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Simultaneous determination of retinol, retinyl palmitate and β-carotene in rat serum treated with 7,12-dimethylbenz[a]anthracene and *Hypericum Perforatum* L. by high-performance liquid chromatography with diode-array detection

Research Article

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Abstract: A new and simple high-performance liquid chromatography method was developed and validated for the simultaneous determination of retinol, retinyl palmitate and β-carotene in rat serum treated with *Hypericum Perforatum* L. and 7,12-dimethylbenz[*a*]anthracene. Furthermore, vitamin C was determined spectrophotometrically. High-performance liquid chromatography analysis was performed utilizing an Inertsil ODS3 reversed phase column with methanol-acetonitrile-tetrahydrofuran (65:30:5, v/v/v) as mobile phase, at a flow rate of 1.5 mL min^{-1} and 40°C. Diode-array detection was conducted at 325 and 450 nm for retinol and retinyl palmitate, and β-carotene, respectively with a running time of 26 min. The high-performance liquid chromatography assay and extraction procedure proposed are simple, rapid, sensitive and accurate. This method was then applied to determine the amounts of retinol, retinyl palmitate and β-carotene in rat serum. Results of this study demonstrated that at 60th day in the 7,12-dimethylbenz[*a*]anthracene-treated group there was a significant decrease ($p<0.001$), ($p<0,01$), ($p<0.05$) in levels of retinol, retinyl palmitate and vitamin C, respectively compared to the control group levels. A significant decrease ($p<0.01$) in retinyl palimitate was observed in the 7,12 dimethylbenz[*a*] anthracene + *Hypericum Perforatum* L. treated group compared to the control group..

Keywords: *Retinol • HPLC • Hypericum Perforatum L. • 7,12-dimethylbenz[a]anthracene*

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants that are potent genotoxic compounds such as mutagens and carcinogens. They are produced mainly as a result of incomplete combustion of organic materials during both natural events and human activities, such as forest fires and volcanic eruptions, and also as a result of burning of fossil fuels and petroleum products during industrial production, food processing, operation of machinery, including automobiles, airplanes and ships [1]. PAHs themselves are chemicals relatively non-reactive toward biological macromolecules under physiological conditions. In mammalian systems, they are metabolized principally in the liver to yield both detoxification products, which are polar and excretable, and bioactivation products, which are more reactive

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and genotoxic. Their biological effects are actually attributed to the oxidation process that occurs during biotransformation of the mother compounds. PAH metabolites, including diol epoxides and free radical cation intermediates formed by monooxygenation and one-electron oxidation, respectively, are molecules that may bind to cellular DNA forming covalent DNA-adducts responsible for carcinogenic process [2-4]. 7,12-dimethylbenz[*a*] anthracene (DMBA) is one of the PAHs used extensively in experimental studies as a presumptive prototype mutagen, a signal transduction modulator and a known breast and skin carcinogen [5]. In our previous study, we demonstrated that the level of trace elements and serum protein patterns in rabbit subjected to DMBA did not shown statistical differences for the parameters affected by *Urtica dioica* [6].

Hypericum perforatum L. (*HP*) commonly named St. John's wort in Western Europe, is a herbaceous perennial plant belonging to the Hypericaceae family found widely distributed in Europe, Asia, and North Africa; it is also naturalized in North America [7]. HP is an increasingly popular alternative to conventional medications used for the treatment of mild to moderate depression [8-10]. Aggressive analysis of this plant over the last three decades has revealed that it possesses several biological properties, including antidepressant, antiviral and antiproliferative activities [11-13]. Although HP is most commonly used as an alternative to standard tricyclic antidepressants, other biological properties possessed by this plant have also been utilized for the treatment of cancer and viral infections [14-17].

Retinoids in the diet are taken up mainly as carotenoids (precursors of retinol), that are exclusively synthesized by photosynthetic microorganisms and by plants. The skeleton of natural retinoids is made up of a non-aromatic six-carbon ring structure with a polyprenoid side chain, which is terminated with a carbon-oxygen functional group. Following uptake, the cleavage of carotenoids to retinol or Vitamin A is the first step of a complex metabolitic process. The retinol is stored in the liver and adipose tissue as lipophilic retinyl esters, mainly consisting of retinyl palmitate [18,19]. Recently, carotenoids possessing antioxidative properties and immunity functions have attracted attention of the public and researchers in the food and nutrition field. Similarly, retinoids with anticarcinogenic and morphologic actions are of major interest [18,20-22]. It is well-known that several carotenoids such as β-carotene are inverted to retinal in the intestinal mucosa of animals and these carotenoids are of the pro-vitamin A type. For research purposes, it is important to determine, as accurately and simultaneously as possible, the amounts of carotenoids and retinoids absorbed and metabolized in the body organs [23].

Clinical studies have shown that supplemental levels of antioxidant vitamins reduce an individual's risk for certain cancers and cardiovascular diseases [24,25]. Moreover, studies have shown that fruit and vegetable consumption (the major source of antioxidant nutrients) has a protective effect against cancer [26].

Several HPLC methods for the analysis of retinol (R), retinyl palmitate (RP) and β-carotene (β-C) levels in tissues and blood serum are described, including UV/DAD [23,25,27-30], fluorescence detection [31-34] and electrochemical detection [35-38]. Carotenoids and retinoids can be monitored by UV/DAD detectors, retinoids by fluorescence detection, and carotenoids by electrochemical detection.

DMBA, which is a PAH, generates free radicals. Plants such as *HP* exert their anti-carcinogenic effects by scavenging the free radicals, detoxification and antioxidant defense system (such as vitamin C, retinol and β-carotene). To our knowledge, in literatüre there has been no report related to effect on the vitamins in the rat treated with *HP* and DMBA. However, no reports for the simultaneous determination of R, RP and β-C in the rat serum are described. Hence, the aim of the present study was to develop and validate an analytical HPLC isocratic method for the simultaneous determination of R, RP and β-C in rat serum treated with *HP* and DMBA.

2. Experimental procedure

2.1. Chemicals and reagents

Vitamin C, R, RP, β-C, DMBA, $CuSO_4$, thiourea, 2,4-dinitrophenylhydrazine and butylated hydroxyltoluene (BHT) were purchased as standard substances from sigma (St Louis, MO, USA). HPLC-grade methanol, asetonitril, ethanol, sulfuric acid, chloroform, *n*-hexane and tetrahydrofuran (THF) solutions were purchased from Merck (Darmstadt, Germany).

2.2. Preparation of standard solutions and calibration

Stock solution of R, RP and β-C was prepared at 10 mg mL^{-1} in chloroform. Stock solution of vitamin C was prepared at 10 mg mL^{-1} in water. Stock solutions (except for vitamin C) were appropriately diluted with mobile phase for the preparation of standard solutions (final concentration ranging between 0.05 and 4 μg mL^{-1}). Calibration curves were calculated by linear regression analysis of the area of the peak versus the concentration in the standard solutions. The slopes, intercepts, correlation coefficient, and related validation parameters such as limit of detection (LOD), limit of quantification (LOQ), slope and intercept were

calculated for each vitamin. All solutions were protected from light and manipulated using amber glass vials, and stored at -20^{0}C.

2.3. Instrument and chromatographic conditions

The chromatographic system consisted of an HP Agilent 1100 series HPLC system with an Agilent series G-1328 DAD detector and an Agilent 1200 series G-1329 ALS autosampler. The data was handled with Agilent Technologies HP 1100 software. Separation was carried out with a 5 µm Inertsil ODS3 reversed phase column (250×4.6 mm ID). Ultra pure water, purified using a Milli-Q system from Millipore was used to prepare the solutions. Spectrophotometric measurements were carried out using a Shimadzu 1700 UV-vis Spectrophotometer. The mobile phase of a mixture of methanol-acetonitrile-THF (65:30:5, v/v/v) used was modified from [24]. The pump was set at a flow rate of 1.5 mL min^{-1}. 100 µL was injected into the HPLC system. The chromatogram was monitored with diode array detection at 325 and 450 nm (R and RP) and β-C, respectively. The chromatographic analysis was carried out at 40^{0}C with isocratic elution. The mobile phase was prepared daily and degassed by ultrasonication before use.

2.4. Extraction of Hypericum Perforatum L.

HP was collected from Van, Turkey and dried at room temperature. Dried material (300 mg) was infused in 30 mL of boiled distilled water for 30 min. After decantation and filtration, the filtrate was again dried in an incubator at a temperature at 50^{0}C. The aqueous extract was then prepared in isotonic physiological solution (0.9% NaCl) (yield 8.4) [39-40].

2.5. Animals treatments

Animal procedures were approved by the care of the institutional Animals and Use Committee. Sprague-Dawley female rats were placed individually in standard cages in temperature controlled rooms, 12 h light/dark cycles were maintained with free access to water and a standard laboratory diet

This study was performed on 24 Sprague-Dawley female rats. Rats were divided into three equal groups. Group 1 was control group (n=8), given as orally (intragastrically) olive oil. Group 2 (n=8), rats treated with a single dose of DMBA (50 mg kg^{-1}) in olive oil given as orally (intragastrically). Group 3 (n=8), treated with a single dose of DMBA (50 mg kg^{-1}) in olive oil followed by aqueous extract of *HP* 100 mg kg^{-1} day given as orally (intragastrically) for 60 days.

2.6. Serum samples preparation

Blood samples were collected at the begining of application (0th) and 60th days of application period by the method of intra cardiac and centrifuged in 500 g-force for 15 min to obtain sera and later stored at -70^{0}C until all experimental procedures is carried out.

2.7. Extraction procedure

Samples were thawed at room temperature under plastic sleeve-covered fluorescent lights to minimize sample degradation from exposure to UV light. R, RP and β-C in plasma were extracted as follows: serum (100 µL) was deproteinized by adding ethanol (100 µL) (containing 0.025% BHT) and the sample was vortex mixed for 1 min. Since carotenoids are easily oxidized, it is useful to add antioxidants such as BHT to the extraction solvent [41]. The sample was extracted twice with *n*-hexane (600 µL). The sample was vortex mixed and centrifuged at 3000 rev. min^{-1} for 15 min. Part (500 µL) of the hexane layer was extracted and evaporated to dry under a stream of nitrogen at 40^{0}C. The residue was dissolved in THF (50 µL) and added methanol (150 µL). The sample was vortex mixed for 1 min. 100 µL injected via the autosampler using amber glass vials.

2.8. Vitamin C analysis

Stock solution of vitamin C was appropriately diluted with ultra pure water for preparation of standard solutions (final concentration ranging between 1 and 7 µg mL^{-1}). Calibration curve was calculated by linear regression analysis of absorbance versus the concentration in the standard solutions. The slope, intercept, correlation coefficient, and related validation parameters such as LOD, LOQ, slope and intercept was calculated for vitamin C (Table 1). The reproducibility of the methods was assessed by running 7 replicate samples, at concentration of 4 µg mL^{-1} (medium concentration)

Vitamin C was determined by the method of Omaye *et al.* [42]. Ascorbic acid is oxidized by Cu(II) to form dehydroascorbic acid in the medium thiourea, which reacts with acidic 2,4- dinitrophenylhydrazine to form a red bis-hydrazone, which gives absorbance at 520 nm.

2.9. Assay validation

The method was validated according to USP 24 requirements for validation of analytical procedures [43,44]. Assay validation involved determination of linearity, accuracy, precision, LOD and LOQ. Intra-day and inter-day precision values were estimated at three different concentrations of vitamins three times on the same day to obtain the relative standard deviation

Table 1. Statistical data for the calibration graph of vitamin C standards

	Vitamin C
Slope	2.373
Intercept	0.033
Correlation coefficient (r)	0.9983
Linearity range (μg mL^{-1})	1-7
Reproducibility, RSD % (n=7)	1.01
LOD (μg mL^{-1})	0.12
LOQ (μg mL^{-1})	0.40

(RSD %). Accuracy was determined with recovery study. In this study, LOD and LOQ were based on the standard deviation of the response and the slope of the corresponding calibration curve using the following equations [44,45]: LOD: 3 sd/m; LOQ: 10 sd/m (sd:standard deviation; m:slope).

Data is presented as mean ($\overline{X}$) ± SEM (standard error of mean). Differences in biochemical parameters were statistically evaluated using one-way analysis of variance (ANOVA) followed by Tukey multiple comparison test.

3. Results and Discussion

3.1. Development of chromatography method

The development of the analytical method was started with selection of the mobile phase composition in order to separate R, RP and β-C. Methanol, acetonitrile, THF and water were used as the basis for the different mobile phase preparations. Various proportions of these solvents were tested. The mobile phase composition consisted of methanol and water, R and RP, which were well separated and β-C, which was not separated. When acetonitrile was used instead of water as part of the mobile phase, not only all vitamins were separated but β-C was eluted in 46 min. Then, THF was added to the mobile phase because THF improves carotenoid separations [46]. Finally, the best mobile phase for the separation of those vitamins was found to be methanol-acetonitrile-THF (65:30:5, v/v/v). At low temperature (20, 25, 30 and 35^{0}C) the vitamins were not well separated. The best separation was performed at 40^{0}C. The retention times for R, RP and β-C were 6.58, 19.79 and 24.79 min, respectively. The chromatograms of R, RP and β-C in mobile phase, spiked and extracted from the serum are illustrated in Figs. 1 and 2.

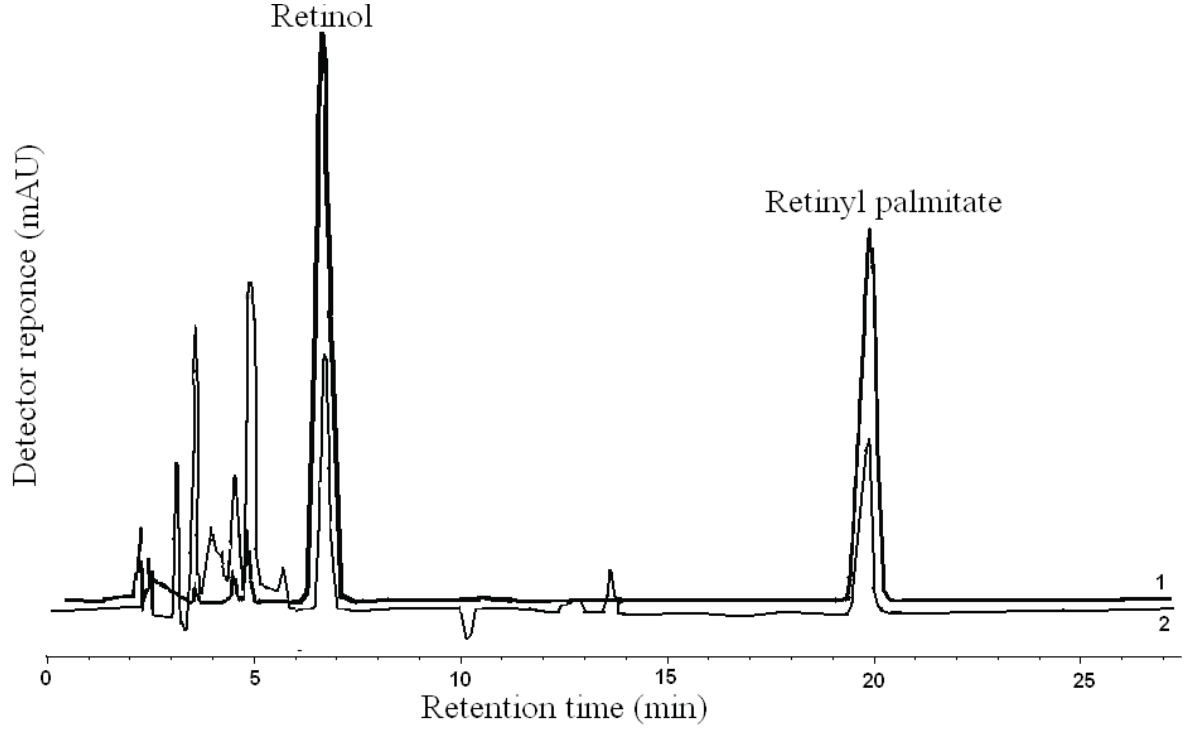

Figure 1. Isocratic elution was performed with methanol-acetonitrile-tetrahydrofuran (65:30:5, v/v/v) as mobile phase at a flow rate of 1.5 mL min^{-1}, 40^{0}C. Inertsil ODS3 reversed phase column (250×4.6 mm 5 μm) was employed. DAD was adjusted at 325 nm. Chromatogram of vitamins: (1) standard of retinol and retinyl palmitate in the mobile phase; (2) retinol and retinyl palmitate the extracted from rat serum.

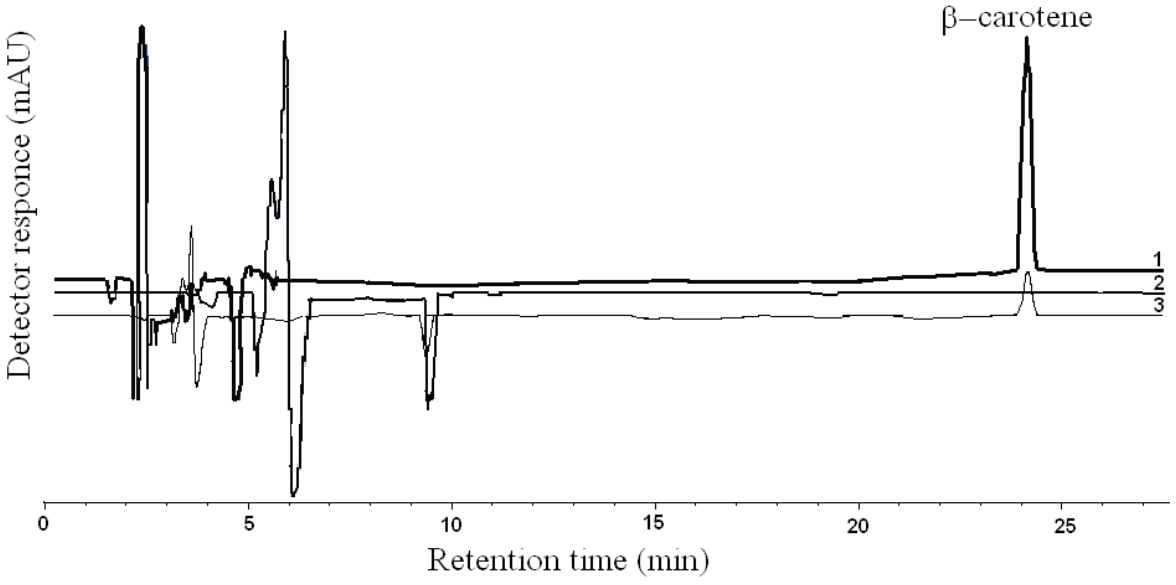

Figure 2. Isocratic elution was performed with methanol-acetonitrile-tetrahydrofuran (65:30:5, v/v/v) as mobile phase at a flow rate of 1.5 mL min^{-1}, 40°C. Inertsil ODS3 reversed phase column (250×4.6 mm 5 μm) was employed. DAD was adjusted at 450 nm. Chromatogram of vitamin: (1) standard of β-Carotene in the mobile phase; (2) β-Carotene the extracted from rat serum; (3) β-Carotene with the spiked from rat serum.

3.2. Linearity of the HPLC method

Resolution was always good in the linearity range studied. Linearity was checked for each vitamin using six standard solutions with concentrations ranging from (0.05-4 μg mL^{-1}) of the theoretical amounts of vitamins in the studied liquid mixture. Peak areas and analyte concentrations were found to be linearly related over this range for all the vitamins (Table 2). Linear regression was used to determine the slope and intercept. The correlation coefficients of the calibration curves were > 0.9970 for all vitamins tested.

3.3. Precision and sensitivity of the HPLC method

The developed method was validated according to the standard procedures [43]. The precision of the method was evaluated by replicate determination of inter-day and intra-day reproducibility. Intra-day repeatability was determined by replicate injection of standard solutions of vitamins (0.05, 0.5 and 4 μg mL^{-1}) 9 times on the same day. Inter-day reproducibility was determined by analysis of standard solutions on three different days. RSD% was calculated to check the precision of the method. Intra- and inter-day precision data for standard solutions of vitamins is summarized in Table 3. These

Table 2. Statistical data for the calibration graphs of Retinol, Retinyl palmitate and β-Carotene standards in mobile phase

Vitamin	Retinol			Retinyl palmitate			β-carotene		
Days	1	2	3	1	2	3	1	2	3
Slope	130.37	131.22	133.17	170.55	172.07	173.78	251.87	255.40	258.49
Intercept	1.42	1.62	1.32	3.22	2.96	2.48	2.76	1.14	1.12
Correlation coefficient (r)	0.9997	0.9996	0.9998	0.9994	0.9993	0.9992	0.9979	0.9991	0.9989
Linearity range (μg mL^{-1})		0.05-4			0.05-4			0.05-4	
Mean of slope		131.59			172.13			255.25	
Mean of intercept		1.453			2.89			1.67	
Mean of r		0.9997			0.9993			0.9986	
LOD (μg mL^{-1})		0.003			0.006			0.011	
LOQ (μg mL^{-1})		0.011			0.021			0.037	

Table 3. Precision of analysis of Retinol, Retinyl palmitate and β-Carotene standards in mobile phase

	Retinol (μg mL^{-1})			Retinyl palmitate (μg mL^{-1})			β-carotene (μg mL^{-1})		
	0.05	0.5	4	0.05	0.5	4	0.05	0.5	4
Intra-day RSD% (n=9)	3.01	2.21	2.54	4.15	3.54	3.79	4.61	4.08	4.76
Inter-day RSD% (n=9)	4.23	2.27	2.32	5.02	4.73	5.23	5.87	5.56	6.24

results indicate that the method developed achieves a high degree of precision, and reproducibility.

3.4. Accuracy of the HPLC method

The accuracy of the method was tested by measurement of recovery. Known amounts of vitamins were added to serum samples and the mixtures were analyzed according to the proposed extraction method. The signal of serum extracts to which increasing amounts of each vitamin was added was compared to the signal of the corresponding standards injected directly into the HPLC system. The recovery of each vitamin was determined at three concentration levels (low, medium and high) for one of the samples from each the groups. The recovery results were calculated using calibration equations. The results are presented in Table 4. Recovery (%) data confirm the accuracy of the method.

3.5. Level of vitamins

Average serum vitamins of the control, DMBA, DMBA+*HP* are shown in Table 5. In statistical analysis, the differences among the groups in the beginning (0th day) were not found to be significant ($p>0.05$). However, on the 60th day study, the levels of Retinol (μg mL^{-1}) between control (1.32 ± 0.11) and DMBA (0.65 ± 0.05), DMBA+*HP* (0.89 ± 0.07) groups were observed to be significantly decreased ($p<0.001$), ($p<0.01$), respectively. In the case of levels of Retinyl Palmitate (μg mL^{-1}) between control (0.72 ± 0.10) and DMBA (0.33 ± 0.07) there was a significant decrease ($p<0,01$). Also, in the case of levels of vitamin C (μg mL^{-1}), a significant decrease ($p<0.05$) between control (4.59 ± 0.37) and DMBA (3.60 ± 0.21) groups was observed. In this HPLC method developed, β-carotene level were not determined, but with the recovery experiment (91-93%), the applicability of the method for determination of β-carotene was demonstrated using rat serum (Fig. 2).

Decreased vitamins levels (Retinol and Retinyl Palmitate) are related to increased free radical production due to DMBA. Vitamin content near the control group levels, potent chemopreventive efficiacy of *HP* might be due to its antioxidant effects.

Aralkumaran *et al.* reported that Vitamin C was found to be significantly ($p<0.05$) decreased in mammary carcinoma bearing rats [47]. In this study, a significant decrease ($p<0,05$) was observed in the levels of vitamin C, between control and DMBA groups, which is consistent with Aralkumaran *et al.* Ascorbic acid protects against the genotoxic effects of several radical-generating mutagens, enzymatic superoxide-generating system (hypoxanthine/xanthine oxidase) DMBA [48]. In another study, researchers demonstrated that Vitamin C, an important extracellular antioxidant, has benefical effects on the conversion of cancerous cells to normal ones and in the lipid peroxidation by product scavenging mechanism [49].

The beneficial effects on antioxidant status observed here on treatment with *HP* in rats subjected to DMBA indicate that the change in vitamin levels

Table 4. Recovery of analysis of Retinol, Retinyl palmitate and β-Carotene in the serum extracted

Compound	Amount added (μg)	Control Recovery (%)	DMBA Recovery (%)	DMBA+HP Recovery (%)
Retinol	0.05	105.23	106.41	107.45
	0.5	98.32	97.54	96.78
	4	99.02	97.36	98.63
Retinyl palmitate	0.05	94.23	93.48	95.37
	0.5	96.85	94.22	92.27
	4	97.74	95.53	93.14
β-carotene	0.05	92.18	93.24	91.79
	0.5	93.41	94.91	92.83
	4	95.72	95.38	93.65

Table 5. Retinol, retinyl palmitate and vitamin C levels at different periods in control, DMBA and DMBA+*HP* groups in serum rat

Groups	Retinol (μg mL^{-1})		Retinyl palmitate (μg mL^{-1})		Vitamin C (μg mL^{-1})	
	0 day, $\overline{X}$ ± SEM	60 days, $\overline{X}$ ± SEM	0 day, $\overline{X}$ ± SEM	60 days, $\overline{X}$ ± SEM	0 day, $\overline{X}$ ± SEM	60 days, $\overline{X}$ ± SEM
Control	0.96 ± 0.09	1.32 ± 0.11^{a,b}	0.62 ± 0.09	0.72 ± 0.10^{b}	3.85 ± 0.30	4.59 ± 0.37^{c}
DMBA	1.15 ± 0.03	0.65 ± 0.05^{a}	0.75 ± 0.06	0.33 ± 0.07^{b}	4.78 ± 0.54	3.60 ± 0.21^{c}
DMBA+*HP*	1.08 ± 0.16	0.89 ± 0.07^{b}	0.61 ± 0.10	0.54 ± 0.08	4.34 ± 0.35	4.15 ± 0.15

$\overline{X}$ *=means*
SEM=standard error of mean
a=p<0,001, b=p<0,01, c=p<0,05

act as chemopreventive agent. We suggest that decreased vitamin levels are related to increased free radical production due to DMBA, and the potent chemopreventive efficiacy of *HP* might be due to its antioxidant effects.

4. Conclusion

The method developed and validated is relatively rapid, simple and showed good linearity, precision, accuracy, and sensitivity, being adequate for the simultaneous determination of R, RP and β-carotene in rat serum. HPLC system equipped with DAD could be used (R and RP) at 325 nm and β-carotene at 450 nm in the same run. The results obtained from this study demonstrated that the vitamin levels decreased in DMBA-treated group, but increased in *HP*-treated group.

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