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Investigation on the Anti-Oxidant, Anti-Radical Capacities and the Reduction Properties, and the Measurement of the Phenolic and Flavonoid Compound Levels of Alcoholic, Hydro-Alcoholic and Aqueous Extracts of the *Galium Aparine* L. Plant

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ABSTRACT

The polyphenolic compounds, in particular, the anti-oxidants with herbal sources, constitute an essential part of a human’s nutritional diet. The anti-oxidants are among the agents neutralizing the free radicals and prevent from the prevalence of chronic diseases and destruction of many nutritional substances. These compounds are also extractable from the *Galium aparine* L. plant. The objective of the present study is to investigate the anti-oxidant, anti-radical capacities and the reduction properties, and the measurement of the phenolic and flavonoid compound levels of alcoholic, hydro-alcoholic and aqueous extracts of the *Galium aparine* L. plant. After extraction, the total phenolic and flavonoid levels of the extracts were measured. Then, the total anti-oxidant capacity was evaluated on the basis pf comparison to synthetic anti-oxidants (BHA, BHT, and TBHQ) and cupric reduction method (CUPRAC). In final, the anti-radical properties of the extracts and the ferric reducing power of the extracts were measured comparatively. The obtained results of the investigation represented that the highest amount of the total phenol for the aqueous extract equaled to 91.5641 ± 0.035 micrograms of Gallic acid per ml of extract, and the highest amount of flavonoid for an alcoholic extract equaled to 363.733±2.186 micrograms of Quercetin per ml of extract. The results of the comparative assay of the common anti-oxidants represented that the highest anti-oxidant capacity pertained to TBHQ with a level of 1.0211 micrograms per ml, and among the extracts, the highest amount pertained to the aqueous extract (0.1742 micrograms per ml). Comparison between the averages of the main treatments of anti-oxidants in terms of the total anti-oxidant capacity based on CUPRAC method represented that the highest amount pertained to the alcoholic extract (0.3814 micrograms per ml). The highest inhibitory power in the DPPH test pertained to TBHQ and alcoholic extract with amounts of 96.0696 and 96 micrograms per ml respectively. The highest amount for TBHQ in the total reduction test was obtained 2.0590 micrograms per ml. In the results of HPLC spectrum, the highest flavonoid compound of Quercetin type pertained to hydro-alcoholic extract with an amount of 6.681±0.03 mg/L of extract, and the lowest flavonoid compound of Quercetin type pertained to aqueous extract with an amount of 2.2401±0.04 mg/L of extract. According to the examined results, the *Galium aparine* L. plant having high anti-oxidant properties may be used as a natural anti-oxidant based on different methods. In this regard, the ethanolic extract has the highest efficiency.

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INTRODUCTION

Free radicals are molecules comprising of one or several uncoupled (single) electrons in their utmost layers and they are very reactive (Birben *et al.*, 2012). These substances are created through lateral reactions of the body's metabolic processes (Kohen & Nyska, 2002). Though there are numerous free radicals in the body, those derived from oxygen and/or nitrogen (Reactive oxygen/nitrogen RONS) (species) are of higher importance in the human's biotic system (Fisher-Wellman K & Bloomer, 2009). the free radicals and other active types of oxygen continuously during the natural process of cell metabolism (Urso & Clarkson, 2003). Despite the beneficial role the oxygen plays in regard to continuation of lives in the organisms, the active types of oxygen (ROS) include: superoxide radicals (O_2^-), single radical (O_2), hydrogen peroxide (H_2O_2), Hydroxyl (OH), nitric oxide, (NO), peroxyl (ROO) and peroxynitrite (ONOO) result in harmful changes like peroxidation of lipids, deactivation of enzymes and harming the DNA through oxidative (Jin et al, 2001; Kulisic et al, 2004; Vagi et al, 2005). The anti-oxides, in particular, those having cyclic phenolic foundation containing OH groups, are compounds which prevent rancidity and color changes of fats and oils by absorbing the free radicals and consequently preventing the oxidation, and they play an important role in preventing the fat oxidations (Fenema, 1996). The anti-oxides with low concentrations postpone the oxidation of the substances significantly by preventing the accomplishment of oxidative chain reactions (Pizzale *et al.*, 2002; Tomaino *et al.*, 2005). The anti-oxides being generated in the body encounter free radicals by both enzyme and non-enzyme defensive systems and on one hand, these compounds cause a reduction in the hazard of affection to cardiovascular diseases and strokes, and on the other hand, they prevent the progression of cancers which cause harming the DNAs (Noguchi *et al.*, 2000). Despite the existence of different anti-oxides in plasma, the body's defensive system is not solely able to annihilate the free radicals generated in the body. This is why it requires the provision of anti-oxides from external sources, which are provided through nutritional sources (Young & Woodside, 2001). Despite the fact that the synthetic anti-oxides act effectively during thermal processes and conservation conditions, there are a lot of evidences confirming the poisonousness and malnutrition effects of the synthetic anti-oxides added to nutritional substances such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert beta hydroxyquinone (TBHQ) and propyl gallate (PG) (Frankel, 1999). In addition to these side effects, among the disadvantages of using the synthetic anti-oxides are the hazard of liver injury and generation of cancer in laboratorial animals (Gao *et al.*, 1999; Williams *et al.*, 1999). The strongest synthetic anti-oxide (TBHQ) is not allowed to be consumed in Japan, Canada and Europe, and BHA is removed from GRAS compounds list as well. Therefore, investigation on natural anti-oxides as replacements for synthetic ones is of particular importance (Shahidi, 1997). Hence, according to the above-mentioned cases, as the side effects of the synthetic anti-oxides are recognized to put poisonous effects on consumers, derange the liver enzymes, and cause the generation of various types of cancers, their consumption is being limited in the world. Therefore, the necessity to have strong anti-oxides with lower poisonousness and higher effectiveness is unavoidable. Today, many nutritional experts recommend consumption of plants, fruits, and vegetables for provision of anti-oxides required for the body, because the consumption of herbal anti-oxides generate fewer side effects and better treatment (Frankel, 1999). Since the plants are one important source of polyphenols (anti-oxides) (Lindberg & Bertelsen, 1995), researches are increasing in this regard. The plants rich of anti-oxide compounds may result in maintaining the cells against oxidative harms (Komaran & Karionakaran, 2006) and this anti-oxide activity degree and the level of increase in herbal anti-oxides depend on the plant type, increment stage, metabolic conditions, duration length, and stress intension. The natural anti-oxides result by an increase of plasma anti-oxide powers and reduction of affection to some diseases such as cancer, cardiac diseases and strokes (Prior & Cao, 2000). The secondary metabolites derived from plants as total phenol and flavonoid have strong potentials to purge the free radicals, which exist in different parts of a plant such as a leaf, fruit, grain, root and skin (Mathew & Abraham, 2006). Therefore, according to the high prevalence of chronic and erosive diseases, it is reasonable to use the plants to provide the anti-oxides required for the body, in particular, those plants, which have high total phenol and flavonoid. Indeed, the phenol compounds existing in herbal substances such as spicery, vegetables and fruits result in the emergence of anti-oxide properties in the, (Ali Mirzaei *et al.,* 2011).

MATERIALS AND METHODS

This research was carried out in the laboratory of the faculty of agriculture and natural resources at Azad University of Sanandaj in spring and summer 2016. The *Galium Aparine* L. plant (at the stage of blossoming) was collected from the northwestern side of Lake Zrebar in Marivan city located in Kurdistan province (the location is represented in figure 1) in May. The center for recognized the studied species agricultural and natural resource researches of Kurdistan province in the herbarium section with corresponding herbarium code. In order to optimize the extraction of phenol and flavonoid compounds and investigation on the anti-oxide properties, the considered plant (all of its sections) was immediately packed in nylon packages after collection and isolation of impurities and it was kept in -20° C until the experimentation time. In the present investigation, the treatments were carried out through three repetitions and the charts were plotted using Microsoft Excel 2010. The statistical analyses were carried out using SAS v.9.2, and in the following, the comparison test of the least significant difference (LSD) mean was utilized to compare the averages, and all data were reported as mean ± SD (standard deviation). The results were considered significant at a confidence value of 0.1%. Also, all consumed substances of the experimentation are summarized in table 1.

Fig.1. The topography of the location of Galium Aparine L. collection

Table.1. table of chemicals and indicators used in the experimentation

Chemicals	
96% medical ethanol	Sulfuric acid
Gallic acid	Sodium phosphate
Folin- Ciocalteus	Ammonium molybdate tetrahydrate
Sodium Carbonate	Potassium dihydrogen phosphate
Quercetin	Dipotassium hydrogen phosphate
Alumonium Chloride	Potassium ferricyanide
Potassium acetate	Trichloroacetic acid
DPPH	Iron chloride
TBHQ	Copper chloride
BHT	Neocuproine
BHA	Acetic acid
Methanol	Ammonium acetate

RESULTS

The results existing in table.2 represent that the extracts which are extracted by different solvents at 5% level on the basis of LSD test are significant in which the highest amount of total phenol concentration by gallic acid pertains to the aqueous extract and is 91.5641 micrograms per ml, and the alcoholic and hydro-alcoholic extracts are in one group and they did not represent any significant difference in terms of the level of total phenol.

The results existing in table.3 represent that the extracts, which are extracted by different solvents at 5% level by LSD test, are significant in which the highest amount of total flavonoid concentration by quercetin pertains to the alcoholic extract and is 363.733 micrograms per ml, and the aqueous extracts have the lowest amount, which equals to 45.067 micrograms per ml.

The results of the chart in figure.2 represents that the highest anti-oxide concentration pertains to TBHQ with an amount of 1.0211 micrograms per ml, and there is no significant difference between the samples of extracts and they are placed in one group statistically, and the highest amount pertains to the aqueous extract. Also, the highest anti-oxide capacity pertaining to 500-micrograms per ml concentration equals to 1.400 micrograms per ml, and the lowest amount pertains to 50-micrograms per ml concentration Based on the results of figure.3, the highest capacity pertains to the alcoholic extract and the lowest one pertains to the aqueous extract.

The results existing in figure.4 represent that all common anti-oxides have stronger and further inhibitory power with significant differences at the maximum allowable concentration of consumption in nutritional substances, i.e. 200 micrograms per liter and even lower concentration of 100 micrograms per liter than most of the *Galium aparine* L. extract concentrations. The highest inhibitory power pertains to TBHQ and alcoholic extract with values of 96.0686 and 96 , which are placed in one group. On the other hand, as the extract concentration increases, the inhibitory power increases significantly. According to the obtained results and plotting the regression lines for different extract concentrations, IC_{50} was determined 681.35, 452.59 and 386.77 for aqueous, hydro-alcoholic and alcoholic extracts respectively.

The results of figure.5 represent that the highest anti-oxide capacity pertains to 800-micrograms per ml concentration with an amount of 1.6146 micrograms per ml, and the lowest amount pertains to the 100-micrograms per ml concentration. Also, the results represented that the highest reduction power pertains to TBHQ with an amount of 2.0590 micrograms per ml, and there is no significant difference between the extract samples and they are placed in one group statistically, and the highest amount pertains to the hydro-alcoholic extract.

Table.2. Comparison between the different treatments of *Galium aparine* L. anti-oxide extracts in terms of the level of total phenol

Type of extract treatment	The average of total phenol concentration by gallic acid (Micrograms per ml)
Aqueous extract	91.5641±0.035 [A]
Hydro-alcoholic extract	24.6410±0.023[B]
Alcoholic extract	24.1282±0.023[B]

Table.3. Comparison between different treatments of *Galium aparine* L. anti-oxide extracts in terms of total flavonoid

Type of extract treatment	The average of total flavonoid concentration by quercetin (Micrograms per ml)
Aqueous extract	45.067±11.345[c]
Hydro-alcoholic extract	202.400±20.817[b]
Alcoholic extract	363.733±2.186[a]

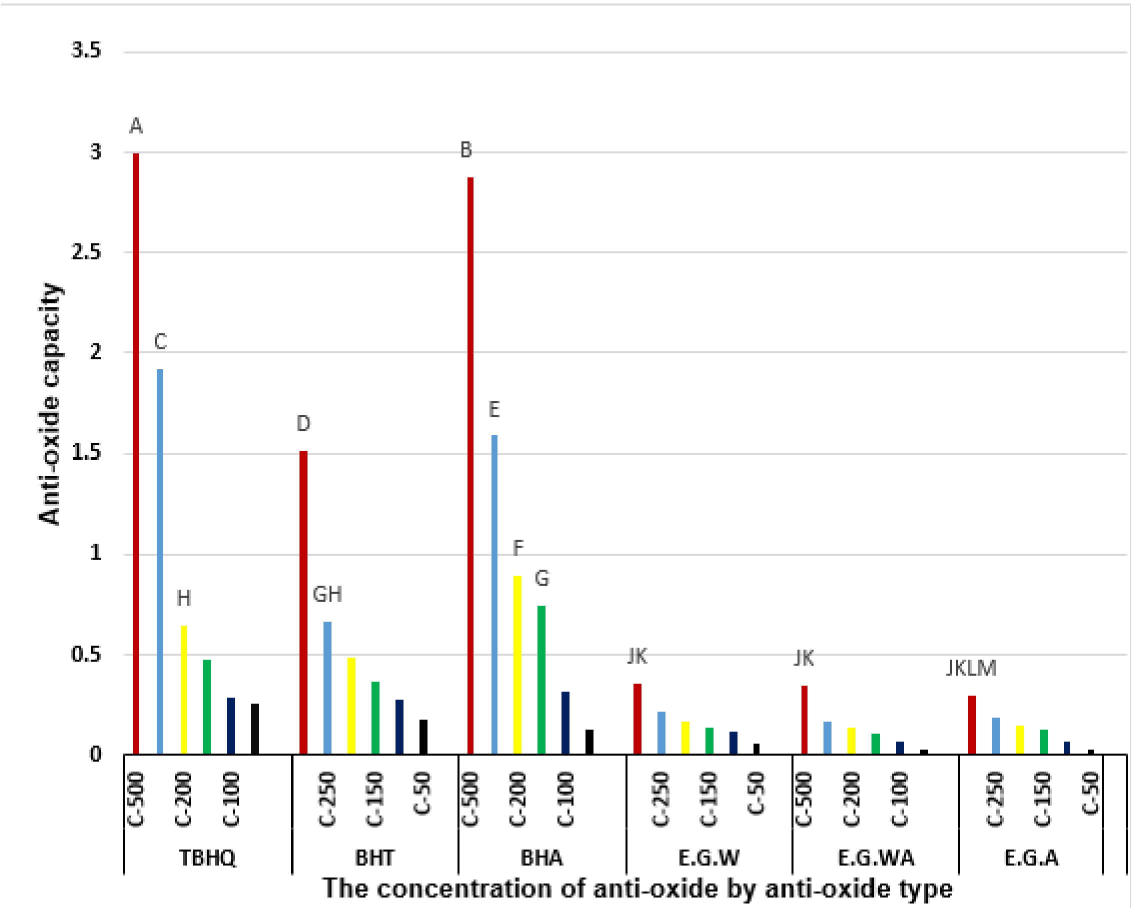

Fig.2. Comparison between the average of counteractive effect of anti-oxide types and different concentrations of anti-oxides in terms of total anti-oxide capacities

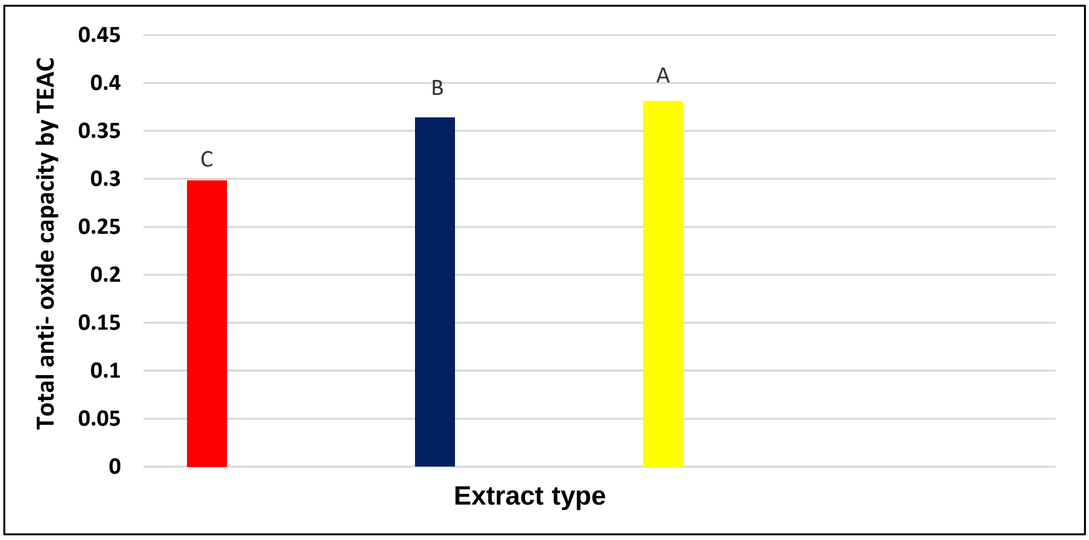

Fig.3. Comparison between different extracts of Galium aparine L. anti-oxides in terms of total anti-oxide capacity using CUPRAC method (red color: aqueous extract, blue color: hydro-alcoholic extract, yellow color: alcoholic extract)

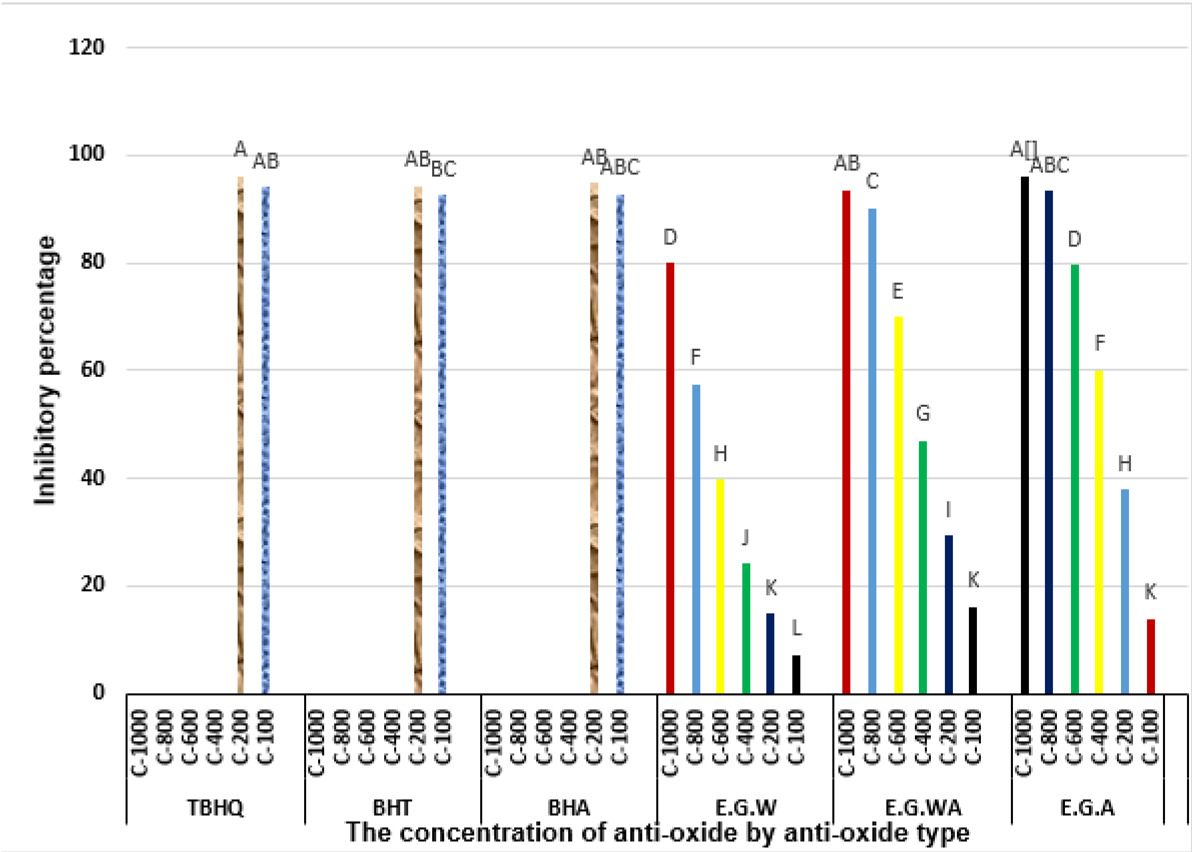

Fig.4. Comparison between the average of anti-oxides and different concentrations of common Galium aparine L. anti-oxide extracts in terms of inhibitory percentage DPPH

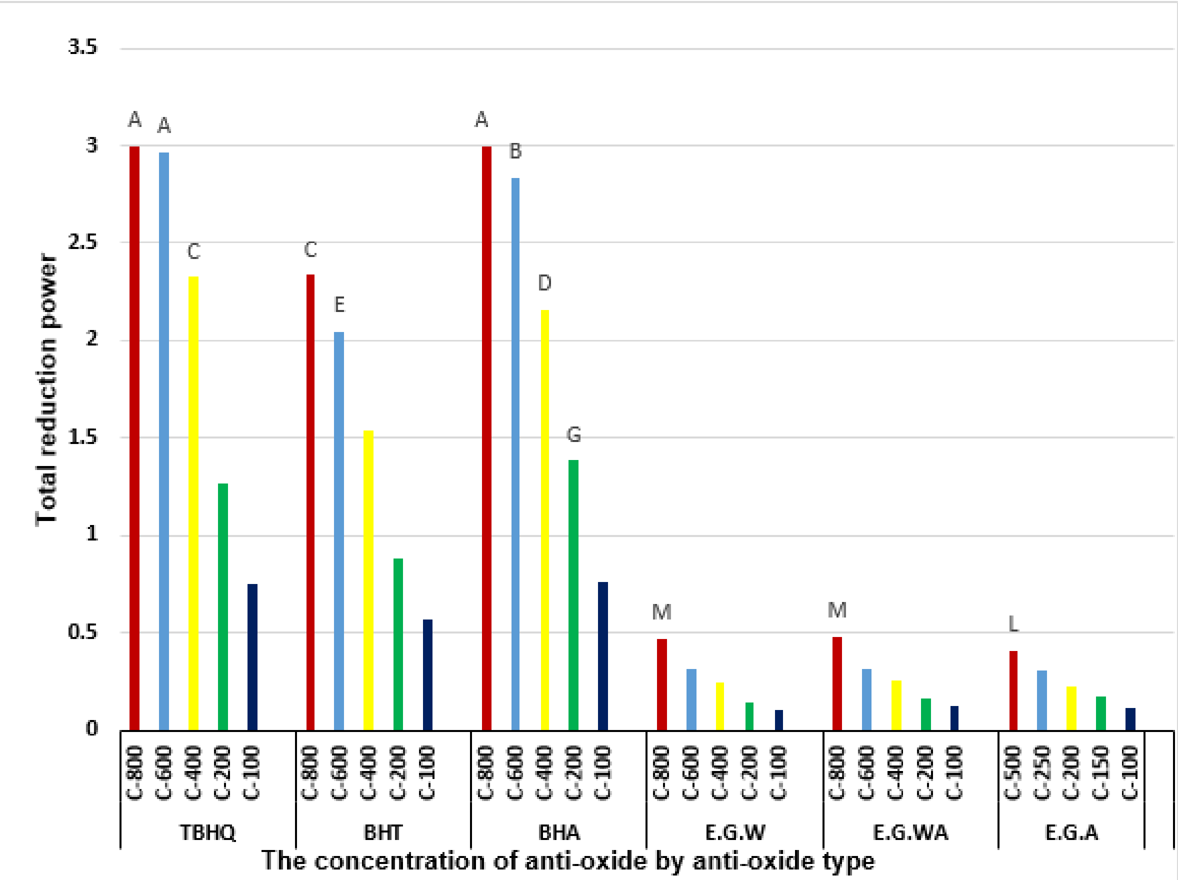

Fig.5. comparison between the average of counteractive effect of anti-oxide type and different concentrations of anti-oxides in terms of total reduction power

DISCUSSION

On one hand, the anti-oxides cause reduction in the hazard of affection to cardiovascular diseases and strokes, and on the other hand, they prevent the progression of cancers, which cause harming the DNAs. Despite the existence of different anti-oxides in plasma, the body's defensive system is not solely able to annihilate the free radicals generated in the body. This is why it requires the provision of anti-oxides from external sources, which is provided through nutritional sources. There areas a lot of evidence confirming the poisonousness and malnutrition effects of synthetic anti-oxides added to nutritional substances such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert beta hydroxyquinone (TBHQ). In addition to this hazard, liver injury and generation of cancer in laboratorial animals are among the disadvantages of using the synthetic anti-oxides.

Therefore, the necessity to have strong anti-oxides with lower poisonousness and higher effectiveness is unavoidable. Today, many nutritional experts recommend consumption of plants, fruits, and vegetables to provide the anti-oxides required for the body, because usually, the consumption of herbal anti-oxides generate fewer side effects and better treatment. Since plants are one important source of anti-oxides, researches are increasing in this regard. Plants rich of anti-oxide compounds may result in maintaining the cells against oxidative harms. Natural anti-oxides cause an increase in the power of plasma anti-oxides and reduction in affection to some diseases such as cancer, cardiac diseases and brain strokes. Secondary metabolites are derived from plants like total phenol and flavonoid, are derived from plants having strong potential to purge free radicals, which exist in different sections of a plant such as a leaf, fruit, grain, root and skin. On the other hand, the *Galium aparine* L. plant is a one-year plant with a foursquare stalk, which is accounted for the class of this type of plants. Hence, according to the cases mentioned in the study, it is proceeded to investigate the anti-oxide, anti-radical capacities, reduction properties, and measurement of the level of phenolic and flavonoid compounds of alcoholic, hydro-alcoholic and aqueous extracts of *Galium aparine* L. plant.

The results obtained from the investigation represented that the highest amount of total phenol for the aqueous extract equals to 91.5641±0.035 milligrams of Gallic acid per gram of extract, and the highest amount of flavonoid for the alcoholic extract equals to 363.733±2.186 milligrams of quercetin per gram of extract. The results of the comparative test with common anti-oxides represent that the highest anti-oxide capacity pertains to TBHQ with an amount of 1.0211 micrograms per ml in which the highest amount pertains to aqueous extract. The comparison between the averages of the main anti-oxide treatments in terms of total anti-oxide capacity by CUPRAC method represents that the highest amount pertains to the alcoholic extract (0.3814 micrograms). The results of DPPH test represent the high power of common anti-oxides in comparison to the studied extract. In the reduction power test, the highest amount was obtained for TBHQ (2.0590 micrograms per ml). The results of HPLC spectrum implicate the appropriate value of flavonoid compounds in the examined extracts, and the highest flavonoid compound of quercetin type pertains to the hydro-alcoholic extract with an amount of 6.681±0.03 milligrams per liter of extract, and the lowest one pertains to the aqueous extract with an amount of 2.2401±0.04. Considering the investigated results, the *Galium aparine* L. plant is a good source of anti-oxide compounds. The results represented that the type of solvent and the level of extract concentration affects the level of polyphenolic compounds and the anti-oxide properties and increases as the concentration, anti-oxide properties, and polyphenolic compounds of the extract increase. Finally, the ethanol extract was determined as the best solvent for the most extraction of phenolic and anti-oxide compounds. Hence, according to the point that the extracts of the *Galium aparine* L. have different levels of anti-oxide activities, they may be considered as a potential source of natural anti-oxides in order to replace the synthetic anti-oxides and even treat some of the diseases. In order to recognize the active chemical compounds of a plant and more comprehensive biologic evaluation, further researches are required.

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