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Comparative study of the antioxidant activity of phytosterols, DL methionine and N-acetylcysteine

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ABSTRACT

The antioxidant activity for phytosterols present in avocado soybean unsaponifiables (ASU), DL methionine and N-acetylcysteine (NAC) was analyzed by using different antioxidant assays such as total antioxidant activity assay, 1, 1-diphenyl-2-picryl-hydrazyl free radical (DPPH·) scavenging method and superoxide anion radical scavenging method. Total antioxidant activity was measured according to ferric thiocyanate method, NAC showed the highest ability to prevent linoleic acid peroxidation followed by phytosterols and DL methionine. In addition, NAC showed an effective DPPH scavenging activity as indicated by high antioxidant activity index (AAI = 4.152) which is higher than that of phytosterols (AAI = 0.495) while DL methionine showed no DPPH scavenging activity (AAI=zero). The superoxide scavenging activity had been studied in vitro using PMS-NADH (phenazine methosulfate- NADH) system, from the values of IC_{50} (concentration providing 50% inhibition), it is apparent that superoxide radical scavenging ability of NAC is 4.66 higher than that of DL methionine. © 2010 Trade Science Inc. - INDIA

INTRODUCTION

Free radicals are produced in normal and/or pathological cell metabolism[1]. Oxidation is essential to many living organisms for the production of energy needed for biological processes[2]. However, the uncontrolled production of oxygen- derived free radicals is involved in the onset of many diseases such as cancer, rheumatoid arthritis, cirrhosis and arteriosclerosis as well as in degenerative processes associated with ageing[3]. Exogenous chemical and endogenous metabolic processes in the human body or in the food system might produce highly reactive free radicals, especially oxygen derived radicals, which are capable of oxidizing biomolecules, resulting in cell death and tissue damage[4]. Almost all organisms are well protected against free radical damage by oxidative enzymes such as superoxide dismutase (SOD) and catalase (CAT), or chemical compounds such as a-tocopherol, ascorbic acid, carotenoids, polyphenol compounds and glutathione[5]. When the mechanism of antioxidant protection becomes unbalanced by factors such as ageing, deterioration of physiological functions, diseases and accelerated ageing occur[2]2. However, antioxidant supplements or antioxidant-containing foods may be used to help the human body to reduce oxidative damage[6]. Natural antioxidants are being extensively studied for their capacity to protect organisms and cells from damage brought on by oxidative stress without any side effect which is shown by synthetic antioxidants[7]. The three drugs studied are phytosterols, DL methionine and NAC. For phytosterols, they are triterpenes that are important structural components of plant membranes, and free phytosterols serve to stabilize phospholipid bilayers in plant cell membranes just as cholesterol does in animal cell

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membranes[8]. They have antioxidant activity[9] and potent anti-inflammatory properties, specially stigmasterol which is able to bind to chondrocyte membrane and possesses potential anti-inflammatory and anticatabolic properties[10]. DL methionine is an essential amino acid with an important role in biological methylation reactions. It constitutes the main supply of sulphur in the diet, preventing disorders in hair, skin or nails[11]. Moreover, it helps to reduce cholesterol levels by increasing the lecithin production in liver and acts as a natural chelating agent for heavy metal[12]. It also acts as hepatoprotectant, antidote in acetaminophen poisoning and urinary acidifier[13]. DL methionine is known as an antioxidant as it acts as a precursor amino acid for important antioxidant molecules such as glutathione, cysteine and taurine which protect the cells from oxidative damage and play vital role in detoxification[14]. In addition, methionine has been shown to chelate lead and remove it from tissues, it also has the hydroxyl and peroxynitrite radicals scavenging ability[4]. For N-acetylcysteine, it is considered to have mucolytic properties, it reduces complications by 22% to 29% in patients suffering from chronic bronchitis[15]. It also exhibits direct and indirect antioxidant properties, its free thiol group is capable of interacting with the electrophilic groups of ROS, this interaction with ROS leads to intermediate formation of NAC thiol, with NAC disulfide as a major end product . In addition, NAC exerts an indirect antioxidant effect related to its role as a GSH precursor[16]. GSH is a tripeptide made up of glutamic acid, cysteine, and glycine[3]. The antioxidant power of DL methionine has been evaluated using different in vivo and invitro techniques[17-19]. It has been reported that phytosterols exert an inhibitory effect against copper-induced lipid peroxidation of low density lipoproteins (LDLs) and have radical scavenging activity[9]. Direct activity of NAC is achieved by counteracting accumulated reactive oxygen species[20], such as hydroxyl radical[21], hypochlorous acid[22], it reacts slowly with H_2O_2[12] also it has scavenging activity against DPPH radical and superoxide anion[23].

In a research program dealing with the comparison of the antioxidant activity of these three drugs, we applied three different methods which are superoxide scavenging spectrophotometric method, Iron thiocyanate method and DPPH scavenging method.

The three methods have been validated. The rules for the measurements and the limits for the acceptance are given by appropriate pharmacopoeias[24,25].

EXPERIMENTAL

Instrumentation

- Absorption spectra were recorded on Double beam Shimadzu (Japan) 1601. Pc UV-VIS spectrophotometer connected to a computer fitted with UVPC personal spectroscopy software version 3.7, using matched quartz cuvettes in a thermostated cell holder. Measurments took place at 25°C (± 0.2)
- Jenway pH meter 3310 pH/ mV/C meter

Materials

Chemicals and reagents

Linoleic acid (sigma), 4×10^{-2} M in ethanol. Ethanol (Merck). Concentrated HCl (Merck). Ammonium thiocyanate (Prolabo). 30% W/V in ethanol. Ferrous chloride (Prolabo). 0.1% w/v in 3.5% HCl. Methanol (Merck). 2,2-Diphenyl-1-picrylhydrazyl radical (Sigma), prepared in two concentrations (0.2Mm, 0.124mM). Phenazine methosulphate (PMS) (Sigma, Germany)60 µM in phosphate buffer. Nitro blue tetrazolium (NBT) (Sigma, Germany) 150 µM in phosphate buffer. Nicotinamide adenine dinucleotide (NADH) (Sigma, Germany) 486 µM in phosphate buffer. Disodium hydrogen phosphate (Adwiac). Potassium dihydrogen phosphate (Adwiac).

Standard materials

DL methionine pure sample was kindly supplied by Hikma pharmaceutical Co., Cairo, Egypt. Purity was reported to be 100 ± 0.3%. NAC was kindely supplied by Mepaco pharma, Cairo, Egypt. Purity was reported to be 100 ± 0.2%.

Unsaponifiables of avocado and soya extract (ASU) were kindely supplied by Expanscience laboratories, Cairo, Egypt. Purity was reported to be 99 ± 0.5%.

Procedures

Superoxide scavenging method

The Superoxide scavenging activity was determined

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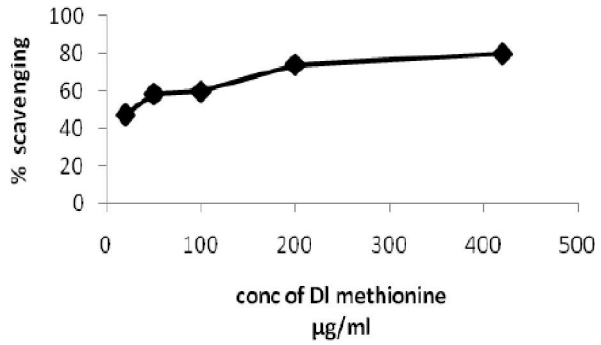

Figure 1 : Scavenging effect of DL methionine on superoxide radical

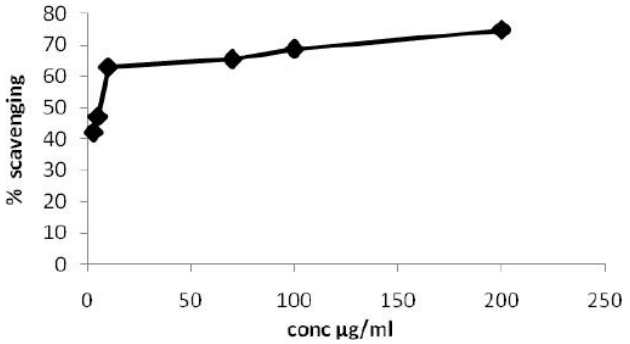

Figure 2 : Scavenging effect of NAC on superoxide radical

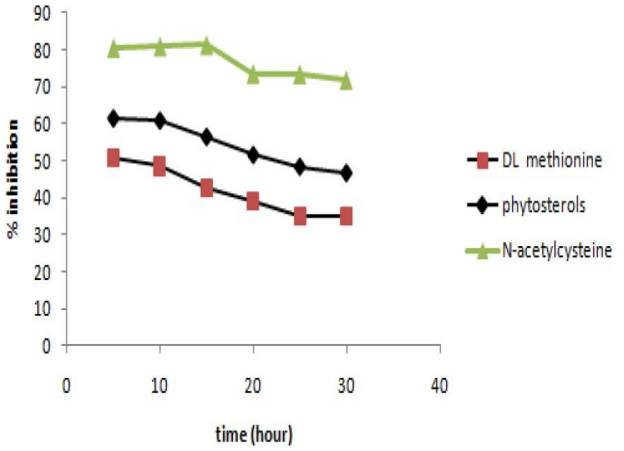

Figure 3 : Percentage inhibition of linoleic acid oxidation by DL methionine, phytosterols and N-acetylcysteine

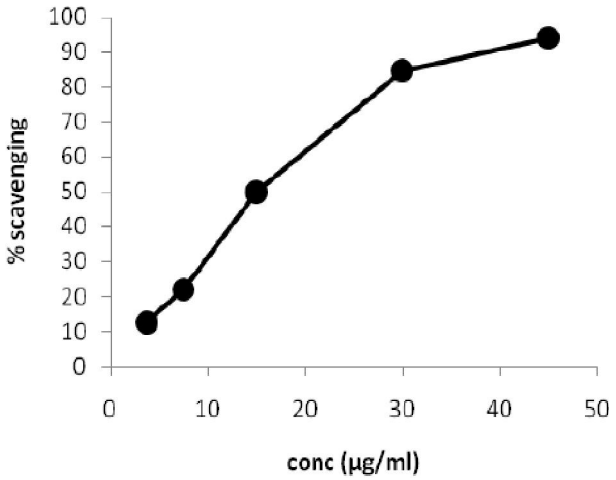

Figure 4 : Scavenging effect of ASU on 0.2mM DPPH

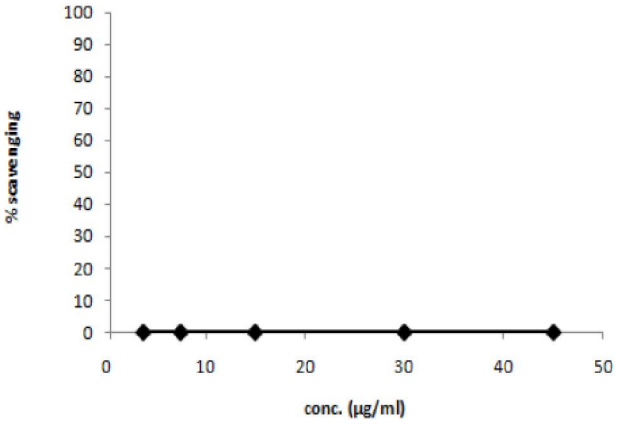

Figure 5 : Scavenging effect of DL methionine on 0.124 mM DPPH

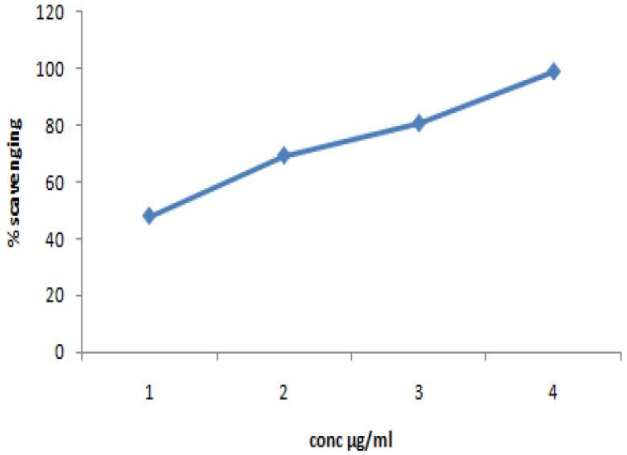

Figure 6 : Scavenging effect of NAC on 0.124 mM DPPH

using PMS-NADH system[26]. In a 10 ml volumetric flask different aliquots (0.2-4.2 ml) of 1000 µg ml^{-1} DL methionine standard solution and (0.03-2 ml) of 1000 µg ml^{-1} NAC standard solution were taken separately, 1 ml of 60µM PMS in 0.1 M phosphate buffer (PH 7.4), 1 ml of 468 µM NADH in phosphate buffer, 1 ml of 150 µM NBT in phosphate buffer, each one separately was added then the volume was completed with phosphate buffer then incubated at room temperature for 5 min and the color was measured at 560 nm. Control solutions were prepared, in which phosphate buffer was used instead of the drug and the same procedure

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was carried out. The reagents solutions were prepared daily.

Method validation

Precision

Repeatability

The intraday variation was evaluated by applying the previously mentioned procedure under Superoxide scavenging method for the analysis of different concentrations of DL methionine and NAC separately (n=6) and standard deviation was calculated.

Intermediate precision

The interday variation was evaluated by applying the previously mentioned procedure under Superoxide scavenging method for the analysis of different concentrations of DL methionine and NAC separately (n=6) and standard deviation was calculated.

Robustness

The robustness as a measure of method capacity to remain unaffected by small, but deliberate, variation in method parameters was carried out by changing the solvent to potassium dihydrogen phosphate buffer and standard deviation was calculated.

Iron thiocyanate method

In a 10 ml volumetric flask, 1 ml of ASU, DL methionine and NAC stock standard (50 mg%) solutions separately were taken, 2ml of 4×10^{-2} M linoleic acid and 0.2 ml of 0.1% ferrous chloride were added and the mixture was left at room temperature in dark for 48 hours . The peroxide content formed was evaluated by measuring the absorbance at 500 nm, after the addition of 0.1 ml of 30% ammonium thiocyanate at different time intervals and the volume was completed to 10 ml with ethanol. Control was carried out using the same procedure without the addition of any drug.

Method validation

Precision

Repeatability

The intraday variation was evaluated by applying the previously mentioned procedure under Iron thiocyanate method (n=6) and standard deviation was calculated.

Intermediate precision

The interday variation was evaluated by applying the previously mentioned procedure under Iron thiocyanate method (n=6) and standard deviation was calculated.

Robustness

The robustness was carried out by changing the solvent to methanol and standard deviation was calculated.

DPPH spectrophotometric method

In a 10 ml volumetric flask different aliquots (0.25 - 3ml) of the ASU stock standard solution (150 $\mu g\ ml^{-1}$), (0.25 - 3ml) of the DL methionine stock standard solution (150 $\mu g\ ml^{-1}$) and (1-4ml) of NAC stock standard solution (10 $\mu g\ ml^{-1}$) were taken separately, 1 ml of 0.2mM, 0.124mM DPPH; each one separately was added then the volume was completed with methanol. The mixture was shaken and left to stand in dark at room temperature for 30 minutes. The absorbances of the resulting solutions were measured at 517 nm. Control solutions were prepared, in which methanol was used instead of the sample and the same procedure was carried out. The assays were carried out in triplicate and the sample solution, as well as the $DPPH^{\cdot}$ solutions were prepared daily.

Method validation

Precision

Repeatability

The intraday variation was evaluated by applying the previously mentioned procedure under DPPH spectrophotometric method (n=6) and standard deviation was calculated.

Intermediate precision

The interday variation was evaluated by applying the previously mentioned procedure under DPPH spectrophotometric method (n=6) and standard deviation was calculated.

RESULTS AND DISCUSSION

Superoxide scavenging method

Nitro blue tetrazolium is a chemical compound com-

posed of two tetrazole moieties, which undergoes direct reduction by superoxide free radical to form blue formazan which has absorption at wavelength 560 nm[27], production of superoxide radical is occurred by reaction of phenazine methosulfate with NADH.

The influence of the sample on the scavenging of superoxide was measured by means of spectrophotometric measurement of the decrease in absorption compare to control. Results are expressed as:

$$\% \text{ scavenging} = ((A_{control} - A_{sample})/A_{control}) \times 100$$

The results of this method showed that DL methionine and NAC have direct superoxide scavenging activity and by calculating IC_{50} value (concentration of drug required to scavenge 50% of superoxide radical) from the curves prepared from the concentrations of drugs and percentage scavenging of superoxide radical as shown in figure 1, 2, IC_{50} values of DL methionine was found to be 27.5 µg/ml and NAC was found to be 5.9 µg/ml, but this method couldn't be applied on ASU as the solvent aqueous and phyosterols aren't soluble in aqueous solvents.

The results of assay validation are presented in TABLE 1, 2. The rules for the measurements and the limits for the acceptance are given by appropriate pharmacopoeias, from these data one can conclude that the proposed method meet all criteria required.

Iron thiocyanate method

During the linoleic acid peroxidation, peroxides are formed and that leads to oxidation of Fe^{2+} into Fe^{3+}. The latter ions form a complex with ammonium thiocyanate and this complex has a maximum absorbance at 500 nm. This step was repeated every 5 h. High absorbance indicates high linoleic acid emulsion peroxidation. Total antioxidant activity determination was performed triplicate. The inhibition percentage of lipid peroxidation in linoleic acid emulsion was calculated by following equation:

$$I\% = [(Abs_0 - Abs_1)/Abs_0] \times 100$$

where Abs_0 was the absorbance of the control and Abs_1 was the absorbance in the presence of the test compound at different concentrations[28].

The ferric thiocyanate method measures the amount of peroxide produced during the initial stages of oxidation which is the primary product of lipid oxidation.

TABLE 1 : Validation parameters of superoxide scavenging assay of DL methionine

Parameter	Value
Repeatability (SD_r)	0.476
Intermediate precision (SD_{int})	0.892
Robustness	0.378

TABLE 2 : Validation parameters of superoxide scavenging assay of NAC

Parameter	Value
Repeatability (SD_r)	0.292
Intermediate precision (SD_{int})	0.748
Robustness	0.246

TABLE 3 : Validation parameters of iron thiocyanate assay of phytosterols

Parameter	Value
Repeatability (SD_r)	0.004
Intermediate precision (SD_{int})	0.009
Robustness	0.071

TABLE 4 : Validation parameters of iron thiocyanate assay of DL methionine

Parameter	Value
Repeatability (SD_r)	0.009
Intermediate precision (SD_{int})	0.01
Robustness	0.065

TABLE 5 : Validation parameters of iron thiocyanate assay of NAC

Parameter	Value
Repeatability (SD_r)	0.004
Intermediate precision (SD_{int})	0.005
Robustness	0.054

A collective data of the antioxidant activity of DL methionine, phytosterols and N- acetylcysteine is shown in figure 3, it is apparent from the figure that N-acetylcysteine has the highest protection against linoleic acid oxidation and this effect increases at first then decreases slowly by time, phytosterols give moderate protection against linoleic acid oxidation and DL methionine has the least protection and this effect in phytosterols and DL methionine decreases by time.

The results of assay validation are presented in TABLE 3, 4, 5, from these data one can conclude that the proposed method meet all criteria required.

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TABLE 6 : Values for the antioxidant activity index (AAI) of phytosterols with different final concentrations of DPPH˙ showing intraday precision

DppH final conc 8 µg/ml	Time	I		II		III		mean IC_{50}	mean AAI	SD_r of AAI
		IC_{50}	AAI	IC_{50}	AAI	IC_{50}	AAI			
ASU	10 minutes	14.5	0.55	15.5	0.52	16.5	0.49	15.5	0.52	0.030
	30 minutes	14.5	0.55	17.5	0.46	17	0.47	16.3	0.49	0.049
DppH final conc 4.9 µg/ml	**Time**	**I**		**II**		**III**		**mean IC_{50}**	**mean AAI**	**SD_r of AAI**
		IC_{50}	AAI	IC_{50}	AAI	IC_{50}	AAI			
ASU	10 minutes	10	0.49	10	0.49	10	0.49	10	0.49	0
	30 minutes	9.5	0.5	9.5	0.5	9.5	0.5	9.5	0.5	0

I, II and III: Different measurements at the same day

TABLE 7 : Values for the antioxidant activity index (AAI) of phytosterols with different final concentrations of DPPH˙ showing interday precision

DppH final conc 8 µg/ml	Time	I		II		III		mean IC_{50}	mean AAI	SD_{int} of AAI
		IC_{50}	AAI	IC_{50}	AAI	IC_{50}	AAI			
ASU	10 minutes	15	0.53	19	0.42	17	0.47	17	0.47	0.055
	30 minutes	18.5	0.43	14.5	0.55	16	0.50	16.30	0.49	0.060
DppH final conc 4.9 µg/ml	**Time**	**I**		**II**		**III**		**mean IC_{50}**	**mean AAI**	**SD_{int} of AAI**
		IC_{50}	AAI	IC_{50}	AAI	IC_{50}	AAI			
ASU	10 minutes	11	0.45	10.5	0.47	10	0.49	10.5	0.47	0.020
	30 minutes	9.5	0.52	9	0.54	10	0.49	9.5	0.52	0.025

I, II and III: Different days of analysis

TABLE 8 : Values for the antioxidant activity index (AAI) of NAC with different final concentrations of DPPH˙ showing intraday precision

DppH final conc 8 µg/ml	Time	I		II		III		mean IC_{50}	mean AAI	SD_r of AAI
		IC_{50}	AAI	IC_{50}	AAI	IC_{50}	AAI			
NAC	10 minutes	1.8	4.44	1.8	4.44	1.9	4.21	1.83	4.31	0.133
	30 minutes	2	4	1.9	4.21	2.1	3.81	2.00	4.01	0.200
DppH final conc 4.9 µg/ml	**Time**	**I**		**II**		**III**		**mean IC_{50}**	**mean AAI**	**SD_r of AAI**
		IC_{50}	AAI	IC_{50}	AAI	IC_{50}	AAI			
NAC	10 minutes	1.1	4.45	1.1	4.45	1.2	4.08	1.13	4.33	0.214
	30 minutes	1.2	4.08	1.15	4.26	1.2	4.08	1.18	4.14	0.104

I, II and III: Different measurements at the same day

TABLE 9 : Values for the antioxidant activity index (AAI) of NAC with different final concentrations of DPPH˙ showing interday precision

DppH final conc 8 µg/ml	Time	I		II		III		mean IC_{50}	mean AAI	SD_{int} of AAI
		IC_{50}	AAI	IC_{50}	AAI	IC_{50}	AAI			
NAC	10 minutes	1.75	4.57	1.85	4.32	1.8	4.44	1.80	4.44	0.125
	30 minutes	2.2	3.64	1.8	4.44	1.9	4.2	1.97	4.09	0.411
DppH final conc 4.9 µg/ml	**Time**	**I**		**II**		**III**		**mean IC_{50}**	**mean AAI**	**SD_{int} of AAI**
		IC_{50}	AAI	IC_{50}	AAI	IC_{50}	AAI			
NAC	10 minutes	1.2	4.08	1.3	3.77	1.35	3.63	1.28	3.83	0.230
	30 minutes	1.25	3.92	1.1	4.45	1.4	3.5	1.25	3.96	0.476

I, II and III: Different days of analysis

DPPH spectrophotometric method

DPPH· (2,2-diphenyl-1-picrylhydrazyl) is a stable radical of organic nitrogen, characterised by a typical deep purple colour and a maximum absorbance in the range of 515–520 nm. The stable free radical DPPH· is well known as a good hydrogen abstractor yielding DPPH-H as byproduct[29].

In the presence of a hydrogen/ electron donor (free radical scavenging antioxidant) the absorption intensity is decreased, and the radical solution is discoloured according to the number of electrons captured. The violet colour of DPPH· faints into the yellow colour of its reduced congener (DPPH-H), with a high λ-shift in the visible spectra (from 517 nm to 330 nm).

The radical scavenging activity was calculated as follows:

$$I\% = [(Abs_0 - Abs_1) / Abs_0] \times 100$$

where Abs_0 is the absorbance of the control and Abs_1 is the absorbance in the presence of the test compound at different concentrations.

The IC_{50} (concentration providing 50% inhibition was calculated graphically by plotting the extract concentration vs. the corresponding scavenging effect as shown in figure 4, 5, 6. The antioxidant activity is expressed as the antioxidant activity index (AAI), calculated as follows as:

$$AAI = \frac{\text{final concentration of DPPHÿ (µg.ml - 1)}}{\text{IC50 (µg.ml - 1)}}$$

Thus, the AAI was calculated considering the mass of DPPH· and the mass of the tested compound in the reaction, resulting in a constant for each compound, independent of the concentration of DPPH· and sample used as shown in TABLE 6-9.When different DPPH· solutions were used for the same sample, the IC_{50} value varied, although the AAI value remained constant, since no significant differences between the AAI values found for each compound tested were observed. As it was mentioned, there is a deficiency to compare the antioxidant potential between samples due to the several ways that of the results are presented. The DPPH· index (I%) only shows the capacity of the sample, in a fixed concentration, to reduce or not the DPPH· radicals, in which many cases, increasing the sample concentration the I% will be increased. The IC_{50} shows the sample concentration necessary to decrease the initial DPPH· concentration by 50%, however, using different DPPH· concentration the results will be different for the same sample. So, the AAI relates the DPPH· concentration used in the assay with IC_{50} of the sample, resulting in a constant data for each compound or plant extract. DL methionine showed no DPPH· scavenging activity.

CONCLUSION

By applying the three spectrophotometric methods which detect the antioxidant activities of the three studied drugs, it is apparent from IC_{50} values that N-acetylcysteine is 4.66 times higher than that of DL methionine in superoxide scavenging activity; also its AAI indicates very strong antioxidant activity while phytosterols show moderate activity and DL methionine shows no scavenging activity of DPPH radical. For iron thiocyanate method NAC has the highest ability to prevent linoleic acid oxidation followed by phyosterols and DL methionine has lowest ability.

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