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Antioxidant activity and improvement in the yield of antioxidant compounds

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ABSTRACT

Antioxidants are the first line of defense against free radical damage and are critical for maintaining optimum health and well-being. Exposure of biological system to xenobiotics, pollutants, ionizing radiations or UV light, and development of certain pathological conditions consequently increases production of oxyradicals. Oxygen radicals such as superoxide anions, hydroxyl radicals, and hydrogen peroxide are cytotoxic and give rise to tissue injuries. According to various evidences, compounds derived from natural sources are helpful in providing protection against free radicals. There is an increasing demand for natural antioxidants due to safety concerns for synthetic antioxidants, in addition to increasing consumer preference for natural products, clean label, and less usage of food additives in food products. Therefore, an attempt has been made to review novel techniques such as microwave-assisted extraction and pressurized liquid extraction for recovery of high yield of antioxidant compounds with less solvent and time consumption and also some *in vitro* and *in vivo* models for determining antioxidant properties of products from medicinal plants.

Key words: Antioxidants, extraction, health, disease, high yield, natural compounds, oxygen radicals

INTRODUCTION

Antioxidants are compounds or systems that can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged.[18]

The use of antioxidants for human health has promoted research in the fields of food sciences and medicinal plants to assess their potential as antioxidants. Role of antioxidant includes free radical scavenging capacity, inhibition of lipid peroxidation (LPO), metal ion chelating ability, and reducing capacity. The term reactive oxygen species (ROS) encompasses all highly reactive, oxygen-containing molecules, including free radicals. Types of ROS include the hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides [Table 1]. All are capable of reacting with membrane lipids, nucleic acids, proteins, enzymes, and other small molecules, resulting in cellular damage. Oxidative damage to DNA, proteins, and other macromolecules has been implicated in the pathogenesis of a wide variety of diseases, most notably heart disease and cancer. A growing body of animal and epidemiological studies, as well as clinical intervention trials, suggest that antioxidants may play a pivotal role in preventing or slowing the progression of both heart disease and some forms of cancer. When the balance between ROS production and antioxidant defenses is lost, "oxidative stress" results, which through a series of events deregulates the cellular functions leading to various pathological conditions.[1] The article tries to take a different perspective on antioxidants for the new researchers and industries involved in this field, seeking for extracting high yield of antioxidant compounds due to their increasing demand.

MEASUREMENT OF ANTIOXIDANT ACTIVITY

The various methods used for the measurement of antioxidant activity are as follows:

In vitro Methods

1. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity
2. Hydrogen peroxide scavenging (H_2O_2) assay
3. Nitric oxide scavenging activity
4. Peroxynitrite radical scavenging activity
5. Trolox equivalent antioxidant capacity method/2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assay
6. Total radical-trapping antioxidant parameter method
7. Ferric reducing antioxidant power (FRAP) assay
8. Superoxide dismutase radical scavenging activity (SOD)
9. Hydroxyl radical scavenging activity
10. Hydroxyl radical averting capacity method

Table 1: Various ROS and corresponding neutralizing antioxidants[1]

ROS	Neutralizing antioxidants
Hydroxyl radical	Vitamin C, glutathione, flavonoids, lipoic acid
Superoxide radical	Vitamin C, glutathione, flavonoids, SOD
Hydrogen peroxide	Vitamin C, glutathione, beta carotene, Vitamin E, CoQ10, flavonoids, lipoic acid
Lipid peroxides	Beta carotene, Vitamin E, ubiquinone, flavonoids, glutathione peroxidase

ROS: Reactive oxygen species, SOD: Superoxide dismutase

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11. Oxygen radical absorbance capacity method
12. Reducing power method (RP)
13. Phosphomolybdenum method
14. Ferric thiocyanate method
15. Thiobarbituric acid method
16. N, N-dimethyl-p-phenylenediamine dihydrochloride method
17. b-carotene linoleic acid method/conjugated diene assay
18. Xanthine oxidase method
19. Cupric ion reducing antioxidant capacity method
20. Metal chelating activity.

In vivo Models

1. Ferric reducing ability of plasma
2. Reduced glutathione (GSH) estimation
3. Glutathione peroxidase estimation
4. Glutathione-S-transferase
5. SOD method
6. Catalase
7. c-Glutamyl transpeptidase activity assay
8. Glutathione reductase (GR) assay
9. LPO assay
10. Low-density lipoprotein assay.

DPPH scavenging activity

The molecule DPPH (a,a-diphenyl-bpicrylhydrazyl; DPPH) is characterized as a stable-free radical by virtue of the delocalization of the spare electron over the molecule as a whole so that the molecule does not dimerize, as would be the case with most other free radicals. The delocalization of electron also gives rise to the deep violet color, characterized by an absorption band in ethanol solution centered at about 517 nm. When a solution of DPPH is mixed with that of a substrate (AH) that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet color. To evaluate the antioxidant potential through free radical scavenging by the test samples, the change in optical density of DPPH radicals is monitored. The sample extract (0.2 ml) is diluted with methanol and 2 ml of DPPH solution (0.5 mM) is added. After 30 min, the absorbance is measured at 517 nm. The percentage of the DPPH radical scavenging is calculated using the equation as given below:

% inhibition of DPPH radical = ([Abr−Aar]/Abr)×100

Where, Abr is the absorbance before reaction and Aar is the absorbance after reaction has taken place.[2]

Hydrogen peroxide scavenging (H_2O_2) assay

Human beings are exposed to H_2O_2 indirectly through the environment nearly about 0.28 mg/kg/day with intake mostly from leaf crops. Hydrogen peroxide may enter into the human body through inhalation of vapor or mist and through eye or skin contact. H_2O_2 is rapidly decomposed into oxygen and water, and this may produce hydroxyl radicals ($OH^{\cdot}$) that can initiate LPO and cause DNA damage in the body. The ability of plant extracts to scavenge hydrogen peroxide can be estimated. A solution of hydrogen peroxide (40 mM) is prepared in phosphate buffer (50 mM pH 7.4). The concentration of hydrogen peroxide is determined by absorption at 230 nm using a spectrophotometer. Extract (20–60 μg/ml) in distilled water is added to hydrogen peroxide and absorbance at 230 nm is determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging is calculated as follows:

% scavenged (H_2O_2) = [(Ai−At)/Ai]×100

Where, Ai is the absorbance of control and At is the absorbance of test.[2]

Ferric reducing ability of plasma

It is one of the most rapid tests and very useful for routine analysis. The antioxidative activity is estimated by measuring the increase in absorbance caused by the formation of ferrous ions from FRAP reagent containing 2, 4, 6-tripyridyl-s-triazine (TPTZ) and $FeCl_2 \cdot 6H_2O$. The absorbance is measured spectrophotometrically at 593 nm. The method involves the use of blood samples that are collected from the rat retro-orbital venous plexus into heparinized glass tubes at 0, 7, and 14 days of treatment. 3 ml of freshly prepared and warm (37°C) FRAP reagent, 1 ml (10 mM) of TPTZ solution in 40 mM HCl, 1 ml of 20 mM $FeCl_2 \cdot 6H_2O$, and 10 ml of 0.3 M acetate buffer (pH 3.6) are mixed with 0.375 ml distilled water and 0.025 ml of test samples. The absorbance of developed color in organic layer is measured at 593 nm. The temperature is maintained at 37°C. The readings at 180 s are selected for the calculation of FRAP values.[2]

GSH estimation

GSH is an intracellular reductant and plays major role in catalysis, metabolism, and transport. It protects cells against free radicals, peroxides, and other toxic compounds. Deficiency of GSH in the lens leads to cataract formation. GSH also plays an important role in the kidney and takes part in a transport system involved in the reabsorption of amino acids. The method can be used for the determination of antioxidant activity. The tissue homogenate (in 0.1 M phosphate buffer pH 7.4) is taken and added with equal volume of 20% trichloroacetic acid containing 1 mM EDTA to precipitate the tissue proteins. The mixture is allowed to stand for 5 min before centrifugation for 10 min at 2000 rpm. The supernatant (200 μL) is then transferred to a new set of test tubes and added with 1.8 ml of the Ellman's reagent (5,50-dithiobis-2-nitrobenzoic acid [0.1 mM] prepared in 0.3 M phosphate buffer with 1% of sodium citrate solution). Then, all the test tubes are made up to the volume of 2 ml. After completion of the total reaction, solutions are measured at 412 nm against blank. Absorbance values were compared with a standard curve generated from known GSH.[2]

NOVEL TECQNIQUES

The techniques used for the high extraction yield of natural compounds are as follows:

Microwave-assisted Extraction (MAE)

The MAE process is a high-speed method used to selectively extract target compounds from various raw materials. MAE uses energy of microwave radiation to heat solvents quickly and efficiently. Using a closed system, extraction can be performed at higher temperatures and extraction times (ETs) can be reduced drastically. It is an innovative solvent extraction technology, offers a superior alternative to several thermal applications owing to its

efficient volumetric heat production, and has many advantages over conventional solid–liquid extraction methods. Applications include the extraction of high-value compounds from natural sources including phytonutrients, nutraceutical and functional food ingredients, and pharma actives from biomass. Two parameters define the dielectric properties of materials. The first, is ε', the dielectric constant which, describes the polarizability of the molecule in an electric field. The dielectric loss factor, ε", measures the efficiency with which the absorbed microwave energy can be converted into heat. The ratio of the two terms is the dissipation factor, δ, ultrasonic extraction.[20]

$\delta = \varepsilon''/\varepsilon'$

The use of microwave irradiation is another way of increasing the efficiency of conventional extraction methods. MAE consists of heating the solvent in contact with the sample by means of microwave energy. The process involves disruption of hydrogen bonds, as a result of microwave-induced dipole rotation of molecules, and migration of the ions, which enhance penetration of the solvent into the matrix, allowing dissolution of the components to be extracted.[19]

Pressurized solvent extraction

Accelerated solvent extraction (ASE) is a solid–liquid extraction process performed at elevated temperatures, usually between 50 and 200°C and at pressures between 10 and 15 MPa. Therefore, ASE is a form of pressurized solvent extraction that is quite similar to supercritical fluid carbon dioxide extraction (SFE). Extraction is carried out under pressure to maintain the solvent in its liquid state at high temperature. The solvent is still below its critical condition during ASE. Increased temperature accelerates the extraction kinetics and elevated pressure keeps the solvent in the liquid state, thus achieving safe and rapid extraction. Furthermore, pressure allows the extraction cell to be filled faster and helps to force liquid into the solid matrix. Elevated temperatures enhance diffusivity of the solvent resulting in increased extraction kinetics. A typical schematic diagram of an ASE system is given in Figure 1. Although the solvent used in ASE is usually organic solvent. Pressurized hot water or subcritical water can also be used in an ASE apparatus, which is usually called pressurized hot water extraction or subcritical water extraction.[3]

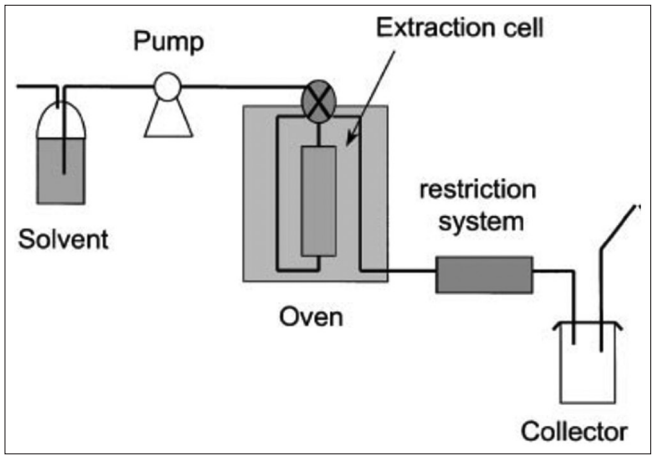

Figure 1: Scheme of a pressurized solvent extraction system

A solid or semi-solid sample is placed into a stainless steel extraction cell which is filled with solvent and heated (50–200°C) in an oven.

The heating process generates solvent expansion and thus pressure in the extraction cell, typically in the region of 500–3000 psi. To prevent overpressurization of the cell, a static valve pulses open and closed automatically when the cell pressure exceeds the set point. The solvent that escapes during this venting is collected in a vial. A static extraction stage of about 5–10 min is followed by pumping fresh solvent through the system to rinse the sample and the tubing. All the solvents present in the system is then purged with a compressed gas, generally nitrogen, and the total solvent volume (extractant and rinsing) is collected in the vial. The Dionex apparatus offers the possibility of automation and up to 24 samples can be sequentially extracted. Cells can be of different sizes: 1, 5, 11, 22, and 33 ml, different pollutants from various environmental matrixes. After comparison with the corresponding reference methods, recoveries and precision were generally found equivalent, or even better. Most papers stress the shorter ETs and lower solvent consumption of ASE in comparison to other conventional preparation techniques of environmental samples with the exception of SFE. ASE and SFE can be considered as complementary, the first allowing the extraction of polar compounds, while the second is more selective for compounds of low or medium polarity. The ASE methodology is accepted in the US EPA SW-846 Method 3545A for selected priority pollutants from environmental matrixes.[4]

Advantages

The main advantages of novel techniques over the conventional extraction techniques are as follows:

- Reduced solvent consumption
- Shorter operational times
- Moderately high recoveries
- Good reproducibility and
- Minimal sample manipulation for extraction process.[20]

Various Investigations

- The MAE of polyphenols from *Equisetum arvense* (horsetail) revealed that more polyphenols can be extracted from waste horsetail aqueous ethanol, for just 80 s, when compared to conventional extraction method (solid–liquid extraction) for 12 h. The optimal conditions were reached with 54.5% of ethanol, 45.1 ml/g liquid/solid ratio, the microwave power (MWP) of 170 W, and 80 s ET. The total polyphenol content in extract reached a concentration of 161.57 mg of gallic acid equivalents per g of extract dry matter and was identified as the predominant antioxidants in horsetail extracts.[5]
- The comparison of antioxidant activity of oil of *Hypericum perforatum* L. was done between solvent-free microwave extraction (SFME) and conventional hydrodistillation (HD) technique (Clevenger apparatus). A central composite design was applied for evaluating the influences of irradiation power, irradiation time, and moisture content of the plant on extraction yield. Under optimal conditions defined by the central composite design, a yield of 0.365 g/100 g extract was achieved. For SFME extraction process, 69 compounds representing 96.6% of the oil were

identified including two major groups: Total oxygenated compounds and sesquiterpenes hydrocarbons accounted up to 66% of oil and the compositions in these two groups were also considered as responses. The irradiation power and moisture content had a high significant effect on all responses while irradiation time has a significant effect only on extraction yield. IC50 values were 462.36 and 40,042.87 μg/ml, respectively, for SFME and HD suggesting that SFME represents an interesting alternative protocol for isolation of *H. perforatum* L. oil. By comparison with HD method, SFME contained a higher proportion of oxygenated compounds. The antioxidant activities of the resulting essential oils have been evaluated by DPPH and RP test.[6]

- In the study of *citrus limon* peels for the recovery of antioxidants, ultrasound-assisted extraction (UAE) and MAE were optimized (by response surface methodology [RSM]) and compared for the recovery of total phenolic compounds (TPC) expressed as gallic acid equivalents (GAEs). The antioxidant activity was determined by the DPPH and RP tests. The optimized conditions used for MAE were ethanol, 48%; solvent:solid ratio, 28:1 ml/g; irradiation time 123 s; and power, 400 W, and for UAE ethanol, 63.93%; liquid/solid ratio 40 ml/g of liquid/solid ratio; holding time, 15.05 min; and amplitude, 77.79%. Maximum predicted TPC recoveries under the optimized conditions for MAE and UAE were 15.74 and 15.08 mg GAE/g, respectively, which were close to the experimental values of 15.78 ± 0.8 and 15.22 ± 0.88 mg GAE/g, indicating suitability of the employed model and the success of RSM in optimizing the extraction conditions.[7]
- The practical feasibility of MAE to substitute the traditional time-consuming techniques (ultrasonic extraction and rotary extraction) for efficient extraction of phenolic compounds (divided into four fractions: Free, ester-bound, glycoside-bound, and insoluble-bound) from *Citrus mandarin* peels was studied. The operating parameters were optimized using central composite design combined with RSM. The optimum extraction conditions used were: MWP, 152W; ET, 49 s; liquid to solid ratio, 16; and methanol concentration, 66%. It has been reported that MAE gives obvious advantages in terms of high extraction efficiency and antioxidant activity of extract within shortest ET.[8,9]
- Extraction of antioxidants from Sweet grass (*Hierochloe odorata* L.) was investigated using Soxhlet extraction, MAE, and SFE at different extraction schemes and parameters. Ethanol ensures high antioxidant recoveries and provides the extracts diluted with undesirable substances, while less polar acetone and ethyl acetate give lower recoveries and more concentrated antioxidant extracts. One-step MAE gave the most concentrated extract with 8.15% of 5,8-dihydroxycoumarin (extract yield 0.42%).[9]
- MAE along with RSM proved to be effective in estimating the effects of three independent variables such as ET, liquid-to-solid ratio, and MWP on the phenolics extraction from espresso spent coffee grounds (SCGs). High level of phenolics was extracted from SCG, using less solvent (ethanol) with considerably shorter ET. Although a detailed cost-benefit analysis is needed to assess the economic practicability of the proposed approach, the analysis reported in this study could be of great significance for future exploitation of espresso SCG as a valuable source of natural antioxidant in industrial scale-up.[10]
- MAE extract of triterpene saponins from defatted residue of yellow horn (*Xanthoceras sorbifolia* Bunge) attained substantial free radical scavenging activity with an IC50 value of 0.782 mg/ml. The highest extraction yield of triterpene saponins reached 11.62 ± 0.37% of defatted kernel, which was increased 1.79- and 1.13-fold to those of UAE and heat reflux extraction (HRE). Furthermore, the optimal extraction duration (7 min) was decreased 8.57- and 12.86-fold to those of UAE and HRE. The optimum extraction parameters include extraction temperature, 51°C; extraction duration, 7min; irradiation power, 900 W; ethanol concentration, 32 ml/g; ratio of solvent to material, 42%v/v; and three extraction cycles. Compared with the UAE and conventional extraction methods like HRE, MAE possessed higher efficiency for the extraction of triterpene saponins.[11]
- A comparison was carried out among non-conventional extraction techniques, such as MAE and PLE, and traditional solid–liquid extraction to test their efficiency toward the extraction of phenolic compounds from leaves of six Tunisian olive varieties. Thus, fast processes could be obtained using MAE and PLE, temperature-driven extraction processes. Therefore, the optimum conditions for each process should be selected depending on the target compound to be isolated and other considerations (such as environmental impact, bioactivity, and final use). MAE seems to be the choice for extracting more polar compounds such as oleuropein derivatives, apigenin rutinoside, and luteolin glucoside isomer, and PLE was more efficient to extract compounds with less polarity such as apigenin, luteolin, or diosmetin.[12]
- *Scutellaria pinnatifida* A. Hamilt. ssp. *alpine* study revealed that the extract which contains highest amount of flavonoid and phenolic compounds (PLE optimum extract) exhibited the greatest antioxidant activity. The high scavenging of PLE extract may be due to hydroxyl groups existing in the flavonoids. The tested plant has moderate to potent antioxidant activity and we could know what components in the PLE extract showed these activities. The temperature, pressure, static time, dynamic time, and the solvent flow rate were adjusted 65.8°C, 39.2 bar, 12.9 min, 18.9 min, and 0.76 ml/min, respectively. The PLE method is quicker and it has more extraction yield than percolation and the roots of this plant are a potential source of natural antioxidants and flavonoids.[13]
- Pressurized liquid extraction may significantly modify the composition of extracts from black bamboo leaves, with a 300% increase in the total phenolic and flavonoid recovered, compared to low temperature extraction. For a broad range of ethanol aqueous solutions and temperatures, 50% EtOH and 200°C (static time: 25 min) gave the best performance, in terms of the TP and TF (75% EtOH) content yield and DPPH scavenging ability (25% EtOH). High temperatures reduced the solvent viscosity and surface tension and disrupted the black bamboo leaves matrix to allow for greater solvent connected with phenolics and flavonoids. At the same time, the modified solvent properties under high temperatures are helpful for extracting more non-polar phenolics and producing ethyl

ester components in extracts. PLE is a powerful method for increasing extraction efficiency and the content of biological compounds. Black bamboo leaves extracts from PLE with high antioxidant content could serve as valuable sources in the nutraceutical and cosmetic industries as natural antioxidants.[14]

- Increased angiotensin-converting enzyme (ACE)-inhibitory activity was observed in hydrolysates when high pressure assisted the proteolysis of the different enzymes, with exception of Alcalase. Moreover, there is also a potential benefit to be gained by application of this technology to increase the antioxidant capacity of lentil hydrolysates through a selection of the appropriate protease and pressure level. Proteolysis at 300 MPa by Savinase gave rise to lentil hydrolysates with the highest ACE inhibitory and antioxidant activities in a relatively short time (15 min). In addition, the biological activity of these hydrolysates was highly retained (ACE-inhibitory activity) or improved (antioxidant activity) on *in vitro* gastrointestinal digestion. The identification of several peptides in these hydrolysates containing bioactive amino acid sequences in their structure support the potential of high pressure treatment as a technology to efficiently release bioactive peptides from lentil proteins.[15]
- Propolis samples were evaluated from important honey producing locations of Anatolia, namely Bingol (BG), Rize (RZ), Tekirdag (TK), and Van for their antiradical capacities, total phenolic contents, and individual phenolic compounds which was recovered by means of PLE. Temperature, 40°C; pressure, 1500 psi; ethanol:water:HCl (70:25:5, v/v/v) containing 0.1% tert-butylhydroquinone as solvent, three extraction cycles within 15 min, and a cell size of 11 ml was the most favorable PLE operating conditions. The recovery was in the range of 97.2% and 99.7%. Major phenolic compounds in all samples were found to be gallocatechin, catechin, epicatechin gallate, caffeic acid, chlorogenic acid (ChA), and myricetin. ChA level of BG propolis was 4.5, 3, and 23 times higher than that of RZ, TK, and VN region, respectively. According to antiradical tests, all propolis samples have superior antiradical capacities up to 500 mg Trolox equivalent activity per gram of extract.[16]
- PLE was employed to extract anthocyanins and other phenolic compounds from jabuticaba skins. PLE conditions resulting in the highest recovery of anthocyanins used were 5 MPa of extraction pressure, 553 K of temperature, and 9 min of static ET, and comparison was done with a conventional low-pressure solvent extraction (LPSE). The attributes compared were yield, content of anthocyanins and phenolic compounds, and economic feasibility. Similar extraction yields were obtained by LPSE and PLE under optimized conditions; however, 2.15 and 1.66-fold more anthocyanins and TPC, respectively, were extracted using PLE, while the cost of manufacturing obtained for the PLE extract was 40-fold lower.[17]

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