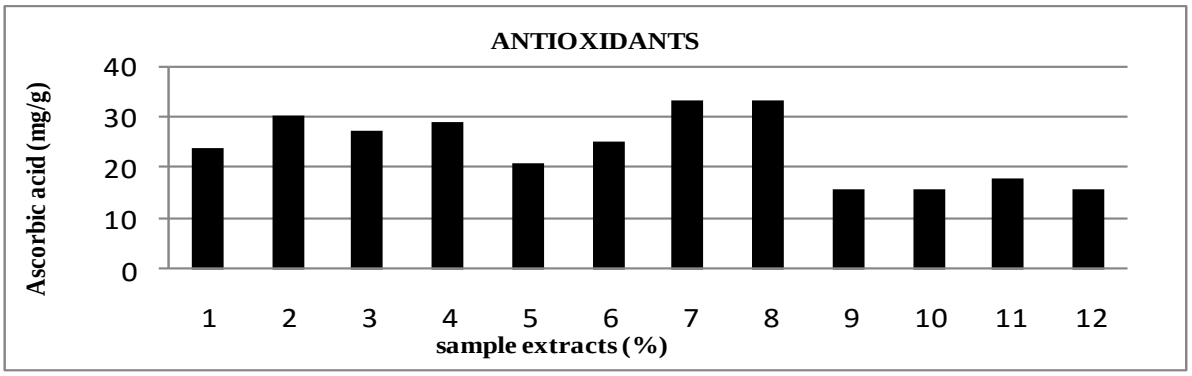

Figure 1: Extraction and estimation of Antioxidants from *Sinapsis alba* (1%, 5%, 10%) using aqueous solvent system

Spectroscopic Analysis of Standard Reaction Mixtures

The spectroscopic readings of the standard reaction mixtures are as seen in the *figure 2*. The graph implies that DNA treated with oxidant showed highest absorbance, this corresponds to the melting of the double stranded DNA which implies that the opening of the double helix DNA strands exposes more Guanine and Cytosine bases to the UV-rays which corresponds to increase in absorbance readings, whereas DNA treated with antioxidant showed very low absorbance which implies that the double helix DNA strands remain intact.

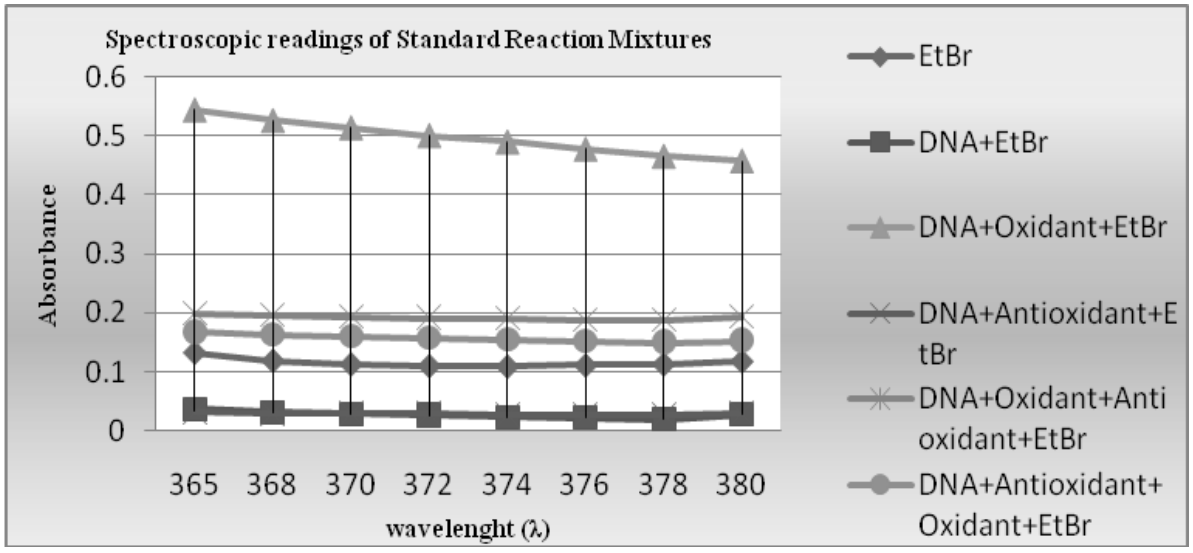

Figure 2 : Spectroscopic analysis of standard reaction mixtures

Despite nearly a quarter of a century of study, and a large number of base- and sugar-derived DNA lesions having been identified, the majority of studies have focused upon the guanine modification, 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-OH-dG). For the most part, the biological significance of other lesions has not, as yet, been investigated. In contrast, the description and characterization of enzyme systems responsible for repairing oxidative DNA base damage is growing rapidly, being the subject of intense study [23].

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Spectroscopic Analysis of DNA Protection by *Synapis alba*

The spectroscopic readings of various aliquots of 5% extract were taken and recorded as in *figure 3*. It was found that 20µl was found to have lowest absorbance readings when compared to the other aliquots - 10µl, 30µl, 40µl and 50µl. The result indicated that 20µl was potent enough to carry out DNA protection over oxidative damage.

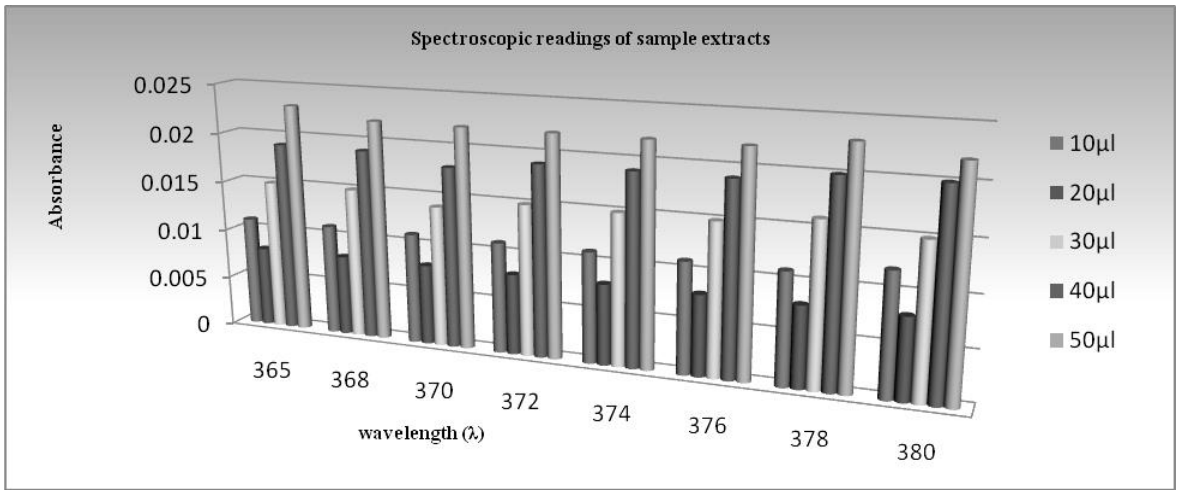

Figure 3: Spectroscopic analysis of sample extracts

The overall spectroscopic readings of the DNA treated with extracts of *Synapis alba* showed absorbance equal to that of DNA treated with the standard antioxidant taken i.e. Ascorbic acid which signifies the potent antioxidant activity and DNA protection by *Synapis alba.* Studies carried out by Uhl M *et al.* showed that Mustard juice was protective against benzo[a]pyrene (B[a]P)-induced DNA damage in human-derived cells in a dose-dependent manner. Chemo-protective properties may be associated with induction of detoxifying enzymes. [24]

Spectroscopic Analysis of DNA Damage Repair by *Synapis alba*

A time based study was carried out and the spectroscopic readings were taken at every 15, 30 and 60 minutes for all the reaction mixtures under study. The spectroscopic readings of DNA treated with oxidant as seen in *figure 4* were thus compared to those of the readings of DNA treated with oxidant and the sample extracts as seen in *figure 5*. It was found that when the sample extract was added the spectroscopic readings gradually decreased with time which signifies the potent DNA damage repair by the source *Synapis alba.* At 15^{th} minute more than 80% DNA damage repair was observed by the drastic decrease in the absorbance readings (i.e. from 0.55 O.D. to 0.025 O.D.) after 60 minutes of incubation it was observed that the absorbance reading drastically decreased to 0.01 O.D. indicating that more than 95% of the oxidatively damaged DNA is repaired. Thus proving that the aqueous extract of *Synapis alba* is an efficient source to combat DNA damage.
The oxidative damage of deoxyribonucleic acid (DNA) has been intensively studied due to its role in the occurrence of some diseases. Electrochemical and spectroscopic studies carried out by Berghian-Grosan C *et al.* showed that the hydrogen peroxide is one of the reactive oxygen species (ROS) which can induce oxidation of DNA bases, sugar lesions or DNA strand breaks [25]. The generation of reactive oxygen species may be both beneficial to cells, performing a function in inter- and intracellular signaling, and detrimental, modifying cellular biomolecules, accumulation of which has been associated with numerous diseases. Of the molecules subject to oxidative modification, DNA has received the greatest attention, with biomarkers of exposure and effect closest to validation. [23]

Oxidative DNA damage and DNA repair may mediate several cellular processes, like replication and transcription, mutagenesis and apoptosis and thus may be important for the organism development as well as its pathogenesis, including cancer. Activity of DNA repair enzymes can depend on many factors, such as gene polymorphism, mRNA and protein level, as well as enzymes activation and inhibition [26]. Diet is thought to influence the incidence of several cancers but it is very difficult to unravel which aspects of diet are important

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[27]. It has been estimated that 30–40% of all kinds of cancer can be prevented with a healthy lifestyle and dietary measures. A low use of fibers, red meat intake, and an imbalance of Omega-3 and Omega-6 fats are known to increase the cancer risk [28]. Antioxidants, vitamins and trace element intakes have been shown to be particularly important in the prevention of cancer [29].

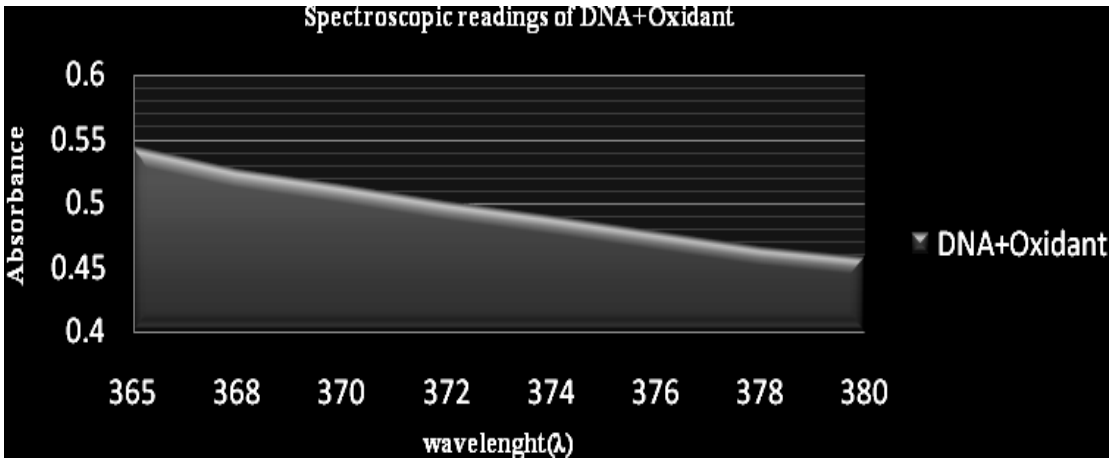

Figure 4 : Spectroscopic analysis of DNA + oxidant

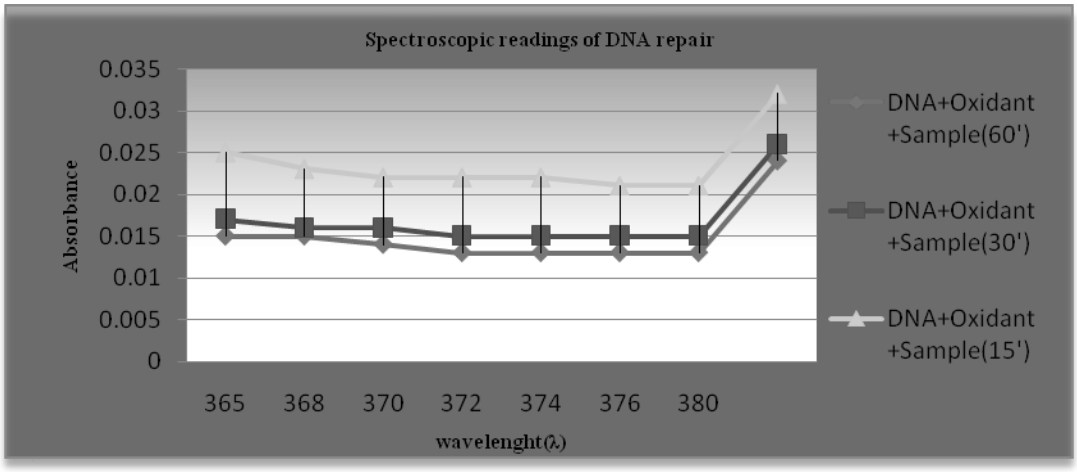

Figure 5 : Spectroscopic analysis of DNA repair

Electrophoresis Confirmation of Oxidative DNA Damage Repair by *Synapis alba*

The DNA bands observed after carrying out electrophoresis is shown in the *figure 6.* It was observed that DNA treated with the oxidant was broken into fragments and the DNA band which was treated with the sample was similar to that of the DNA treated with the standard antioxidant. The above results obtained prove that *Synapis alba* is a potent natural antioxidant which helps in protection and repair of damaged DNA by oxidative stress.

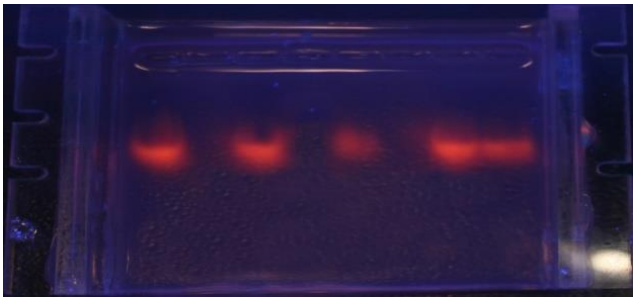

Figure 6 : Electrophoretic bands

Similar results from most observational studies provide support for a protective association of high dietary intakes and/or blood levels of antioxidant vitamins, especially β-Carotene and Vitamin C, on cancer risk and oxidative DNA damage. Several interventions with supplemental doses of antioxidants resulted in significant decrease in endogenous DNA damage. [30][31](Duthie *et al,* 1996 and Brennan *et al,* 2000)

CONCLUSION

In recent years there is great demand for antioxidants from natural sources. Currently, many studies devote to explore and utilize natural antioxidants to remove excessive free radicals in human body, thus realizing the prevention and treatment of many diseases, which are highly correlated with free radicals and cellular redox imbalance. The main aim of this project was to extract potent natural phenolic antioxidant from a simple source such as *Synapis alba* and to evaluate its extent of protection and repair of the oxidative stress on DNA. Oxidative DNA damage is a natural phenomenon which occurs during cellular metabolism, if without a repair mechanism it would result in subsequent harmful consequences in an organism.

According to our current study the seeds of *Synapis alba* have proven to have high antioxidant activity, of which most of the antioxidant compounds belongs to the phenolic group such as p-hydroxybenzoic acid, sinapic acid, sinapine, etc. The antioxidant activities such as the protection and repair mechanism of the DNA by these phenolic groups were determined with the help of spectroscopic analysis and electrophoresis. In the spectroscopic analysis, effective results were obtained which proved the extent of antioxidant activity of our source. In electrophoresis it was virtually observed that the DNA treated with our source was able to prevent DNA damage hence no band fragments were observed.

With the results obtained from various assays we could conclude that *Synapis alba* is a potent natural antioxidant which can be consumed for the treatment of diseases involving free radicals such as protection against cancer, reducing the buildup of atherosclerotic plaque, delaying some effects of aging and decreasing the chance of cataract formation in the lens of the eye and other damages to the DNA and the cellular structures.

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