

Biological Evaluation of Natural Antioxidants from Residual Sources

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Abstract: This work has been carried out to evaluate biologically the safety utilization of the by- products, namely orange peel and permeate and investigate the antioxidant role of them against the oxidative stress in lipid peroxidation induced in adult albino rats by the oral ingestion of CCL₄. The lipid profile namely total cholesterol, HDL, LDL and triglycerides were performed and the results indicated that the reactive oxygen (RO) and oxidative stress associated with ccl₄- induced hepatotoxicity. In this case the lipid peroxidation level was induced in liver by the increase of MDA level in hepatotoxicity rats after 4 and 8 weeks. The obtained data revealed the safety utilization of both orange peel and permeate. The data also showed the prophylactic effect of reconstituted orange peel and permeate on ccl₄- induced hepatotoxicity treatment after zero, 4 and 8 weeks time intervals. On the other hand after treatment by pre- oral ingestion of both orange peel and permeate for 8 weeks, the data exerted a prophylactic effect against the oxidative stress caused by CCL₄ as indicated by the recovery in the levels of lipid profile and MDA. In addition, histopathological study was performed, similar trend of results were corresponding to the biochemical parameters.

Key words: By- products • Orange peel • Permeate • Antioxidants • CCL₄ • MDA

INTRODUCTION

The oxidative deterioration of fats and oils in foods responsible for rancid odours and flavours, with a consequent decrease in nutritional quality and safety caused by the formation of toxic compounds. on the other hand, oxidative stress is involved in the pathology of cancer and other diseases [1].

Humans are subjected to various foreign chemicals such as drugs, food additives and pollutants. Free radical originating from the metabolism of a number of pollutants can directly attack critical target molecules or attack poly unsaturated fatty acids in membranes and initiate lipid peroxidation and liver necrosis.

Free radicals originating from carbon tetrachloride (CCL₄), can act directly on liver [2, 3].

On the other hand, Reports revealing that synthetic antioxidant such as Butylated hydroxyanisole (BHA) and Butylated hydroxytoluene (BHT) could be toxic, with regard to food additives safety, created a need for identifying alternative natural and probably safer sources of food antioxidants [4].

The replacement of synthetic antioxidants by natural ones may have benefits due to health implications and functionality such as solubility in both oil and water, of interest for emulsions, in food system. Caution regarding an assumption of safety of natural antioxidants has been repeatedly advised.

The growing interest in the substitution of synthetic food antioxidants by natural ones has fostered research on vegetable, fruit and animal sources and the screening of materials for identifying new antioxidants. Milk permeate, which penetrates the membrane during ultra filtration process of milk has been regarded as waste product, although it contains high level of lactose, soluble proteins, vitamins and minerals. However, because of its high nutrient content and its disposal can pose environmental problems, thus milk permeate could be used for the manufacture of some functional foods or in finding a value- added utilization for it [5]. Orange peel, is a part of the orange removed as a waste product when oranges are juiced. A joint study identified a class of compounds isolated from orange peel that show a promise in animal studies as a potent natural

alternative for lowering lipid profile, without the side effect, such as liver diseases of conventional cholesterol-lowering drugs [6].

The compounds, called vitamins A and C, hesperidins and polymethoxylated flavones (PMFS), are increasing linked to health benefits as antioxidants, including protection against cancer, heart diseases and immune deficiency.

The most common (PMFS) are found in the peel orange. They are found in smaller amounts in the juice of orange. Taking (PMFS) as a supplements to meals, desert and beverages could be easier way to lower cholesterol by feeding on citrus food food containing 1% (PMFS) [7]. Therefore, the present study was conducted to investigate the safety utilization of two residues namely, milk permeate and orange peel and evaluate their antioxidant, radical scavenging *in vitro* and their prophylactic effects against ccl_4 toxicity *in vivo*.

MATERIALS AND METHODS

Materials

Raw materials:

- Orange peel : was obtained from Americana Company for Food Chemistry
- Permeate: Fresh buffalo's milk permeate was obtained from Animal Production Research Institute Pilot Unit, Agric. Res. Cent. Dokki, Giza, Egypt.

Chemicals: All chemicals used throughout this work were biochemical grade. Kits for biochemical assay were obtained from Sigma.

Experimental Animals: A total of 32 – adult male albino rats were obtained from animal house of the National Organization for Drug Control and Research (NODCAR), Giza, Egypt.

Methods:

- Chemical analysis of orange peel and milk permeate : moisture, protein, ash, fat and carbohydrate were determined according to A.O.A.C [8].

Determination of Antioxidant Content:

- Determination of total phenols in orange peel and permeate was determined according to Arnous *et al.* [9].
- Determination of total flavonoid (TF) Total flavonoid content in orange peel and permeate was measured according to the method described by Chang *et al.* [10].

Determination of Antioxidants Activities:

- Evaluation of the free radical scavenging capacity In this method, the 2, 2 diphenyl-1-picrylhydrazyl (DPPH) radical was used to measure the antioxidant activity of orange peel and permeate according to Lim *et al.* [11].
- Reducing power (RP) Reducing power for orange peel and permeate according to the method described by Athukorala *et al.* [12].
- Total antioxidant activity. The antioxidant activity of orange peel and permeate was determined by the conjugated diene method [13].

Experiment: A total of 32- adult male albino rats weighting about 120-140g were used in this experiment. Animal were kept under normal laboratory condition in the animal house of NODCAR for 2 weeks before the initiation of the experiment. Negative control rats were fed on basal diet. Rats were randomly divided into 3 groups (8 rats each) and submitted to the following treatments: Group 1: (Positive Control) rats were fed on basal diet and carbon tetrachloride (100mg/kg rats) orally.

Group 2: Rats were fed on basal diet, orange peel extract and CCL_4 (100mg/kg rats) orally.

Group 3: Rats were fed on basal diet, permeate extract and CCL_4 (100mg/kg rats) orally.

Venous blood samples (2ml from each rat) were collected from retro-orbital plexus vein before treatment (zero time), 4 weeks and then after 8 weeks.

Statistical Analysis: The results were statistically analyzed according to statistical analysis system [14] Duncan's at 5% level of significance was used to compare between means according to Sendecor and Cochran [15].

RESULTS AND DISCUSSION

Tables (1 & 2) show the obtained data concerning the composition and antioxidant activity values, respectively of orange peel and permeate extract. Antioxidant activity and scavenging capacity were observed being 87 and 81% in case of orange peel and 90.55 and 1.89% for permeate, respectively. The obtained data evidenced that both by products could be used as a natural antioxidants against the toxic effect of pollutants which may find their way to human.

The preceding data agree with that found by Lancer *et al.* [16] who suggested that UF permeate could be used as a natural antioxidant. Also, Hernandez *et al.* [17] found

Table 1: Proximate composition of antioxidant sources (% dry basis)

Antioxidant source	% Dry basis				
	Moisture	Protein	Fat	Ash	*carbohydrate
Orange peel	8.3±0.40	8.7±2.10	13.0±2.10	3.0±2.10	75.3±2.10
Permeate	3.2±0.10	5.8± 1.40	10.2±0.20	4.0±0.50	80.0± 1.81

*Calculated by difference

Table 2: Antioxidant content and antioxidant activity in different sources

Antioxidant sources	Antioxidant content			Antioxidant activities	
	Total flavonoid mgQE/ml	Total phenols (mg GAE/ml)	Antioxidant activity %	Reducing power O.D	Scavenging capacity %
Orange peel	0.771±0.020	152±3.20	87.51±2.32	2.231±0.20	81±2.49
Permeate	0.004±0.001	2.45±0.20	90.55±0.87	1.89±0.07	1.89±0.07

QE: Quercetin equivalents, GAE: Gallic acid equivalents, O.D: optical density.

Table 3: Effect of orange peel and permeate on the toxicity of CCL₄ on liver function and total protein

Animal groups	Parameters											
	GPT			GOT			Alkaline phosphatase			Total protein		
	0 time	4 weeks	8 weeks	0 time	4 weeks	8 weeks	0 time	4 weeks	8 weeks	0 time	4 weeks	8 weeks
Group 1 (Positive control)	26.5 ±4.4 ^a	59.0±6.1 ^b	104.3 ±8.1 ^a	111.5 ±5.1 ^a	125.0±7.3 ^b	179.0±7.4 ^a	128.4±7.4 ^a	153.0±8.3 ^b	170.3±7.5 ^a	7.08±0.4 ^a	7.95 ±06 ^a	5.30±0.6 ^a
Group 2	30.6 ±4.5 ^b	100.1 ±8.1 ^a	37.8 ±8.3 ^b	120.3 ±4.3 ^a	115.5±4.3 ^a	111.5±5.5 ^a	129.3±8.2 ^b	130.8±6.9 ^b	155.0±8.1 ^a	6.50±0.4 ^b	7.90±0.5 ^a	7.83±0.5 ^a
Group 3	32.1 ±5.2 ^a	29.5 ±6.2 ^a	45.1 ±7.1 ^a	111.1 ±7.1 ^a	114.1±7.4 ^a	117.5±6.7 ^a	128.5±7.4 ^b	124.5±8.8 ^b	141.1±8.0 ^a	7.11±0.5 ^b	7.81±0.5 ^{ab}	8.25±0.6 ^a

In the same column with different superscripted letters are significantly different (p>0.05)

Group 1 (Positive control): Fed on basal diet and carbon tetrachloride

Group 2: Fed on basal diet, orange peel extract and carbon tetrachloride

Group 3: Fed on basal diet, permeate extract and carbon tetrachloride

that they hydrolysate could be suitable as a natural antioxidant. In addition, Kroyer [18] reported that orange peel shows the highest antioxidant activity than peel of lemon and grapefruit. Also, Firas *et al.* [19] reported that orange peel methanolic extract possesses moderate antioxidant activity could be used as natural antioxidant.

Toxicity of CCL₄: Drugs or contaminants are metabolized by the body in much the same way as foods and environmental pollutions, they are broken down by liver and gut enzymes so they can be absorbed and eliminated in the urine [20]. CCL₄ are considered to be one of the group which causes hepatic toxicity and disfunction of this important organ. ccl₄ finds it' way to human body through many routes such as refrigrant, free extinguishes, cleaning fluids, solvent and insecticides [21].

According to the preceding view; the objective of this experiment was to investigate the role of orange peel and permeate as antioxidant to extenuate the toxicity of CCL₄. To achieve this object, two categories were used including biochemical analysis and histopathological examination. CCL₄ dose caused a remarkable increase in the activity of (GPT & GOT) after 8 weeks being 170% for the first enzyme against 54.9% for the second in comparison with the initial group (Table 3).

As regards alkaline phosphatase, it can be observed from Table (3) that this parameter was markedly affected by CCL₄.

Results concerning the effect of CCL₄ on total protein revealed the negative effect of CCL₄ on this parameter, very significant effect was noticed.

The obtained data are in agreement with those of Robins and Kumer [22] who found the CCL₄ poisoning increases liver enzymes and causes necrotic liver. Also, Parola *et al.* [23] and Kanter *et al.* [24] reported that the metabolisms of CCL₄ causes liver necrosis.

The obtained results concerning lipid profile showed that CCL₄ dose (100mg / k.b.w) increases cholesterol level markedly during the course of experiment about 15.8 and 43.5% after 4 and 8 weeks comparing with control, respectively (Table 4). Concerning, HDL cholesterol it could be seen that a remarkable decrease did occur, been 34.3% after 8 weeks compared with control. On the other hand an increase of 25% after 8 week of treatment was noticed. As for triglycerides level (Table 4), a remarkable increase did occur and been 51.5% after 8 weeks in comparison with control.

It can be assumed from these results that the toxicity of CCL₄ during the course of treatments may be attributed to its free radicals which can act on lipid fractions and

Table 4: Effect of orange peel and permeate on the toxicity of CCL₄ on lipid profile

Animal groups	Parameters											
	Cholesterol			HDL			LDL			Triglycerides		
	0 time	4 weeks	8 weeks	0 time	4 weeks	8 weeks	0 time	4 weeks	8 weeks	0 time	4 weeks	8 weeks
Group 1 (Positive control)	57.5±4.4 ^a	67.1 ±5.3 ^b	83.0 ±5.4 ^c	31.3±3.1 ^a	33.3±4.1 ^a	23.3±3.1 ^b	24.0 ±2.1 ^b	27.0±1.4 ^b	83.0±2.4 ^a	99.8±7.4 ^b	140.0±7.3 ^c	150.0±6.5 ^c
Group 2	59.4 ±4.2 ^b	59.1±5.7 ^b	70.0±5.5 ^a	32.2± 4.9 ^a	30.0±3.5 ^a	27.0±4.1 ^a	23.5 ±2.9 ^a	21.0±1.2 ^a	21.6±2.5 ^a	70.5±8.4 ^b	123.6±7.5 ^a	115.0±8.1 ^b
Group 3	59.6 ±3.2 ^b	62.4±4.8 ^b	75.0±4.2 ^a	31.0±3.3 ^a	28.2±3.8 ^a	26.0±4.1 ^a	26.2 ±2.4 ^a	25.0±3.2 ^a	23.2±1.7 ^a	67.5±8.4 ^b	123.0±7.3 ^a	121.0±7.7 ^a

In the same column with different superscripted letters are significantly different (p>0.05)

Group 1 (Positive control): Fed on basal diet and carbon tetrachloride

Group 2: Fed on orange peel extract and carbon tetrachloride

Group 3: Fed on permeate extract and carbon tetrachloride

consequently promotion of lipid peroxidation [25]. The obtained data are agreement with Sherlock [26] who reported that free radicals of CCL₄ can act with the membrane proteins and lipids. According to the preceding view about the toxic effect of CCL₄, it is of interest to study the substances which may suppress its toxicity .i.e. antioxidants the herein study was conducted to clarify the role of orange peel and permeate as antidote against the toxicity of CCL₄.

In this respect oral daily dose (1ml) of both orange peel and permeate separately for 8weeks. At day 14 the rats were orally fed with ccl₄(100mg/kg.b.w) twice weekly until the end of experiments. the toxicity of ccl₄ orange peel and ccl₄-permeate was detected by testing lipid profile and malondialdehyde (MDA) of manipulated animals (biochemical analysis).

It was evident that the ingestion of orange peel - CCL₄ (group 2) caused a remarkable decrease in the toxicity of CCL₄on cholesterol level after 8 weeks by 17.8 against 44.3% for positive control (fed on CCL₄ only). On the other hand, HDL level was decreased by 25.6 against 17.2% for positive control. As for LDL (bad cholesterol) a remarkable increase by 245% after 8 weeks was detected against insignificant increase in the manipulated group with orange peel. The obtained data revealed that orange peel to some extent reduced the toxicity of ccl₄ on these fractions (Table 4).

With regard to the fore mentioned effect on triglycerides, table (4) shows that this fraction was increased by 63.12 against 50% for positive control after 8 week, thus orange peel does not reduce the toxicity on this fraction.

With regard to the group 3 fed on permeate -CCL₄, Table (4) shows that cholesterol level was decreased by 25.8% after 8 weeks of treatment. The obtained results concerning HDL and LDL were 31, 28.2, 26, 26.2, 25 and 32.2 mg/at zero, 4 and 8 weeks of treatment, respectively. These values were statistically considered to be insignificant.

Concerning, triglycerides, it could be seen in the group manipulated with orange peel, that an increase did occur and been 38.2 against 50% after 8 weeks for positive control (Table 4).

As for the effect CCL₄ associated with permeate, triglycerides value was increased by 44.3% compared with the control (CCL₄ only) after 8 weeks of treatments.

It can assumed from these results that the association of both orange peel and permeate did not overcome completely ccl₄ toxicity but to some extent reduced its detrimental effect.

The obtained results concerning the toxicity of CCL₄ on lipid are in agreement with Kaplowitz *et al.* [27] and Lee *et al.* [28] who reported that reaction of CCL₄ with membrane lipids leads to lipid peroxidation.

Effect of Orange Peel and Permeate on Malondialdehyde (MDA) Concentration in Plasma of Rats: According to the available literatures CCL₄ is an extensively used to induce lipid peroxidation and toxicity [29]. Well established that ccl₄is metabolized in the liver which initiates free radical mediated lipid peroxidation and causes functional and morphological changes leading to accumulation of lipid derived oxidants causing liver injury. Malondialdehyde (MDA), resulting in structural changes endoplasmic reticulum and other bio membranes and loss of metabolic activity leading to liver damage [30].

Fig. 1 illustrates the effect of reconstituted orange peel and permeate as antioxidant materials on MDA contents of ccl₄ induced hepatotoxicity and non-induced rat liver.

As indicated from the results, MDA was enhanced in rat induced hepatotoxicity by 13.n mol /ml of plasma after 8weeks against 8 and 7 n mole/ml for the groups manipulated with orange peel and permeate incorporated with CCL₄, respectively However, aforementioned results indicated clearly that the moderate extenuative effect for ccl₄ toxicity by using orange peel and permeate may be attributed to the nature of both materials as including

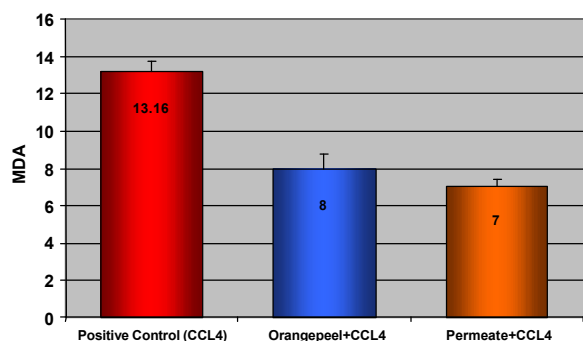


Fig. 1: The effects of orange peel and permeate on MDA content of CCL₄ induced hepatotoxicity.

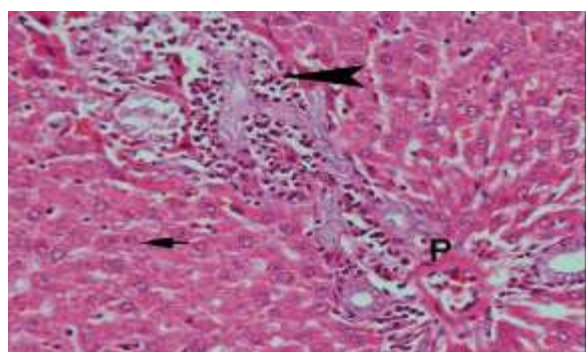


Fig. 2: Liver of a rat received CCL₄ only. (H&E X80)

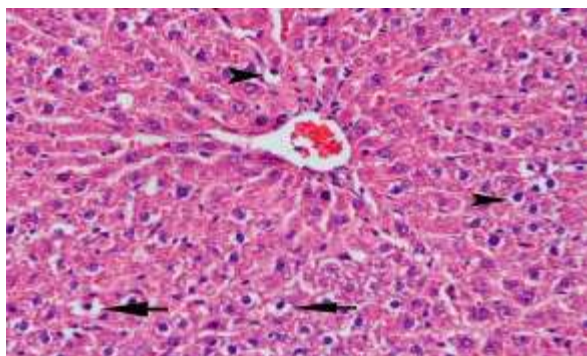


Fig. 3: Liver of a rat received CCL₄ + orange peel (H&E, X80)

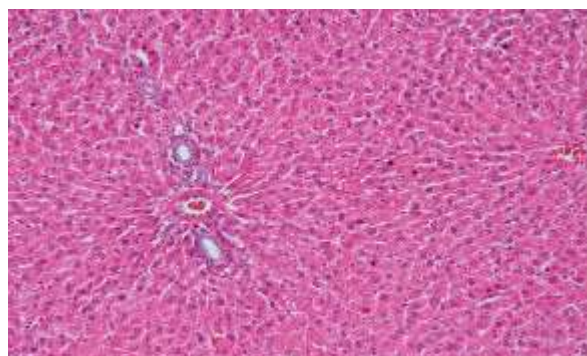


Fig. 4: Liver of a rat received CCL₄ + permeate (H&E, X40)

antioxidant active components which caused a decrease in the concentration of MDA in plasma as compared with positive control. (manipulated with CCL₄).

Effect of Orange Peel and Permeate on the Toxicity of CCL₄ as Detected by Histopathological Examination:

Liver often shows high concentrations of different chemicals namely drugs, environmental pollutants and their metabolites. Therefore this organ is mostly subjected to degenerations due to the presence of these chemical metabolites. These changes are generally in the form of necrosis followed by hyperplasia, thus it is worthy to examine ccl₄ toxicity by the detecting the injuries which may be caused by the ingestion of it. On the other hand, it seems beneficial to clarify the antioxidant role of both orange peel and permeate against histopathological toxicity of CCL₄ on liver tissues.

The microscopical examination of the rats received 100 mg /kg.b.w CCL₄ revealed that liver was affected. Liver showed inflammatory cell infiltration in the portal area and damaging effect on hepatic tissue (Fig. 2).

The microscopical alternation in the rats received CCL₄ and reconstituted orange peel revealed diffuse kupffer cells proliferation in between the hepatocytes (Fig. 3).

The microscopical examination of rats received and reconstituted permeate showed a normal histological structure of the portal area and surrounding hepatocytes (Fig. 4).

It can be assumed from these findings that the incorporation of CCL₄ with both orange peel and permeate led to some extent a prophylactic effect against the detrimental effect of CCL₄.

Our results are in agreement with Galati *et al.* [31] who evaluated histopathological of rat liver and reported that the fruit juice containing many phenols compounds, ascorbic acid and flavonoid fraction which cause a reduction in ccl₄ toxicity. Also, the pre mentioned data are in agreement with Fahim *et al.* [32], who examined liver ccl₄ intoxicated rats and reported that it shows an increase in the the number and size of van kupffer cells [33].

CONCLUSION

It is evident from our results that some residues are antioxidant sources, perhaps by-products such as orange peel and permeate are the most promising. From the *in vitro* study, it has been observed that orange peel and permeate have good antioxidant activity and

scavenging capacity. These results demonstrate the applicability of these naturally occurring antioxidant as an ingredient in food in order to increase their oxidative stability. Biologically, orange peel and permeate offer a promising approach to prophylactic effect against disease conditions through a regulated diet. Regarding hepatotoxicity and lipid oxidation induced by CCL₄, the addition of both by-products under study was effective against the hepatotoxicity and lipid oxidation caused by CCL₄. It is evident from our results that safety utilization of both orange peel and permeate. Therefore, to derive health benefits from these effective natural antioxidants, their application in dairy and food industry may be very valuable and desirable.

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