ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 6, Issue, 6, pp.4421-4435, June, 2015

International Journal of Recent Scientific Research

RESEARCH ARTICLE

PROTECTIVE EFFECT OF SALICIN ISOLATED FROM EGYPTIAN WILLOW LEAVES (*SALIX SUBSERRATA*) AGAINST GAMMA-RADIATION-INDUCED ELECTROPHORETIC AND MOLECULAR CHANGES IN EPIDIDYMAL TISSUE IN RATS

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ARTICLE INFO

Article History:

Received 14th, May, 2015
Received in revised form 23th, May, 2015
Accepted 13th, June, 2015
Published online 28th, June, 2015

Key words:

Gamma irradiation, Rats, Epididymis, Protein pattern, Lipoprotein, Enzyme electrophoresis, DNA pattern.

ABSTRACT

The study aimed to investigate efficiency of salicin which was isolated from willow leaves to resist irradiation effect on electrophoretic protein, lipoprotein, isozymes and genomic DNA patterns in epididymal tissue of male rats. Irradiation caused significant ($P < 0.05$) elevation in the MDA level in the epididymal tissue. Salicin administration reduced the MDA level in all irradiated salicin treated rats. It showed the most suitable antagonistic effect in the irradiated salicin prepost-treated group. Irradiation caused various abnormalities in all electrophoretic patterns (protein, lipoprotein and isozymes). It caused qualitative alterations represented by disappearance of some or all normal bands with appearance of abnormal bands and /or deviation of normal bands to be appeared with another data (Rfs, Mwts and B % values). It caused quantitative alterations represented by changing B % of the bands appeared with normal Rf and Mwts. Salicin administration improved the SI values in protein pattern of all groups except irradiated salicin post-treated group. It could not prevent the qualitative or the quantitative effect of irradiation on lipoprotein pattern of all irradiated salicin treated groups. It showed the highest antagonistic effect against irradiation on electrophoretic esterase pattern of the irradiated salicin pre-treated group (SI = 1.00) and electrophoretic catalase pattern of the irradiated salicin pre-treated and simultaneous treated groups (SI = 0.89). It minimized the qualitative effect of irradiation on the electrophoretic peroxidase pattern of all irradiated salicin treated groups especially in the irradiated salicin prepost-treated (SI = 0.55) and post-treated group (SI = 0.50). At the molecular level, salicin administration showed obvious antagonistic effect against irradiation on the DNA pattern in all irradiated salicin treated groups except irradiated salicin simultaneous treated group (S = 0.17). The study concluded that salicin administration prevented or minimized the mutagenic effect of irradiation at the biochemical, electrophoretic and molecular patterns in the most irradiated salicin treated groups.

INTRODUCTION

Germ cells are killed or damaged within a short time of radiation exposure. Among the long-term side effects of radiation, injury to the reproductive system is of particular concern (Damewood and Grochow, 1986). Disruption of normal cyclic process of spermatogenesis and therefore, impairment of fertility in both animals and man by radiation, has been reported (Jagetia *et al*., 1998).

Irradiation causes damage to living tissue through a series of molecular events. The formation of reactive oxygen species (ROS) as a result of interaction of irradiation with cellular macromolecules is the cause of dysfunction and death, in both normal as well as tumor cells exposed to radiation (Mobbs *et al*., 2011; Moores and Regulla, 2011). The energy exchange between the rays and the targeted molecules leads to changes produced in deoxyribonucleic acid (DNA), lipids, and proteins and then cell inactivation (Burlakova *et al*., 2001 ; Di Pietro *et al*., 2006).

Irradiation causes damage of cells directly by ionizing DNA and other cellular targets and indirectly by effect through ROS (Borek, 2004). It produces oxygen-derived free radicals in tissue environment by mean of water radiolysis (Arora *et al*., 2005) : these include hydroxyl radicals (the most damaging), superoxide anion radicals and other oxidants such as hydrogen peroxide (Konopacka and Rogolinski, 2004). These radicals increase the oxidative stress (OS) which leads to enhancing

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lipid peroxidation as evidenced by increased lipid peroxidation product (MDA) (Di Pietro *et al*., 2006 ; Nwozo *et al*., 2012).
The gamma-rays are absorbed directly by DNA, leading to single or double-strand breaks, base damage, and DNA–DNA or DNA-protein cross-linkages (Zimmermann *et al*., 2001; Eric and Giaccia, 2012). This gives rise to genomic instability and increase the incidence of cancers, cell death, genetic damage and numerous forms of body tissue pathology (Elshazly *et al*., 2012; Rubner *et al*., 2012).

Irradiation caused cytological, genetic, biochemical, physiological, and morphogenetic changes in the cells and tissues (Gunckel and Sparrow, 1961). It causes genomic DNA damage**,** cellular biomacromolecules (Pillai *et al*., 2008), increases hydrogen peroxide accumulation and lipid peroxidation (El-Beltagi *et al*., 2011) and peroxidation of membrane lipids, protein oxidation and gene expression alteration (Pillai *et al*., 2008).

Whole-body irradiation showed significant increase in protein carbonyls by 73% (Smutná *et al*., 2013). The radiation-induced alteration of the protein structure was observed by measuring the changes in the molecular properties of the proteins (Cho and Song, 2000 ; Moon and Song, 2001). It is worthy to note that each protein type has a biological role, due to this role, the DNA secrets enzymes which act as catalysts to produce specific type of protein. Oxidative protein damage could also affect the activity of DNA repair enzymes. Another possible mutagenic effect of ROS involves their attack on lipids, to initiate lipid peroxidation. The peroxides can decompose to a range of mutagenic carbonyl products (Cheeseman, 1993).

Recently, the studies confirmed that catalase (CAT) is an antioxidant enzyme that destroys H_2O_2 which can synthesize a highly reactive OH. On participation of the glutathione redox cycle, GSH together with glutathione peroxidase (GPx) converts H_2O_2 and lipid peroxides to non-harmful products (La Falci *et al*., 2011; Strzezek *et al*., 2012).

Radioprotective agents are compounds that are administered before exposure to ionizing radiation to reduce its damaging effects, including radiation-induced lethality (Stone *et al*., 2004). The radioprotection is possibly a synergistic effect of the phytochemicals present in the herbal extract, rather than any single component (Paul *et al*., 2012). Presence of the potent antioxidants either in free form or bound form in the extract may be responsible for the overall antiradical, antioxidant and radioprotective ability of the plant (Dixit *et al*., 2013).
Although synthetic radioprotectors such as the aminothiols have yielded the highest protective factors, typically they are more toxic (Rades, 2004) than naturally occurring protectors (Weiss *et al*., 2003). In general, the best radioprotective agents also have been reported to result in the highest behavioral toxicity (Landauer *et al*., 2001).

Although the antioxidant activity of the total aqueous extract of willow leaves was much more than salicin alone (Arab and Steck, 2000), it contains high levels of heavy metals including Pb, Al, Fe, Cd, Ni, Zn, Co, Cr and Mn as reported by Aboulthana *et al*. (2011). So, salicin which was the most abundant active ingredient in the willow leaves was extracted and isolated to be under the present study. Salicin is considered as natural aspirin. It is very possible to be digested without side effects in the stomach and kidneys. Scientists believe that this is because salicin is converted to salicylic acid after the stomach has absorbed it (Vane *et al*., 1990). It is a pro-drug that is gradually transported to the lower part of the intestine, hydrolysed to saligenin by intestinal bacteria, and converted to salicylic acid after absorption. It thus produces an antipyretic action without causing gastric injury (Akao *et al*., 2002).

The present study aimed to reveal role of salicin as a radioprotector against effect of gamma irradiation on the epididymal tissue in male rats in the hope that this compound may be further explored as novel antioxidative radioprotector.

MATERIALS AND METHODS

Plant material

Salicin was isolated from the leaves of the willow trees (*Salix subserrata*, *Salix safsaf*) that collected fromOrman garden, Giza, Egypt. This species was well authenticated by qualified specialists in plant taxonomy.Dried fresh leaves were extracted with methanol at the concentration 10 % weight of the fresh leaves per volume of the solvent. Salicin with some derivatives was isolated in n-butanol solvent. The n-butanol extract was subjected to paper chromatography using ethyl acetate: methanol : water (77:13:10) v / v as solvent system according to method describe by Mabry *et al.* (1970). The solution of the material required to be purified was applied on top of the column glass which packed with sephadex LH-20. Elution was started using methanol (50 %) (Kur'yanov *et al*.,1991). The desired compound was visualized during elution using UV lamp and followed till eluted and taken from the column (Nahrsted *et al*., 2007). After separation and purification process, salicin was identified by advanced chromatographic techniques.The aqueous solution of salicin was prepared by dissolving salicin in distilled water to obtain the concentration that used in the experiment.

Acute toxicity test

The safety of salicin orally was evaluated by determination it's LD_{50}. Forty eight adult albino mice weighing 20-25 g was used to study acute toxicity. It was divided into 6 groups each of 8 mice. The groups were treated orally with rising doses of 500, 1000, 2000, 3000, 4000 and 5000 mg/kg body weight of aqueous solution of salicin. Mortality was recorded 24 hrs post treatment. The LD_{50} was calculated according to the equation suggested by Paget and Barnes (1974).

Animals

Seven groups of male rats weighing between 150-200 gm per one obtained from the animal house laboratory of national research centre. Ten rats in each group. All the animals were kept under normal environmental and nutritional conditions. The animal groups were divided as the following : rats were non-irradiated and non-treated with salicin representing control group, rats were non-irradiated but treated with the safe dose of salicin (was about 150 mg / Kg) taking in the consideration weight of each rat representing salicin treated group, rats were irradiated at the dose 7 Gy and non-treated with salicin representing irradiated group, rats were treated with salicin for 15 days followed by irradiation at the 15th day representing

irradiated salicin pre-treated group, rats were treated with salicin for 15 days followed by irradiation at the 15th day then the treatment was continued daily for another 15 days representing irradiated salicin prepost-treated group, rats were irradiated and treated with salicin at the same time of irradiation and continue daily for 15 days representing irradiated salicin simultaneous treated group and rats were irradiated at the same gamma dose then left without treatment for 15 days. At the 15th day, the rats were treated with salicin for another 15 days representing irradiated salicin post-treated group.

Irradiation

The rats were exposed to single dose of 7 Gy delivered at the dose rate of 1.167 Rad / Sec. at Middle Eastern Regional Radioisotope Centre for the Arab Countries, Dokki, Egypt using Cobalt 60 (Co^{60}) as a suitable gamma source.

Lipid peroxidation product

Lipid peroxidation level was measured as thiobarbituric acid reactive substance in homogenate of epididymal tissue according to method of Ohkawa *et al*. (1979).

Statistical Analysis

All the grouped data were statistically evaluated with SPSS/16.00 software. The results were expressed as mean ± SE of studied groups using the analysis of variance test (one-way ANOVA) followed by student's t-test. P values of less than 0.05 were considered to indicate statistical significance. The means of irradiated groups and the salicin treated groups were individually compared with those of control group. The irradiated group was compared with irradiated salicin treated groups.

Electrophoretic protein and lipoprotein patterns

Total protein was determined in the epididymal homogenate according to Bradford, (1976). The sample was mixed with the sample buffer. The protein concentration in each well should be about 70 µg protein. Proteins were separated through polyacrylamide gel electrophoresis (PAGE). Polyacrylamide stock, electrode and gel buffer were prepared according to method suggested by Laemmli, (1970). After electrophoretic separation, the gel was gently removed from the apparatus and put into a staining solution of coomasie brilliant blue for native protein pattern (Hames, 1990) and staining solution of sudan black B for lipoprotein pattern according to method of Chippendale and Beck (1966).

Electrophoretic isozymes

For electrophoretic esterase pattern, native protein gel was stained using certain stain prepared according to the method suggested by Baker and Manwell (1977). It was stained for catalase pattern according to the method described by Siciliano and Shaw (1976) and for peroxidase pattern; the native gel was stained according to the method suggested by Rescigno *et al*. (1997).

DNA assay

The DNA extraction was carried out by using the EZ-10 Spin Column Genomic DNA Minipreps Kit for animal tissues purchased from BIO BASIC INC Co.The PCR reaction was carried out using the PCR kit purchased from Promega with 9 different primers (OPA-04, OPA-05, OPA-07, OPA-10, OPA-11, OPA-12, OPA-14, OPA-15 and OPA-17) purchased from Operon A with melting Temp. 32° C and concentration 100 Pmol. / µl.

The PCR was performed for amplification of the genomic DNAusing DNA thermal cycler (Progeny 30, Techno, Cambridge Ltd. Dux ford Cambridge, UK) and according to method described by Rapley (1998).

Data analysis

The polyacrylamide gel plate was photographed, scanned and then analyzed using Phoretix 1D pro software (Version 12.3). The agarose gel plate was analyzed using Quantity One software (Version 4.6.2).The similarity index (S.I.) compares patterns within, as well as, between irradiated and non-irradiated samples. The similarity values were converted into genetic distance (GD) according the method suggested by Nei and Li (1979).

RESULTS AND DISCUSSIONS

Lipid peroxidation

As compared to control, irradiation caused significant ($P < 0.05$) elevation in the MDA level in the epididymal tissue. Salicin administration showed the ameliorative effect against irradiation by reducing MDA level in all irradiated salicin treated rats. As compared to the irradiated group, it was found that salicin showed the most suitable antagonistic effect against irradiation on epididymis of irradiated salicin prepost-treated group (Fig. 1).

During results of the present study, the MDA level elevated significantly as a result of irradiation in the tissue. This was in accordance with the results obtained by Saada and Azab (2001) that showed that the MDA level increased due to production of ROS associated with increasing lipid peroxidation. ROS are known to attack the highly unsaturated fatty acids of the cell membrane to induce peroxidation reactions which considered a key process in many pathological events and are one ofthe reactions induced by OS (Schinella *et al*., 2002).

The increase in the MDA level might be due to elevation of the intracellular ROS concentration leads subsequently to OS (Maurel *et al*., 2003) and decrease in activity of antioxidant enzymes with possible damage of cellular membranes (El Habit *et al*., 2000 ; Das *et al*., 2012). In addition, Dixit *et al*. (2012) showed that the doses 2, 6 and 10 Gy of irradiation enhanced the MDA level. This may be due to reducing the antioxidant enzymes as superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST) and / or due to imbalance between ROS production and antioxidant defenses at the cellular level (Salomon *et al*., 2013).

Also, Hui *et al.* (1996) demonstrated that the increase in lipid peroxidation was related to the decrease in the biooxidase activities after irradiation. They mentioned that the anion radicals formed by irradiation react with polyunsaturated fatty acids in biological membrane forming lipid peroxides which result in severe damage to cellular membrane, organelles and their associated enzymes.

The current results showed that irradiation enhanced the MDA level in epididymal tissue. This was in accordance with Ourique *et al*. (2013) who stated that irradiation disrupts the prooxidant and antioxidant balance in this tissue. This reduced the epididymal efficiency to produce sperms and hence reducing the fertility potential. The peroxidation reaction in this tissue could lead to the damage of lipid matrix structure in spermatozoa membranes, and could be associated with impaired motility (Aitken *et al*., 2013).

Salicin is phenolic glycoside and characterized by its antioxidant activity in biological systems. The antioxidant activity of this compound refers to their ability to scavenge free radicals (Madrigal-Carballo *et al*., 2009). The authors suggested that the phenolic molecules undergo redox reactions because phenolic hydroxyl groups readily donate hydrogen to reducing agents. The antioxidant activity of salicin fraction refers to the presence of hydroxyl group (Meyer *et al*., 1998).
Salicin hydrolyzes in the gastrointestinal tract to give D-glucose and salicyl alcohol. Upon absorption, salicyl alcohol is oxidized into salicylic acid (Chrubasik and Eisenberg, 2004). Thus in the current study, the effect of salicin was attributed to its hydrolysable form salicylic acid.The effect of salicylic acid was compatible with an antioxidantprofile: it inhibited lipid peroxidation and increasedglutathione synthesis, but did not modify the activitiesof glutathione-related enzymes (De La Cruz *et al*., 2004).

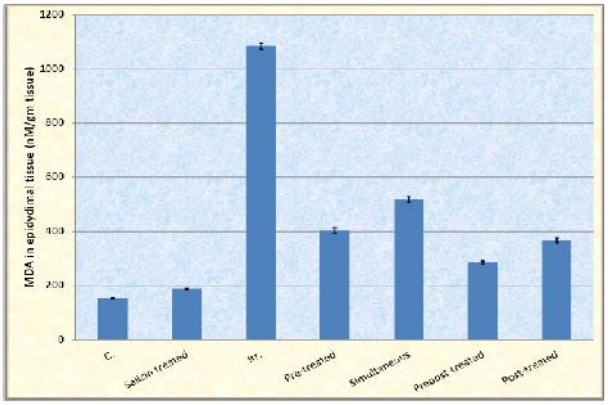

Fig. 1 Effect of irradiation, salicin and their combination at various therapeutic modes on MDA level on epididymal tissue of rats.

Electrophoretic protein pattern

As revealed in table 1 and illustrated in fig 2, protein pattern in control epididymis tissue produced 12 bands with Rfs ranged between 0.08 – 0.88 (Mwts 11.03 – 238.23 KDa and B % values 4.80 -16.89). There were 2 common band appeared in all groups with Rfs 0.44 and 0.82 (Mwts 33.45 and 13.55 KDa and B % values 6.25 and 7.89). Irradiation caused various qualitative mutation represented by disappearance of 5 normal bands and appearance of 2 abnormal bands with Rfs 0.11 and 0.48 (Mwts 215.78 and 28.81 and B % values 6.69 and 7.31). The 2nd band might be deviated to be appeared with Rf 0.18 (Mwt 165.72 and B % 6.35).

It was found that the lowest SI value (SI = 0.40) was recorded with irradiated salicin post-treated group and the highest SI value (SI = 0.73) was recorded with irradiated salicin prepost-treated group. Salicin administration minimized the qualitative alterations occurred as a result of irradiation effect in all irradiated salicin treated groups except irradiated salicn post-treated group as compared to SI value of the irradiated group (SI = 0.57). The epididymal tissue was selected to be under study because the pididymal proteins are very important for the fertility and motility of spermatozoa in all mammalian species (Matoušek, 1985). The current experiment showed that irradiation decreased the ordered structure of proteins. This was in agreement with Moon and Song, (2001) who suggested that radiation caused initial fragmentation of polypeptide chains and, as result, subsequent aggregation and degeneration of proteins.

The difference in the protein fractions separated electrophoretically after irradiation might be due to a rise of protein carbonyl only in the cytoplasm and mitochondria and this was followed by activation of histone – specific proteases in nuclei of the irradiated rats (Pleshakova *et al*., 1998). Irradiation affected protein conformation in the different tissues appeared to depend on several factors, such as protein concentration, the presence of oxygen and an oxygen scavenger, and the quaternary structure of the proteinsresulting in both non-random and random fragmentations (Kempner, 1993). The hydroxy and superoxide anion radicals that are generated by radiation could modify the primary structure of the proteins, which would result in distortions of the secondary and tertiary structures (Davies and Delsignore, 1987) and irreversible changes at the molecular level by breakage of the covalent bonds of the polypeptide chains (Kempner, 1993). The protein fragmentation is affected by the local conformation of an amino acid in the protein, its accessibility to the water radiolysis products, and the primary amino acid sequence (Filali-Mouhim *et al*., 1997). It was reported that irradiation caused aggregation and cross-linking of proteins. Covalent cross linkages are formed between free amino acids and proteins, and between peptides and proteins in solution after irradiation (Garrison, 1987 ; Filali-Mouhim *et al*., 1997). The similarity index between the control and all the irradiated samples and between the irradiated samples themselves recorded low values, indicating to apparent effect of the irradiation and the differences in the protein pattern. It was stated by many previous studies that the irradiation created a great genetic distance between the control and the irradiated samples that may be due to the activation of some genes. These genes produce different types of proteins not produced in the control. These protein types may lead to variation of the different biological processes. The maintenance of normal protein levels after the treatment with salicin may be due to trapping of these free radicals by this compound, thus preventing DNA damage (Sharada *et al*., 2015 ; Abdalla *et al*., 2015).

Table 1 Data of the electrophoretic protein pattern in epididymal tissue of control, irradiated and irradiated salicin treated groups at different therapeutic modes in rats.

Control			Salicin			Irradiated			Irradiated salicin treated												
									Pre-treated			Simultaneous			Prepost-treated			Post-treated		
Rf.	**Mwt**	**B. %**	**Rf.**	**Mwt**	**B. %**	**Rf.**	**Mwt**	**B. %**	**Rf.**	**Mwt**	**B. %**	**Rf.**	**Mwt**	**B. %**	**Rf.**	**Mwt**	**B. %**	**Rf.**	**Mwt**	**B. %**
0.08	238.23	10.86	0.08	238.23	21.47	0.06	251.22	9.17	0.06	248.86	18.75	0.08	238.23	19.11	0.07	246.50	17.57	0.12	209.88	23.88
0.14	193.30	7.17	0.16	178.89	14.32	0.11	215.78	6.69	0.12	207.51	3.32	0.15	182.49	6.44	0.20	147.87	11.88	0.21	137.25	9.93
0.22	131.39	8.60	0.24	115.13	5.75	0.18	165.72	6.35	0.16	174.10	6.25	0.23	129.05	8.52	0.27	99.17	5.57	0.28	89.26	8.08
0.27	93.61	5.76	0.34	54.77	8.15	0.23	129.05	6.56	0.25	110.53	9.94	0.30	78.88	7.47	0.31	70.36	6.90	0.33	59.20	15.62
0.31	68.60	4.80	0.43	35.22	8.48	0.42	36.55	20.41	0.31	72.17	9.30	0.36	48.73	5.80	0.41	38.52	11.55	0.44	33.45	9.27
0.40	39.69	8.27	0.48	28.81	7.11	0.48	28.81	7.31	0.43	35.74	6.39	0.43	35.74	6.78	0.48	28.24	7.12	0.59	19.97	15.87
0.44	33.45	6.25	0.59	19.83	13.44	0.58	20.34	8.96	0.48	27.86	7.24	0.50	25.83	9.11	0.58	20.19	12.88	0.73	16.06	8.47
0.60	19.49	16.89	0.81	13.83	11.13	0.80	14.04	22.77	0.59	19.97	12.69	0.57	20.74	10.67	0.72	16.23	6.73	0.80	14.20	8.89
0.69	16.88	9.86	0.88	11.03	10.14	0.89	10.54	11.78	0.70	16.71	8.30	0.71	16.47	7.63	0.81	13.83	11.46	—	—	—
0.76	15.19	6.05	—	—	—	—	—	—	0.79	14.30	8.84	0.80	13.93	9.25	0.88	11.03	8.34	—	—	—
0.82	13.55	7.89	—	—	—	—	—	—	0.87	11.50	8.99	0.86	11.83	9.23	—	—	—	—	—	—
0.88	11.03	7.60	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Rf. : Rate of Flow, **Mwt. :** Molecular Weight, **B. %** : Band Percent.
Arrangement of the bands at each lane is not correlated with the other bands in the other lanes.

The antioxidative role of salicin may be related to enhancing gene expression of antioxidant enzymes. The recovery and regeneration were faster in the irradiated salicin-treated rats than the irradiated alone. It might be added to the major radioprotectors such as Panax ginseng (Pande *et al*., 1998), Tinospora cordifolia (Jagetia and Baliga, 2002), Podophyllum hexandrum (Samanta *et al*., 2004) and Mentha piperita (Samarth and Samarth, 2009) which reported as radio-protectors against radiation-induced male reproductive dysfunctions for the modulation of testicular injuries after irradiation.

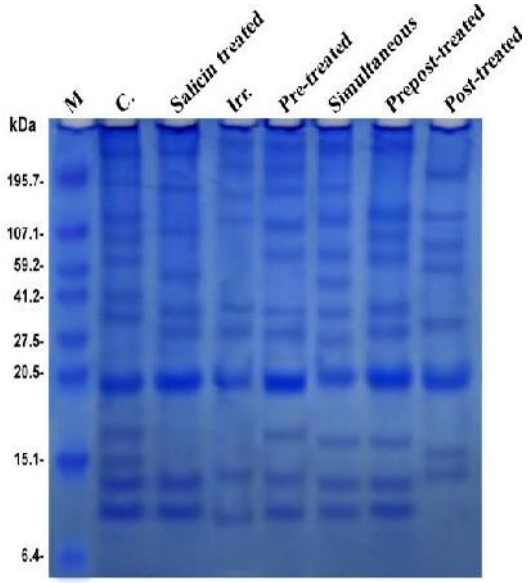

Fig. 2 Electrophoretic pattern showing effect of salicin against the irradiation effect on protein pattern in epididymal tissue of rats.

Electrophoretic lipoprotein pattern

Lipoprotein pattern in control sample produced 4 bands with Rfs 0.06, 0.63, 0.69 and 0.95 (B % 2.44, 71.78, 25.14 and 0.64) respectively. There was only one common band appeared in all groups with R_f 0.95 (B % 0.64). As showed in Table 2 and illustrated in Fig. 3.It was found that the qualitative and quantitative alterations occurred with the same degree in salicin treated and irradiated salicin prepost-treated groups. These alterations were represented by disappearance of 3 normal bands with increasing B % of the normal band appeared with R_f 0.96(B % 100.00).

Irradiation caused alteration represented qualitatively by disappearance of 2 normal bands with appearance of one abnormal bands with R_f 0.75 (B % 18.39) and quantitatively by increasing B % of the other 2 normal bands appeared with R_f 0.07 and 0.96 (B % 78.57 and 3.04).Salicin could not prevent the qualitative and quantitative effects of irradiation in all irradiated salicin treated groups.

From the SI values, it was showed that the lowest SI value (SI = 0.40) was noticed with salicin treated and irradiated salicin prepost-treated groups. While the highest SI (SI = 0.75) was observed with irradiated salicin post-treated group. Values of SI (SI = 0.57) were approximately equal in the irradiated and irradiated salicin pre-treated groups. Salicin minmized that irradiation effect and improved the SI value in the irradiated salicin simultaneous treated and post-treated groups.

Lipoproteins are lipid–protein complexes that contain large insoluble glycerides and cholesterol with a superficial coating of phospholipids and proteins synthesized in the liver (Havel and Kane, 1995). All lipoproteins carry all types of lipid, but in different proportions, so that the density is directly proportional to the protein content and inversely proportional to the lipid content (Bass *et al*., 1993). They were more susceptible to oxidative modifications (Tsumura *et al*., 2001). The ROS can initiate one-electron oxidation or one-electron reduction reactions on numerous biological systems. The oxidative hypothesis classically admits the involvement of the lipoproteins oxidation radiolytically (Bonnefont-Rousselot, 2004).

There was natural binding between protein and lipoproteins in the rat tissues (Fidge, 1986). So, the alterations in the protein pattern were associated with altering the lipoprotein pattern in these tissues. It was well established that ROS has been implicated in tissue dysfunction leading to reproductive disorders (Turner and Lysiak, 2008). The ROS including superoxide anion radical, hydroxyl radical, hydrogen peroxide, nitric oxide and peroxynitrite can cause damage to membranes (lipid peroxidation) and molecular modifications of proteins including protein carbonyl formation, nitration, and covalent modification by lipid aldehydes (Ichikawa *et al*., 1999). Salicin administration showed protective effect against the irradiation. This may be due to its antioxidative effect against attack of the free radicals. It prevented the alterations in the proteins and hence the lipoproteins fractions.

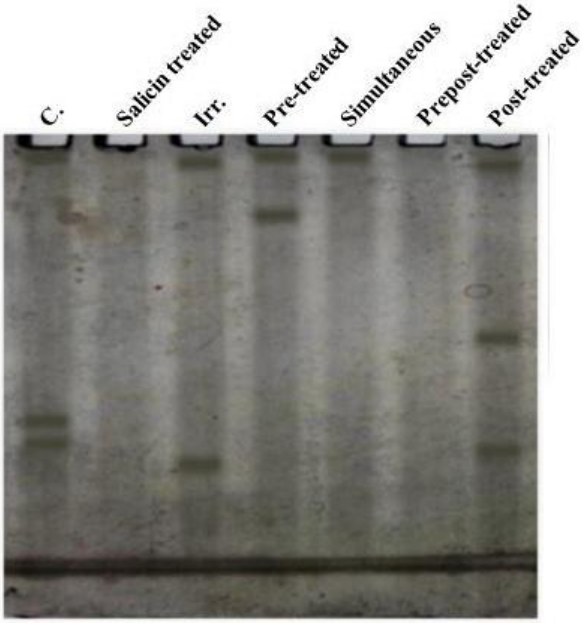

Fig. 3 Electrophoretic pattern showing effect of salicin against the irradiation effect on lipoprotein pattern in epididymal tissue of rats.

Electrophoretic esterase pattern

As shown in Table 3 and illustrated in Fig. 4, it was found that there were 5 types of esterase patternproduced with Rfs ranged between 0.12 - 0.82 (B % values 9.12 – 27.05)in control sample. There were 3 common bands appeared in all the groups with R_{fs} 0.12, 0.31 and 0.54 (B % 25.20, 27.05 and 25.81). Irradiation caused qualitative alterations represented by disappearance of the 3rd and 5th normal bands without appearance of abnormal bands.

Table 2 Data of the electrophoretic lipoprotein pattern in epididymal tissue of control, irradiated and irradiated salicin treated groups in rats.

Control		Salicin		Irradiated		Irradiated salicin treated							
						Pre-treated		Simultaneous		Prepost-treated		Post-treated	
Rf.	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**
0.06	2.44	0.96	100.00	0.07	78.57	0.05	2.14	0.05	96.38	0.95	100.00	0.08	49.04
0.63	71.78	—	—	0.75	18.39	0.19	94.81	0.96	3.62	—	—	0.45	27.21
0.69	25.14	—	—	0.96	3.04	0.96	3.06	—	—	—	—	0.71	20.06
0.95	0.64	—	—	—	—	—	—	—	—	—	—	0.96	3.69

Rf. : Rate of Flow, **B. %** : Band Percent.

Salicin administration prevented the mutagenic effect of irradiation in the irradiated salicin pre-treated group. While in the other irradiated salicin treated groups, it could not prevent the qualitative and quantitative mutagenic effect of irradiation completely.

From the SI values, salicin showed the highest antagonestic effect against irradiationeffect on number and arrangement of the bands in the irradiated salicin pre-treated group (SI = 1.00). It also minmized the irradiation effect in the irradiated salicin simultaneous treated and prepost-treated groups (SI = 0.89). It could not prevent the irradiation effect in the irradiated salicin post-treated group.

During the current study, the epididymal tissue was selected to show irradiation effect on the electrophoretic esterase pattern because the mammalian gonads are a comparatively rich source of these enzymes (Masters and Holmes, 1972). According to results of the present study, irradiation caused electrophoretic alterations in the esterase pattern in the epididymis. This was in agreement with Mikhailov and Torrado (2000) who showed that irradiation caused inhibition in activity and expression of testicular esterases (especially carboxylesterase) which lead to significantly reduction in plasma testosterone concentration. This may lead to lack of puberty growth which correlated with the esterase activity in the gonad.

The carboxylesterases serve as specific proteases involved in the breakdown of bioactive peptides or their precursors (Small *et al*., 1987). They play a vital role in the catabolic pathway (Ajami and Riddiford, 1973) and required to maintain integrity of the male reproductive system. The hyper-expression of carboxylesterases in the male reproductive tract proved to be characteristic of rodents. Their genes were recruited for specific functioning in the male reproductive tract. The functional role of carboxylesterases in the male reproductive organs is apparently determined by their involvement in testosterone biosynthesis and the protection of leydig cells (Mikhailov and Torrado, 2000).

This may refer to effect of irradiation on the protein pattern. As regards changes in electrophoretic mobility demonstrated in the present study, it seemed that free radicals affect the integrity of the polypeptide chain in the protein molecule causing fragmentation of the polypeptide chain due to sulfhydral-mediated cross linking of the labile amino acids. The changes in the fractional activity of different isoenzymes seemed to be correlated with changes in the rate of protein expression secondary to DNA damage initiated by free radicals (El-Zayat, 2007).

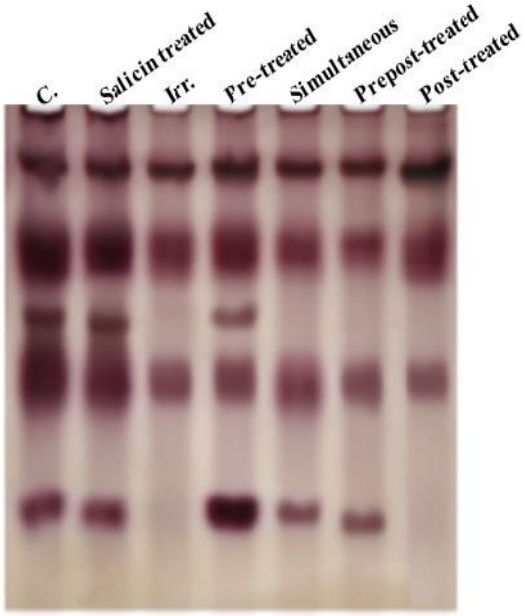

Fig. 4 Electrophoretic pattern showing effect of salicin against the irradiation effect on esterase pattern in epididymal tissue of rats.

Electrophoretic catalase pattern

As compiled in Table 4 and illustrated in Fig. 5, 9 types of catalase enzyme were produced in control sample with R_{fs} ranged between 0.22 - 0.98 (B % 5.93 - 29.99). There was only one common band appeared in all groups with R_{fs} 0.56 (B % 11.87).

Table 3 Data of the electrophoretic esterase pattern in epididymal tissue of control, irradiated and irradiated salicin treated groups in rats.

Control		Salicin		Irradiated		Irradiated salicin treated							
						Pre-treated		Simultaneous		Prepost-treated		Post-treated	
Rf.	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**
0.12	25.20	0.14	26.75	0.14	36.71	0.14	28.25	0.14	33.98	0.14	39.40	0.14	43.12
0.31	27.05	0.30	25.67	0.30	36.86	0.30	26.52	0.31	27.87	0.30	25.65	0.31	37.06
0.43	9.12	0.44	11.08	0.56	26.43	0.43	8.32	0.57	26.03	0.56	20.85	0.56	19.82
0.54	25.81	0.57	24.34	—	—	0.55	18.09	0.83	12.12	0.84	14.10	—	—
0.82	12.82	0.82	12.17	—	—	0.83	18.81	—	—	—	—	—	—

Rf. : Rate of Flow, **B. %** : Band Percent.
Arrangement of the bands at each lane is not correlated with the other bands in the other lanes.

Bed well *et al*. (1989) demonstrated that irradiation caused alterations in the electrophoretic esterase pattern.

Irradiation caused severe qualitative alteration in the catalase pattern represented by disappearance of 4 normal types. Salicin

showed radioprotective effect against irradiation in the irradiated salicin pre-treated and simultaneous treated groups. It could not prevent the irradiation effect completely in the irradiated salicin post-treated groups and it minimized the qualitative mutagenic effect of irradiation leading to appearance of one abnormal unique band appeared with R_f 0.62 (B % 7.49) in the irradiated salicin prepost-treated group.

It was found that the lowest SI value (SI = 0.46) was observed with irradiated and irradiated salicin post-treated groups and the highest value (SI = 0.89) noticed with irradiated salicin pre-treated group. Salicin minimized the irradiation effect in all treated groups except irradiated salicin post-treated group as comapred to irradiated group (SI = 0.46).

Li *et al*. (2007) documented that irradiation caused alterations in the electrophoretic isozymes due to that irradiation-induced ROS markedly alters the physical, chemical and immunologic properties of endogenetic antioxidant enzymes (CAT and GPx), which further increase oxidative damage in cells. The cytotoxic effect of free radicals is deleterious to mammalian cells.

In the present study, irradiation caused severe alterations in electrophoretic CAT pattern. This was in accordance with the results reported by De Freitas *et al*. (2012) who showed that irradiation changes CAT pattern. Salicin administration minimized the alterations in CAT pattern occurred as a result of radiation exposure. This may refer to effect of salicin on stimulation the CAT activity through enhancing expression of the mRNA of catalase (Yeh and Yen, 2003). The increase in the activity of CAT in the irradiated salicin treated groups might be attributed to increased expression of these enzymes as a self-defense mechanism against oxidative stress (Ezz, 2011).

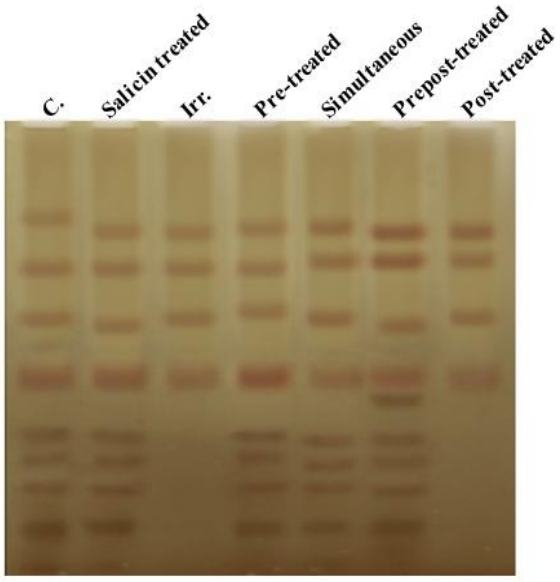

Fig. 5 Electrophoretic pattern showing effect of salicin against the irradiation effect on catalase pattern in epididymal tissue of rats.

Electrophoretic peroxidase pattern

Five types of peroxidase enzyme were produced in control group with R_{fs} ranged between 0.27 - 0.87 (B % 10.14 – 43.58). As revealed in Table5 and illustrated in Fig. 6, there were no common bands. Irradiation causedno quantitative mutations but it caused qualitative alterations represented by deviation of the 1st and 2nd type to be appeared with R_{fs} 0.33 and 0.51 (B % 45.96 and 14.40).Salicin administration could not prevent the abnormalities in the irradiated salicin pre-treated, simultaneous treated and prepost-treated groups. While in the irradiated salicin post-treated group, salicin administration prevented the quantitative mutation but it could not prevent the qualitative abnormalities which were represented by disappearance of the 4th and 5th types with appearance of one abnormal band with R_f 0.37 (B % 75.15).

It was showed that the lowest SI value (SI = 0.2) was observed in irradiated group and the highest value (SI = 0.80) noticed with salicin treated group. As compared to SI of the irradiated group (SI = 0.20), salicin administration minimized the qualitative effect of irradiation in all irradiated salicin treated groups especially. in the irradiated salicin prepost-treated (SI = 0.55) and post-treated group (SI = 0.50).

Peroxidases (GPxs) in the male gonads are attracting much attention (Schneider *et al*., 2009). Indeed, several GPxs have been found to be present on and around epididymal transiting sperm cells and the precise localization of the various GPxs in, on and around sperm cells argues in favor of specific roles for these enzymes. In particular, GPxs could function as H_2O_2 sensors to regulate its concentration and to find a proper balance between the physiological actions of ROS on sperm cells and their detrimental activities on cell physiology (Drevet, 2006). The mature spermatozoa depend on GPxs as a structural protein, to maintain the proper integrity (Chabory *et al*., 2010).

The current study showed that irradiation affected electrophoretic peroxidase pattern.

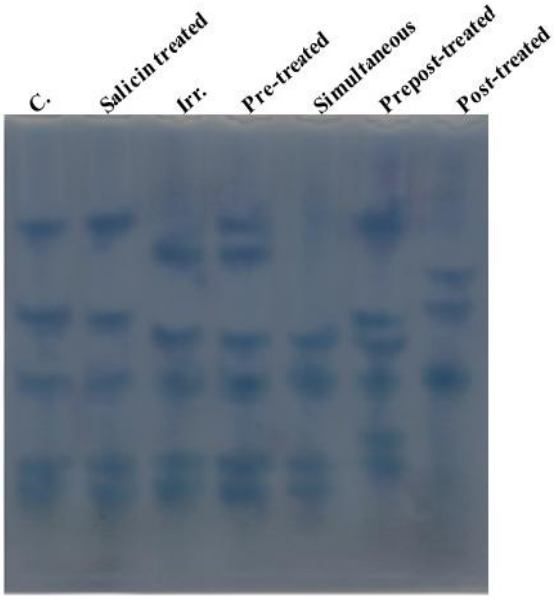

Fig. 6 Electrophoretic pattern showing effect of salicin against the irradiation effect on peroxidase in epididymal tissue of rats

This was in agreement with the study performed by Bhatia and Manda (2004) who reported that irradiation-induced depletion in the level of reduced GSH, as well as GSH peroxidase. This leads to elevation of the hydrogen peroxide and hence generation of the free radicals (Mills, 1960). The disturbances occurred as a result of irradiation effect on the electrophoretic GPx pattern could be ameliorated by salicin administration.

Table 4 Data of the electrophoretic Catalase pattern in epididymal tissue of control, irradiated and irradiated salicin treated groups in rats

Control		Salicin		Irradiated		Irradiated salicin treated							
						Pre-treated		Simultaneous		Prepost-treated		Post-treated	
Rf.	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**
0.22	29.99	0.24	21.58	0.25	54.16	0.24	26.27	0.24	22.26	0.24	31.89	0.25	51.52
0.33	8.85	0.33	7.50	0.33	13.11	0.33	9.71	0.32	6.81	0.32	2.23	0.32	13.38
0.43	7.62	0.46	11.01	0.44	13.96	0.43	10.36	0.43	11.38	0.46	7.34	0.43	11.94
0.56	11.87	0.57	12.32	0.56	18.77	0.56	18.96	0.56	13.06	0.56	9.56	0.56	23.16
0.69	9.42	0.69	11.88	—	—	0.69	6.29	0.71	14.19	0.62	7.49	—	—
0.75	9.16	0.75	6.83	—	—	0.74	5.70	0.75	5.82	0.70	7.15	—	—
0.80	7.08	0.81	7.09	—	—	0.80	5.89	0.81	6.33	0.75	6.25	—	—
0.89	10.07	0.90	10.44	—	—	0.89	6.82	0.89	10.79	0.81	6.63	—	—
0.98	5.93	0.98	11.35	—	—	0.98	10.00	0.97	9.35	0.89	8.48	—	—
—	—	—	—	—	—	—	—	—	—	0.97	12.98	—	—

Rf. : Rate of Flow, **B. %** : Band Percent.

Table 5 Data of the electrophoretic peroxidase pattern in epididymal tissue between control, irradiated and irradiated salicin treated groups in rats

Control		Salicin		Irradiated		Irradiated salicin treated							
						Pre-treated		Simultaneous		Prepost-treated		Post-treated	
Rf.	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**	**Rf.**	**B. %**
0.27	43.58	0.27	43.49	0.33	45.96	0.26	35.81	0.52	72.88	0.26	46.39	0.37	75.15
0.47	16.01	0.47	11.77	0.51	14.40	0.33	10.50	0.61	12.75	0.46	10.55	0.46	15.08
0.61	13.47	0.61	13.91	0.62	13.99	0.52	13.14	0.79	9.70	0.54	11.68	0.61	9.77
0.79	16.80	0.79	12.70	0.78	9.88	0.62	13.48	0.85	4.67	0.61	12.41	—	—
0.87	10.14	0.87	18.14	0.86	15.77	0.80	12.35	—	—	0.73	10.79	—	—
—	—	—	—	—	—	0.86	14.72	—	—	0.79	8.19	—	—

Rf.: Rate of Flow, **B. %** : Band Percent.
Arrangement of the bands at each lane is not correlated with the other bands in the other lanes.

Genomic DNA pattern

As revealed in Table 6 and illustrated in Fig. 7. The data showed that the DNA pattern in control sample produced 10 bands with Rf values ranged between 0.41 – 0.87 (Mwts 800 – 1969 bp, B % 8.23 – 10.86 and quant. 133.05 – 215.07). There were no common or characteristic bands in all groups. As compared to control, irradiation caused severe qualitative alterations with the same degree in irradiated and irradiated salicin simultaneous groups. In these groups, it was observed that all the bands were not matched with all bands of the other groups.

Salicin administration improved the electrophoretic DNA pattern in the irradiated salicin pre-treated, prespost-treated and post-treated groups. Although salicin caused no disappearance or appearance of bands, it could not prevent the irradiation effect on arrangement of the bands in these groups.

As recorded in the tentative Table 7 which showed mean averages of similarity indecies and genetic distances in the DNA electrophoretic pattern for all primers. It was showed that the SI values in the epidydemis tissue were ranged between 0.17 - 0.76. The lowest SI value (S = 0.17) was noticed in the irradiated salicin simultaneous treated and the highest value (SI = 0.76) was noticed in the salicin treated group. As compared to SI value of the irradiated group, salicin administration showed obvious antagonistic effect against irradiation on the DNA pattern in all irradiated salicin treated groups except irradiated salicin simultaneous treated group.

DNA was the primary vital target for cellular inactivation of living systems by irradiation (Pasupathy *et al*., 2001). The present study showed that the DNA alterations were detected electrophoretically.

This was in agreement with Nackerdien *et al*. (1992) who postulated that irradiation caused DNA alterations due to effect of OH radicals which attack DNA. The purine and pyrimidine bases represent the most suitable target to attack of the OH radicals. The OH radicals react very easily with deoxyribose and the bases and cause DNA damage by extracting hydrogen from nucleic acids or reacting with double bonds (Milligan and Ward, 1994).

Kaneko *et al*. (2002) reported that activity of DNA polymerase α was little changed, while DNA polymerases γ and β were induced in the irradiated rats. This indicated that the decline in repair activity leads to the accumulation of oxidative damage and DNA mutations in aged tissues. The DNA repair is the major defense mechanism of cells against DNA damage and its deleterious effects. The major repair strategy is excision repair. It involves excision of the damaged region from DNA, followed by re-synthesis using the complementary undamaged strand as a template. Accumulation of oxidative DNA damage may be related to the decline in repair activity (Kaneko *et al*., 2003). It was found that there was possibility that proteins that bind lesions on DNA act as inhibitors of mutagenesis by directly inhibiting translesion replication. These effects can be explained by the binding of the DNA damage-binding proteins to the damaged site in DNA, forming a physical obstacle to polymerization by the DNA polymerases (Paz-Elizur *et al*., 1997).

Table 6 Data of the genomic DNA pattern in epididymal tissue of control, salicin treated, irradiated and irradiated salicin treated groups at different therapeutic modes in rats.

Control				Salicin				Irradiated				Irradiated salicin treated														
												Pre-treated				Simultaneous				Prepost-treated				Post-treated			
Rf.	BP.	B. %	Quat.	Rf.	BP.	B. %	Quat.	Rf.	BP.	B. %	Quat.	Rf.	BP.	B. %	Quat.	Rf.	BP.	B. %	Quat.	Rf.	BP.	B. %	Quat.	Rf.	BP.	B. %	Quat.
0.41	1969	10.81	203.67	0.41	1959	9.93	211.29	▬	▬	▬	▬	0.41	1969	11.07	217.59	▬	▬	▬	▬	0.50	1687	10.79	231.91	0.42	1939	10.78	230.75
0.44	1880	10.86	215.07	0.44	1869	9.93	221.54	▬	▬	▬	▬	0.49	1700	11.02	230.41	▬	▬	▬	▬	0.53	1606	10.37	226.81	0.49	1700	10.82	240.47
0.50	1694	10.77	203.97	0.50	1694	9.89	215.64	▬	▬	▬	▬	0.59	1412	11.07	232.62	▬	▬	▬	▬	0.59	1424	10.75	230.50	0.54	1593	10.78	240.27
0.58	1462	10.81	200.16	0.59	1437	9.93	216.99	▬	▬	▬	▬	0.62	1343	10.49	195.53	▬	▬	▬	▬	0.62	1343	10.71	218.88	0.60	1394	10.78	230.92
0.64	1284	10.28	202.47	0.61	1355	7.49	190.35	▬	▬	▬	▬	0.72	1082	8.35	167.41	▬	▬	▬	▬	0.67	1215	9.54	207.31	0.66	1238	10.17	209.51
0.67	1222	10.21	209.62	0.65	1276	8.56	200.30	▬	▬	▬	▬	0.76	1000	7.15	143.20	▬	▬	▬	▬	0.69	1149	8.86	169.49	0.71	1107	8.59	172.07
0.68	1171	10.48	159.71	0.67	1200	7.95	190.52	▬	▬	▬	▬	0.79	941	8.15	155.24	▬	▬	▬	▬	0.75	1006	8.34	167.93	0.75	1017	8.17	167.19
0.77	984	8.23	145.07	0.70	1135	8.05	151.78	▬	▬	▬	▬	0.83	862	10.98	197.73	▬	▬	▬	▬	0.78	957	9.27	184.78	0.77	967	8.66	175.96
0.84	844	8.77	133.05	0.84	844	9.67	163.66	▬	▬	▬	▬	0.87	796	10.94	231.28	▬	▬	▬	▬	0.82	876	10.71	219.17	0.81	895	10.61	217.58
0.87	800	8.77	148.59	0.88	784	9.90	213.81	▬	▬	▬	▬	0.90	748	10.78	163.87	▬	▬	▬	▬	0.85	822	10.66	196.45	0.84	839	10.65	196.70
▬	▬	▬	▬	0.91	737	8.71	132.49	▬	▬	▬	▬	▬	▬	▬	▬	▬	▬	▬	▬	▬	▬	▬	▬	▬	▬	▬	▬

Rf. : Rate of Flow, **BP.** : Base Pair, **B. %** : Band Percent, **Quant.** : Quantity
Arrangement of the bands at each lane is not correlated with the other bands in the other lanes.

Table 7 Averages of similarity index (SI) and genetic distance (GD) for the genomic DNA pattern in epididymal tissue between control, salicin, irradiated and irradiated salicin treated groups using all the primers.

			Control	Salicin	Irradiated	Irradiated salicin treated			
						Pre-treated	Simultaneous	Prepost-treated	Post-treated
						SI			
	Control		▬	0.76	0.40	0.55	0.17	0.49	0.50
	Salicin		0.24	▬	0.38	0.58	0.14	0.49	0.47
	Irradiated		0.60	0.62	▬	0.40	0.13	0.34	0.33
Irradiated salicin treated	Pre-treated	GD	0.45	0.42	0.60	▬	0.17	0.53	0.42
	Simultaneous		0.83	0.86	0.87	0.83	▬	0.17	0.19
	Prepost-treated		0.51	0.51	0.66	0.47	0.83	▬	0.59
	Post-treated		0.50	0.53	0.67	0.58	0.81	0.41	▬

The current study showed that irradiation caused severe abnormalities in the DNA pattern in the male reproductive tissues. This was in accordance with Pillai *et al.* (2008) who suggested that the lesions in DNA produced by irradiation include single and double strand breaks, DNA base damage, apyrimidinic / apurinic site formation and inter and intra strand crosslinks and DNA protein crosslinks.

In 2013, Eshak and Osman supported findings of the present study which showed that irradiation increased the DNA fragmentation and the DNA damage leading to genotoxicity. It was reported that radiofrequency radiation induce genetic toxicity (Zotti-Martelli *et al*., 2000). It was known that the membrane lipids are sensitive to the effects of free radicals. While proteins and nucleic acids are more resistant to these detrimental effects. However, DNA molecules can be easily damaged if free radicals are located in an area very close to the DNA molecules (Kayal and Cakatay, 2004).

After using various primers with different sequences, it was showed that DNA was protected by salicin and remains integrated after the exposure to the deleterious effects of irradiation. This idea was supported by Maurya *et al.* (2007) who showed the DNA lesions protected by repairing single-strand breaks induced in DNA and by scavenging oxygen free radicals. It has been shown that salicin is capable of scavenging oxidizing free radicals efficiently. The present study revealed that the widely used this compound showed potent radioprotective effect of salicin to DNA under *in vivo* conditions. Salicin exerts its radio-protective abilities by modulating the activities of radiation-sensitive enzymes. These results may prove useful in developing salicin based radioprotection regime. The data obtained *in vivo* represent a possible strategy to reduce oxidative stress and protect mammalian cells from the damage caused by ROS using a natural compound like salicin.

The effect of the salicylic acid on lipid peroxidation may be explainable by the ability of salicylic acid to absorb hydroxyl ions (Sagone and Husney, 1987) and thus impede a main step in the process of membrane lipid peroxidation. Salicylic acid might spare glutathione stores by avoiding factors that stimulate glutathione depletion. Two observations support this notion: the percentage of oxidized glutathione was reduced, and the activities of enzymes associated with maintaining glutathione levels were not modified substantially (De La Cruz *et al*., 2004). Salicylic acid showed a direct effect on the glutathione system. This effect may be related with the ability of both to react with hydroxyl radicals (Sagone and Husney, 1987 ; Li *et al*., 1999).

On the other hand, Rebouch and Seim (1998) and Ibrahim *et al*. (2007) recorded that salicin might induce elevation in activities of the antioxidants as glutathione peroxidase in these tissues. It might act by improving the turnover of fatty acids peroxidated by the free oxygen radicals during normal metabolism.It might be added to category of the natural products as olive oil, Nigella sativa oil and pomegranate extract which play vital role in male fertility (Aitken *et al*., 2013).

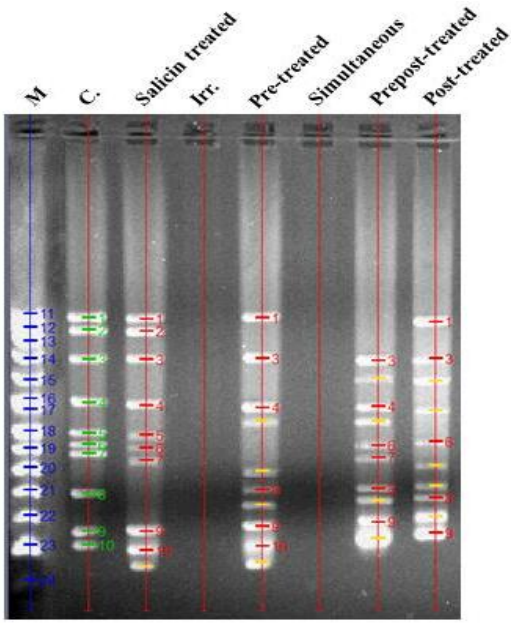

Fig. 7 Genomic DNA pattern showing effect of irradiation on the epididymal tissue and effect of salicin against this irradiation effect.

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How to cite this article:

Ibrahim Abulyazid *et al*., Protective Effect Of Salicin Isolated From Egyptian Willow Leaves (Salix Subserrata) Against Gamma-Radiation-Induced Electrophoretic And Molecular Changes In Epididymal Tissue In Rats. *International Journal of Recent Scientific Research Vol. 6, Issue, 6, pp.4421-4435, June, 2015*

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