

Effect of Apocynin on Liver Toxicity Induced by Microwaves in Rats

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ABSTRACT

Background: electromagnetic waves could cause oxidative stress and generate ROS that lead to free radical production and lipid peroxidation which damage liver tissue. Apocynin (APO) exerts its antioxidant effect by reducing ROS production via inhibition of NADPH oxidase. The present study intended to demonstrate effects of microwaves exposure on hepatic pro-oxidant/antioxidant systems and to investigate protective effects of APO against microwaves induced hepatotoxicity. **Aim of the work:** The aim of the present work is to evaluate the effect of Apocynin on liver toxicity induced by exposure to microwaves in rats of local strain. **Subjects and Methods:** Thirty local strain rats were randomly assigned into three equal groups: a) control group; b) microwave exposed group (frequency of 2.45 GHz for 15 minutes once daily for a period of 16 days); c) apocynin treated prior to microwave exposure group (apocynin 20 mg/kg intraperitoneally injected 1 hour before microwave exposure and continued during the other 16 days). Liver tissue was biochemically assessed in all groups biochemically through the determination of tissue MDA, MPO, GPx and iron in liver tissue. Also, serum alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and Tumor necrosis factor alpha (TNF α) levels were estimated in all groups.

Results: The microwave exposed group showed that there was a significant increase in serum ALT, AST, ALP, TNF- α , as well as significant increase in the level of MPO, MDA and iron in the liver tissue. However, there was a significant decrease in the GPx and zinc level in the liver tissue. These results were estimated and calculated in comparison with those of control group. Moreover, Apocynin treated group showed a significant decrease in serum ALT, AST, ALP, TNF- α , in addition to a significant decrease in the level of MPO, MDA and iron, along with a significant increase in the GPx level in the liver tissue, as compared with microwave exposed group.

Conclusion: It is possible to conclude considering the abovementioned results that apocynin has a significantly protective effect against hepatotoxicity induced by microwaves exposure.

Keywords: apocynin, hepatotoxicity, microwaves, oxidative stress.

INTRODUCTION

Microwaves (MWs) are electromagnetic waves with wavelengths ranging from one millimeter to one meter or equivalently, with frequencies between 0.3 GHz and 300 GHz. During the last few decades, there has been an increase in the number of devices that emit microwaves. Such devices are used predominantly in telecommunications, but also in other society sectors and in households^[1].

Nowadays, we are facing growing public concerns regarding the potential hazard to man from exposure to microwaves. By far the greatest public concern has been that exposure to microwaves may cause cancer. However, most of the studies on animals regarding the *in vivo* effect of MW exposure have reported evidence of changes in cellular biochemistry such as increased reactive oxygen species generation^[2].

Reactive oxygen species (ROS) are generated as products of cellular metabolism, primarily in the mitochondria. When cellular production of ROS overwhelms its antioxidant capacity, damage to cellular macromolecules such as lipids, proteins, and DNA may ensue. Normally, the reactive

oxygen species (ROS) are neutralized by highly efficient antioxidant systems that catalyze the decomposition of hydrogen peroxide to water and oxygen, therefore preventing generation of highly toxic hydroxyl radicals^[3]. Trace elements play an important role as activators of enzyme systems or as constituents of organic compounds. For example, zinc and copper play a role in quenching of free radicals through reduction of the peroxidation ratio and breaking the free-radical production chain. On the other hand, iron and copper possess the ability to generate reactive radicals, resulting in cellular damage like depletion of enzyme activities, damage to lipid bilayer and DNA^[4].

Apocynin, also known as acetovanillone (4-hydroxy-3-methoxy-acetophenone), is the major active constituent of Chinese medicinal herb *Picrorhizakurroa*. Traditionally *P. kurroa* has been used to treat hepatic diseases, respiratory tract disorders, diarrhea, epilepsy and fever. Apocynin is a specific inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and exhibits potent anti-inflammatory activity^[5].

Inhibition of this enzyme may represent an attractive therapeutic target for the treatment of many diseases^[6].

MATERIALS AND METHODS

The present work was carried out on 30 male albino rats ranging in weight between 150-200 gm. The rats were housed in isolated animal cages, under controlled environmental conditions and exposed to alternate cycles of 12h light and darkness, and had free access to tap water and received pelleted standard diet all over the experimental period. All procedures were done according to the ethical committee of Faculty of Medicine, Tanta University.

The animals were divided into three groups (10 rats in each group):

1- Control group:

The animals of this group received saline (one ml) intraperitoneal injection once daily for 16 days.

2- Microwave exposed group:

The animals of this group were exposed to microwaves at frequency of 2.45 GHz for 15 minutes once daily for a period of 16 days^[7].

3- Apocynin treated and Microwave exposed group:

The animals of this group received apocynin (product of sigma) intraperitoneal, injection 1 hour before microwave exposure and continued during the other 16 days at a dose of 20mg/kg per day^[8].

At the end of the experimental period (16 days) the animals were fasted overnight, then, all rats were anaesthized by (IP) injection of pentobarbital (50 mg/kg)^[9] and blood samples were obtained by cervical dislocation.

a) Blood samples:

Blood samples were collected in clean plastic test tubes, and centrifuged at 3000 rpm for 15 minutes and the separated sera were then transferred into clean storage plastic tubes and stored at -30°C for estimation of the following parameters:

1. Serum aspartate aminotransferase (AST) according to the method described by *Harold V.*^[10]
2. Serum alanine aminotransferase (ALT) according to the method described by *Harold V.*^[10]
3. Serum alkaline phosphatase (ALP) according to the method of *Tietz et al.*^[11]
4. Tumor necrosis factor alpha (TNFα) according to the method described by *Brouckaert et al.*^[12]

b) Tissue sampling

Liver of all rats was dissected carefully in each group to avoid mechanical trauma and each liver was weighed and cut transversely into three parts, one part for histopathological examination and the other two parts were kept at -30°C for the tissue biochemical assays.

Homogenates.

After dissection, each liver weighed then homogenized in the suitable buffer for each parameter. The homogenates were collected in clean plastic test tubes and then centrifuged at 3000 r.p.m for 15 minutes at 4°C and the supernatants were separated in a clean storage plastic indorfens and stored at -80°C for estimation of the following parameters:

1. MDA; Liver malondialdehyde (nmol/gm tissue) was estimated according to the method of *Janero's*^[13]
 2. GPx; Liver glutathione peroxidase (U/gm protein) was measured according to the method of *Mannervik's*^[14]
 3. Liver Myeloperoxidase enzyme (U/gm tissue) was assayed according to the method described by *Franck et al.*^[15]
 4. MPO; Liver iron (µg/gm tissue) was estimated according to the method of *Dreux*^[16]
- Then the sacrificed rats were packed in a special package according to safety precautions and infection control measures and sent with hospital biohazard.

The study was approved by the Ethics Board of Tanta University.

Statistical Analysis

Statistical analysis of the results was carried out depending on the following conventional standard equations^[17].

1. **Mean value (X)** =
$$\frac{\sum X}{n}$$

Where $\sum x$ = the Sum of all observations.
n = the number of observations.

2. **Standard Deviations (S.D)** =

$$\sqrt{\frac{\sum X^2 - \frac{(\sum x)^2}{n}}{n - 1}}$$

Where $\sum x^2$ = sum of observations.
 $(\sum x)^2$ = Square of the sum of observations.
N = the number of observations.

3. **Standard error (S.E.)** =
$$\frac{S.D}{\sqrt{n}}$$

A One-way Analysis Of Variance (ANOVA) was calculated with Tukey-Kramer multiple comparison follow-up test calculation when $p \leq 0.05$:

- $SS_{Total} = SS_{Error} + SS_{Treatments}$
- $DF_{Total} = DF_{Error} + DF_{Treatments}$
- $MS_{Treatments} = SS_{Treatments} / (I-1)$
- $MS_{Error} = SS_{Error} / (n_T - I)$
- $F = MS_{Treatments} / MS_{Error}$

Where:

DF: degrees of freedom

SS: sum of squares

MS: is mean square

I: number of treatments

n_T: total number of cases

Data were analyzed using Statistical Program for Social Science (SPSS) version 17.0. Quantitative data were expressed as mean \pm standard deviation (SD).

The following tests were done:

ANOVA when comparing between more than two means.

Significance of the results

$p \leq 0.05$	Significant difference
$p \geq 0.05$	Insignificant difference

RESULTS

1. Serum AST , ALT and ALP (U/ml) level in control group, microwave exposed group, apocynin and microwave exposed group (Table 1, Table 2 and Table 3):
 - **Microwave (MW) Group versus Control group:** significant increase in AST, ALT and ALP when compared to control group ($p < 0.05$).
 - **MW + Apocynin Group versus MW Group:** significant decrease in AST, ALT and ALP ($p < 0.05$).
 - **MW + Apocynin Group versus Control Group:** there is insignificant change in AST, ALT and ALP when compared to control group ($p > 0.05$).

Table (1): Serum AST (U/ml) in all studied groups

Group No	Control	MW Group	MW + Apocynin Group
Mean	96.6	183.6	109.1
\pm S.D	± 16.06	± 29.49	± 20.16
f. test	15.365		
p. value	0.001*		
Tukey's test			
Control group Vs Microwave exposed group	Control group Vs Apocynin and Microwave exposed group	Microwave exposed group Vs Apocynin and Microwave exposed group	
< 0.05*	>0.05	< 0.05*	

*Denotes statistical significance at $p < 0.05$

Table (2): Serum ALT (U/ml) in all studied groups

Group No	Control Group	MW Group	MW + Apocynin Group
Mean	37.50	65.20	41.0
\pm S.D	± 6.04	± 10.56	± 9.75
f. test	28.076		
p. value	0.001*		
Tukey's test			
Control group Vs Microwave exposed group	Control group Vs Apocynin and Microwave exposed group	Microwave exposed group Vs Apocynin and Microwave exposed group	
< 0.05*	>0.05	< 0.05*	

*Denotes statistical significance at $p < 0.05$

Table 3: Serum ALP (U/L) in all studied groups

No \ Group	Control Group	MW Group	MW + Apocynin Group
Mean	80.2	167.2	93.50
± S.D	± 20.56	± 49.74	± 23.07
f. test	8.420		
p. value	0.001*		
Tukey's test			
Control group Vs Microwave exposed group	Control group Vs Apocynin and Microwave exposed group	Microwave exposed group Vs Apocynin and Microwave exposed group	
< 0.05*	>0.05	< 0.05*	

*Denotes statistical significance at $p < 0.05$

2. Serum tumour necrosis factor alpha (TNF α) (ng/L) in control group, microwave exposed group, apocynin and microwave exposed group (Table 4):

- **MW Group versus Control group:** significant increase in TNF α when compared to control group ($p < 0.05$).
- **MW + Apocynin Group versus MW Group:** significant decrease in TNF α ($p < 0.05$)
- **MW + Apocynin Group versus Control Group:** there is insignificant change in TNF α when compared to control group ($p > 0.05$).

Table (4): Serum tumour necrosis factor alpha (TNF- α) (ng/L) in all studied groups

No \ Group	Control Group	MW Group	MW + Apocynin Group
Mean	8.59	34.11	9.09
± S.D	± 0.91	± 3.67	± 1.14
f. test	41.063		
p. value	0.001*		
Tukey's test			
Control group Vs Microwave exposed group	Control group Vs Apocynin and Microwave exposed group	Microwave exposed group Vs Apocynin and Microwave exposed group	
< 0.05*	>0.05	< 0.05*	

*Denotes statistical significance at $p < 0.05$

3. Liver Malondialdehyde level (MDA) (nmol/gm tissue) in control group, microwave exposed group, apocynin and microwave exposed group (Table 5):

- **MW Group versus Control group:** significant increase in MDA when compared to control group ($p < 0.05$).
- **MW + Apocynin Group versus MW Group:** significant decrease in MDA ($p < 0.05$)
- **MW + Apocynin Group versus Control Group:** there is insignificant change in MDA when compared to control group ($p > 0.05$).

Table 5: Liver (MDA) in nmol/gm tissue in all studied groups

Group	Control Group	MW Group	MW + Apocynin Group
No			
Mean	10.68	27.86	11.69
± S.D	± 0.94	± 8.91	± 1.61
f. test	22.743		
p. value	0.001*		
Tukey's test			
Control group Vs Microwave exposed group	Control group Vs Apocynin and Microwave exposed group	Microwave exposed group Vs Apocynin and Microwave exposed group	
< 0.05*	>0.05	< 0.05*	

*Denotes statistical significance at p<0.05

4. Liver Glutathione peroxidase level (GPx) (u/gm protein) in control group, microwave exposed group, apocynin and microwave exposed group (Table 6):

- **MW Group versus Control group:** significant decrease in **GPx** level when compared to control group (p<0.05).
- **MW + Apocynin Group versus MW Group:** significant increase in **GPx** level (p<0.05)
- **MW + Apocynin Group versus Control Group:** there is insignificant difference in **GPx** level when compared to control group (p>0.05).

Table 6: Tissue GPx (U/gm protein) in all studied groups

Group	Control Group	MW Group	MW + Apocynin Group
No			
Mean	230.66	152.28	220.75
± S.D	± 61.56	± 12.69	± 26.61
f. test	11.730		
p. value	0.001*		
Tukey's test			
Control group Vs Microwave exposed group	Control group Vs Apocynin and Microwave exposed group	Microwave exposed group Vs Apocynin and Microwave exposed group	
< 0.05*	>0.05	< 0.05*	

*Denotes statistical significance at p<0.05

5. Liver Myeloperoxidase level (MPO) (U/gm tissue) in control group, microwave exposed group, apocynin and microwave exposed group (Table 7):

- **MW Group versus Control group:** significant increase in **MPO** level when compared to control group (p<0.05).
- **MW + Apocynin Group versus MW Group:** significant decrease in **MPO** level (p<0.05)
- **MW + Apocynin Group versus Control Group:** there is insignificant difference in **MPO** level when compared to control group (p>0.05).

Table 7: Liver (MPO) in U/gm tissue in all studied groups

Group No	Control Group	MW Group	MW + Apocynin Group
Mean	68.78	203.85	74.82
± S.D	± 12.95	± 9.01	± 10.38
f. test	89.727		
p. value	0.001*		
Tukey's test			
Control group Vs Microwave exposed group	Control group Vs Apocynin and Microwave exposed group	Microwave exposed group Vs Apocynin and Microwave exposed group	
< 0.05*	>0.05	< 0.05*	

*Denotes statistical significance at p<0.05

6. Liver iron level (µg/dl) in control group, microwave exposed group, apocynin and microwave exposed group (Table 8):

- **MW Group versus Control group:** significant increase in **Liver iron level** when compared to control group (p<0.05).
- **MW + Apocynin Group versus MW Group:** significant decrease in **Liver iron level** (p<0.05)
- **MW + Apocynin Group versus Control Group:** there is insignificant difference in **Liver iron level** when compared to control group (p>0.05).

Table 8: Liver iron in (µg/gm tissue) in all studied groups

Group No	Control Group	MW Group	MW + Apocynin Group
Mean	72.18	90.36	75.71
± S.D	± 9.99	± 9.19	± 8.60
f. test	10.799		
p. value	0.001*		
Tukey's test			
Control group Vs Microwave exposed group	Control group Vs Apocynin and Microwave exposed group	Microwave exposed group Vs Apocynin and Microwave exposed group	
< 0.05*	>0.05	< 0.05*	

*Denotes statistical significance at p<0.05

DISCUSSION

The liver is a vital organ which plays a major role in metabolism with numerous functions in the human body. The term “liver insufficiency” denotes a breakdown in the functions of the liver. The syndrome of functional liver failure covers a wide spectrum of clinical, biochemical and neurophysiological changes^[17].

When damage to the hepatic parenchyma is so severe that the liver is no longer able to meet the metabolic requirements of the body, the syndrome of ALF develops. The outcome is characterized by

coagulopathy, hepatic encephalopathy, often associated with cerebral edema, hemodynamic changes, electrolyte disturbance, and renal failure^[18]. Microwaves (MWs) are electromagnetic waves with wavelengths ranging from one millimeter to one meter or equivalently, with frequencies between 0.3 GHz and 300 GHz. During the last few decades, there has been an increase in the number of devices that emit microwaves. Such devices are used predominantly in telecommunications, but also in other society sectors and in households^[19].

However, most of the studies on animals regarding the *in vivo* effect of MW exposure have reported evidence of changes in cellular biochemistry such as increased reactive oxygen species generation^[20].

Apocynin (4hydroxy-3methoxy-acetophenone) is naturally occurring methoxy substituted catechol, phenolic compound, extracted from the roots of *Apocynum cannabinum* (Canadian hemp) and *Picrorhiza kurroa* (Scrophulariaceae)^[21]. The results of the present work reveal that exposure of the rats to microwave radiation, induced a significant increase in ALT, AST, ALP, TNF alpha in serum, and a significant increase in the level of MPO, MDA and iron in the liver tissue. However, there was significant decrease in the GPX and zinc level in the liver tissue.

Thapa and Anuj^[22] indicated that the aminotransferases enzymes of the liver are the most frequently utilized to assay liver functions and specific indicators of hepatocellular necrosis. Elevation in levels of serum liver markers, especially AST and ALT, is attributed to damaged liver cells, since these enzymes are located in the cytosol and released into the blood following liver cell damage so injury of hepatocyte wall produce leakage of the liver enzymes into blood stream and increase their levels in the blood.

Chen et al.^[23] also, mentioned that the significant increase in liver enzymes could be due to liver cell injury that disrupted the plasma membrane and consequently their leakage through extracellular fluid.

Moussa^[24] has also postulated that rats exposed to microwave irradiation induced toxic liver cell damage that was evidenced by significant increase in liver enzymes (ALT, AST and ALP). One of the possible mechanisms explaining liver insufficiency and elevated liver enzymes may be due to oxidative stress, which is demonstrated in the present work by significant decrease of GPx and increased MDA after exposure of rats to microwave irradiation.

In accordance with our work, **Kim et al.**^[25] postulated that there was decrease in the GPx activity after exposure of the rats to microwaves for 16 days. This would seem to indicate that the peroxidation of the unsaturated fatty acid in the existing membranes was activated by oxidative stress, in this case free radicals resulting from microwave irradiation, thereby accelerating the cell tissue damage and decreasing the enzyme activities.

Hao et al.^[26] have also demonstrated that there was significant decrease in the level of glutathione peroxidase (GPx) and increased the

level of MDA after microwave exposure. They stated that MW radiation activates the NADH oxidase-mediated increase in ROS, and in turn, excessive ROS damage the mitochondrial electron transport chain, which is the main source of ROS, ultimately forming a vicious cycle and aggravating the disturbance in energy metabolism.

Moreover, our study stated a significant increase in the level of MDA after exposure to microwaves. MDA is a lipid peroxidation product that is released specifically due to the toxic effects of active ROS. ROS is produced as a result of oxidation of unsaturated fatty acids in cell membranes, and thus serves as a good marker for oxidative stress.

APO treatment increased the level of GPx when compared with the microwave exposed group. **Cagin et al.**^[27] have also evidenced the increase in the activity of GPx after apocynin treatment. This is because it is known that the hepatocytes have enzymatic and non-enzymatic antioxidant systems in order to maintain the integrity of the biological membranes in case of oxidative stress. The imbalance between the pro- and antioxidant system plays an important role in the development of several diseases. The GSH cycle is the most important intracellular antioxidant defense mechanism. It is used as a substrate for the activity of several antioxidant enzymes. In particular, GPx is a glutathione-dependent enzyme which is considered endogenous enzymatic antioxidant that protects the cells against cytotoxic free oxygen radicals^[28].

In the presence of glutathione, H₂O₂ is detoxified by GPx via conversion to H₂O and O₂ molecules. Then, GSSG is formed by receiving hydrogen from glutathione which, in turn, is converted to GSH by glutathione reductase. Decreased GPx activity leads to the accumulation of toxins by increasing the oxidative stress^[29].

Chauhan et al.^[30] stated significant increase in lipid peroxidation after microwave exposure. This was explained that microwave radiation is suggested to have the potential of changing the biological lipid membranes and the outcomes of these changes can be seen in the structural and functional properties of a cell.

Rifat et al.^[31] have reported that microwave exposure causes a significant elevation in lipid oxidation products level in mice spleen, leading to oxidative stress, thereby weakening the efficacy of various defense mechanisms. Microwave-induced lipid peroxidation not only damages cell membranes, but also induces antioxidant enzymes and DNA damage.

In contrast with the current results, *Djordjevic et al.*^[32] failed to demonstrate the significant change in MDA level in animals exposed to MWs for 20 days. However, they demonstrated that the increase of MDA occurred in liver tissue of rats exposed to microwaves after prolonged exposition period (40 and 60 days). The observed increase in MDA level was dependent on the exposure duration.

Another explanation for liver insufficiency is the inflammatory effect of microwaves as evidenced by increased MPO activity and TNF- α in the present work after exposure to EMF.

Megha et al.^[33] postulated that oxidative stress produced by generation of free radicals following microwave radiation may lead to generation of pro-inflammatory cytokines such as TNF- α , thus the induced oxidative stress may lead to inflammatory imbalance and disturbance in functions. *They* have also stated that there was significant increase in the level of TNF- α after exposure to microwave irradiation.

On the other hand, significant decrease of MPO activity and TNF- α was noticed after administration of apocynin in comparison with the microwave exposed group. *El-Sawalhi and Ahmed*^[34] also demonstrated that apocynin significantly decreased the markers of inflammation such as TNF alpha and MPO.

Apocynin inhibited NADPH oxidase which is the major pathway for modulation of ROS production by MPO in activated neutrophils. Apocynin is also proved to be a potent anti-inflammatory agent, based on the selective inhibition of superoxide anion production from activated neutrophils and thus the pro-apoptotic pathway activation^[35]. This conclusion agrees with that found by *Atlantis et al.*^[36] who stated that MPO is a particular oxidase in polymorph nuclear leukocyte (PMN) and the MPO activity in tissue is used to estimate the PMN chemotaxis and infiltration. PMN infiltration during the reperfusion period may cause generation and releasing of additional large amount of oxidants that exacerbate this harmful cascade. Apocynin, which is also activated by MPO, reduces the generation of inflammatory mediators by inhibiting NOX. However, it does not impress the defensive property of PMN.

Another explanation for liver insufficiency is due to disturbance in the level of trace elements. Significant increase in iron level had been noticed in the microwave group. In accordance with our results, *Gharib*^[4] who demonstrated that iron showed a significant increase in their concentration

due to electromagnetic radiation exposure. This may be due to oxidative stress resulted from electromagnetic field exposure. This increase leads to depletion of enzyme activities accompanied with a generation of reactive species.

CONCLUSION AND RECOMMENDATION

In the present study, we presented sufficient body of evidence suggesting that Apocynin guard against oxidative stress makes it interesting as promising therapeutic candidate with significant clinical applications in organs toxicity.

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