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CLASSIFICATION, MECHANISM AND ASSAY OF ANTIOXIDANT COMPOUNDS ISOLATED FROM NATURAL SOURCES ITS APPLICATIONS AND FUTURE ASPECTS#

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Abstract

In recent era, applications of natural antioxidants from numerous plants and microorganisms are amplified extensively due to harmful effects of synthetic compounds in the natural environment. A large community of microorganisms are isolated from diverse parts of flora, as an instance, *Azotobacter*spp., *Actinobacter*spp., *Lactobacillus* spp. and Plant Growth Promoting *Rhizobacteria*. Plant extracts, traditionally used for their flavoring characteristics, often have strong H- donating activity which makes them extremely productive antioxidant. Some of them are phenolic compounds, flavonoids, volatile oils and some plant pigments like anthocyanin. Antioxidants are worked on the principle to scavenge free radicals and protect organisms from its noxious effects. Scavenging capacity of antioxidants are analyzed by using diverse kind of assay techniques such as DPPH Scavenging assay, GC-MS assay,Cupric Iron reducing antioxidant capacity assay (CUPRAC assay) etc. Now a days, antioxidants are largely exploited in therapeutical, medicinal and food industries to improve human health and life-style. Many researches in novel aspects of antioxidants are on-going and may be in nearer future it will be expanded in its next level.

Key Words: Antioxidants, Scavenging activity, ROS and RNS, Ascorbic acid

#General Article

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Introduction

The development of antioxidant field is escalated day by day largely due to the fact that oxidative stress is widely connected to the origination of plethora of diseases including diabetes, cancer, various aging related and central nervous system disorders. Recently, the 18th International Congress of the International Society of Antioxidants and Health (ISANH), held in Beirut on 3-4 May 2017 to discuss about the problem of the onset and role of oxidative stress (OS) in very serious diseases. During rigorous oxidative processes, various free radicals are produced which can be potent precursors of systemic cell and tissue damage. To protect the body from free radicals and harmful chain reaction, antioxidants act as an inhibitor of oxidation process by removing free radical intermediates such as Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) and oxidize themselves.Even though they are present in minute concentration, they can perform assorted physiological functions in the body to stop the harmful oxidation reaction. Antioxidant is not a new terminology, since ancient times herbal plants are considered as good antioxidants. Most widely studied antioxidants in the field of Medicine and Pharmacology are tocopherol, ascorbic acid and flavonoids because of their anticancer, antimutagenic and antitumor activities.

Classification of Antioxidants

Antioxidants can be bifurcated into two major categories such as Natural and Synthetic. The main focused site of these free radicals damage and defensive approach of antioxidants in the body is at the cellular level. Based upon this, these antioxidants can also be classified as enzymatic and non-enzymatic antioxidants. Antioxidants are also classified into microbial, fruit & vegetables and medicinal plant antioxidants based upon sources. A detailed classification and subclassification has been displayed in figure.

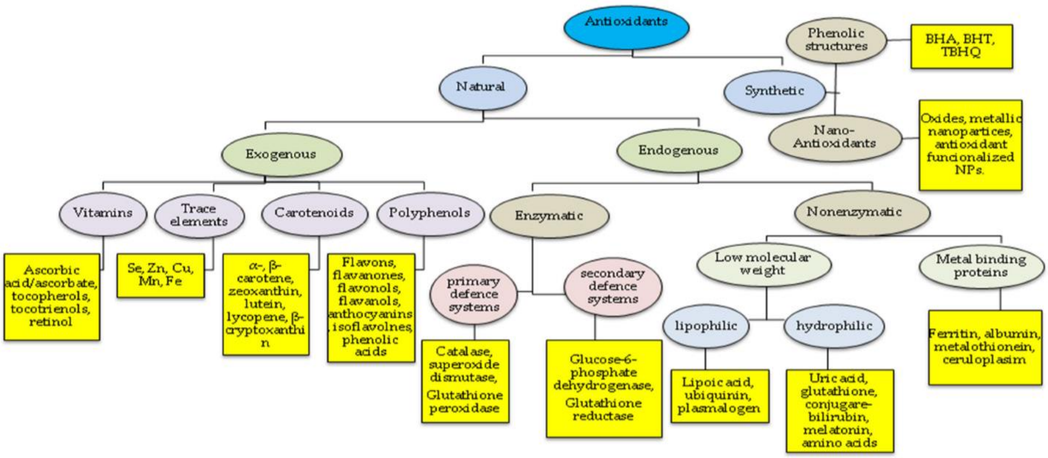


Figure 1: Classification of Antioxidants

Antioxidants from Herbal and Medicinal plants

All parts of plants such as seeds, nuts, leaves, roots and barks contain many of the antioxidants but phenolic compounds and carotenoids are major once. Medicinal and Herbal plants have large impact on pharmaceutical and therapeutical industries because they produce various secondary metabolites which are largely contributed in control of diseases. As an example, clove, turmeric, ginger etc.

Table 1: Some important plants having antioxidant constituents

No.	Common Name	Scientific Name	Antioxidant compounds
1	Turmeric	*Curcuma longa*	Curcumin
2	Ginger	*Zingiber officinale*	Gingerol, Shogaol
3	Clove	*Syzygiumaromaticum*	Eugenol
4	Mint	*Mentha arvensis*	Rosmarinic acid
5	Cumin	*Cuminum cyminum*	Cuminaldehyde
6	Black pepper	*Piper nigrum*	Piperine
7	Lemon	*Citrous limon*	Limon

Antioxidants from Fruits and Vegetables

Fruits and vegetables are potent source of vitamins A, C (ascorbic acid), E(α-tocopherol), β- and α-carotene and glutathione. Alkaloids, terpenoids, sulfur containing compounds, phenolic and polyphenolic compounds are other antioxidants present in them, which reduced oxidative damage by neutralizing activities of free radicals. Carotenoids, a significant bioactive present in fruits and vegetables are mainly effective at inhibiting the oxidation caused by single oxygen. Fruits and vegetables possess a number of flavonoid compounds including anthocyanin, anthocyanidins, flavonols, flavones and flavonones which have properties of anticarcinogenic, anti-inflammatory and antimutagenic.

Table 2: Some important fruits having antioxidant constituents

No.	Commonname	Antioxidant Compounds	Health functions
1	Apple	Ascorbic acid, β-carotene, Polyphenols, Mangiferin, Anthocyanins, Protocatechuic acid	Protect human cells against damage, helps to combat degenerative diseases like cancer and heart disease.
2	Grapes	Phenolic acid, Stilbenes, Flavonols, Flavanols, Viniferin and Anthocyanins	Reduction of low-density lipoprotein, inhibition of platelet aggregation and antifungal agent
3	Guava	Vitamin C, Polyphenols	Normalizes body blood pressure
4	Pineapple	Epicatechin, Ferulic acid, Gallic acid, Catechin, Sterols	Anti-inflammatory, anti-invasive, anti-metastatic and anti-tumor
5	Mango	Ascorbic acid, β-carotene, Polyphenols, Catechins, Mangiferin, Anthocyanin, Gallic acid, Rhamnetin	Protect human cell against damage, helps to combat degenerative diseases like cancer and heart disease.

Table 3: Some important vegetables having antioxidant constituents

No.	Common Name	Antioxidant Compound	Health Functions
1	Allium (onions & garlic)	Diallyl sulfide and allyl methyl trisulfide	Inhibit LDL oxidation, cell proliferation and growth, enhance the immune system, alters carcinogenic activation, stimulate detoxification enzyme.
2	Beet root	Betalains, Polyphenols	Inhibition of LDL oxidation
3	Carrot	β- carotene, linolenic acid and vitamin-A	NA
4	Spinach	Polyphenols	Inhibition of LDL oxidation
5	Tomato	Vitamin-A, C, E, β-carotene, Lycopene, Potassium flavonoids, Folic acid	Prevention of cancer and heart diseases, act as prooxidant in a lipid environment
6	Cruciferous vegetables	Glucosinolates and S-methycysteine sulfoxide	Cancer chemo-preventive effects, prevention of lipid peroxidation.

Antioxidant from Microorganisms

Generally, microorganisms which are intracellularly or intercellularly associated with various parts of plants (endophytic microorganisms)are capable of producing different types of antioxidant compounds like alkaloids, glycosides, phenolic acids, xanthones, steroids, terpenes, tetralones, coumarins and quinones. Such bioactive metabolites have a vast application in agrochemicals like insecticidal and growth promoting chemicals, pharmaceutical and therapeutical industries. As an example, *Methylobacteriumradiotolerans*isolated from *Combretum erythrophyllum* seeds can produce a class of fatty acids (9-octadecene, 3-eicosene, 11- tricosene, hexadecane) and several phenolic compounds which are utilized as antioxidants. Other microorganisms such as *Paenibacillus alvei* which was isolated from *Curcuma longa* rhizome, also have capability to produce commercially important antioxidant compounds. Other than endophytes, *Lactobacillus rhmnosus* which is present in yogurt, kefir and other fermented dairy products is also a good source of antioxidant compounds.

Basic Mechanism of Antioxidants

In human body, antioxidants conduct actions as a second line of defense against the origination of free radicals through oxidative chain reaction processes. Free radicals have a potential to trap molecules that can donate electron to free radicals which are able to trap other molecules wandering in the cells to cause harmful effect to biomolecules and ultimately damage the genetic material. Antioxidant compounds act through some specific mechanism: Hydrogen Atom Transfer (HAT), Single Electron Transfer (SET) and the ability to chelate transition metals. Mechanism of some of the commercially important antioxidant compounds is mentioned here.

Mechanism of Phenolic Compounds

Phenolic compounds have hydroxyl groups in their structures. Thus, they are able to transfer their hydrogen atom to reduce or inhibit free radicals. The reaction mechanism of a phenolic compound with a peroxyl radical ($ROO^{\bullet}$) involves a continuous transfer of the hydrogen cation from the phenol to the radical, constructing a transition state of an H-O bond with one electron. The antioxidant capacity of the phenolic compounds is strongly reduced when the reaction medium consists of a solvent prone to the formation of hydrogen bonds with the phenolic compounds. For example, alcohols have a double effect on the reaction rate between the phenol and the peroxyl radical.Leopoldini et al. conducted a theoretical study to identify the dissociation energy of OH bonds and the adiabatic ionization potential of phenolic compounds of different structures. Some of them aretocopherol, tyrosol, hydroxytyrosol, gallic acids and caffeic acids.HAT mechanism was observed highest in tocopherol, followed by hydroxytyrosol, gallic acid, caffeic acid and epicatechin. On the other hand, kaempferol and resveratrol follow SET mechanism.

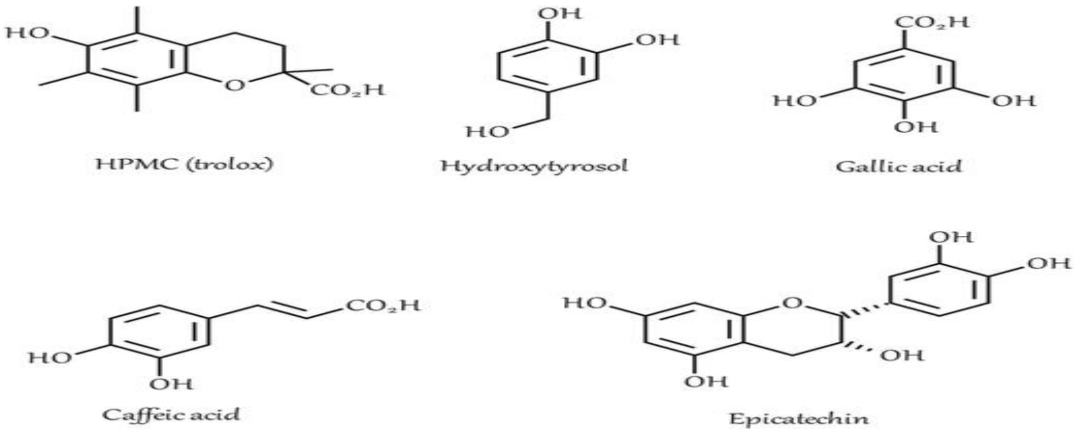


Figure-2: Phenolic compounds which follow HAT mechanism

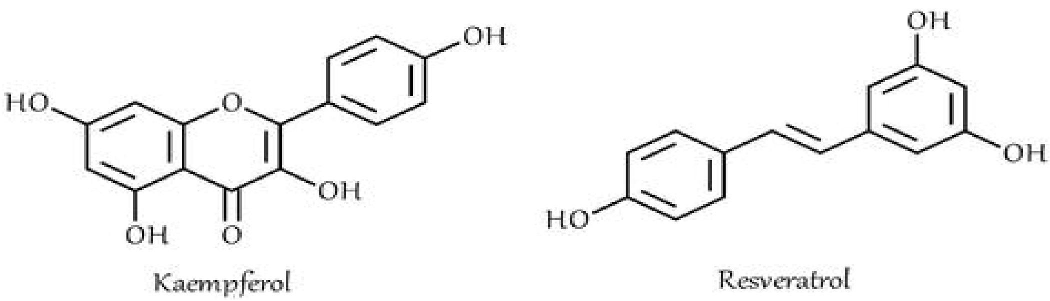


Figure-3: Phenolic compounds which follow SET mechanism

Mechanism of Carotenoids

High capacity of electron donation is associated with antioxidant properties of carotenoids. Carotenoids follow all the three basic mechanisms of antioxidants. Everett et al. discovered that β-carotene reacts with NO_2•by following SET mechanism. In the carotenoids studies with benzyl peroxyl radical which has low reactivity involved SET mechanism. Chemical structure of carotenoids also affects the reactivity towards free radical. Carotenoids with withdrawn electron groups are more resistant to oxidation than carotenoids substituted with electrons. Order of reactivity study of carotenoids with phenoxy radicals is lycopene > β-carotene > zeaxanthin > lutein> echinenone > astaxanthin.

Mechanism of Ascorbic Acid

Ascorbate being a reducing agent, produce ascorbate radical and dehydroascorbic acid by losing two electrons in two subsequent reactions. Due to delocalization of unpaired electron by resonance, the ascorbate radical is comparatively stable. Plasma of healthy humans possesses 10 μg/ml concentration of ascorbic acid. At these concentrations, vitamin-E is co-antioxidant with ascorbate to protect LDL from peroxyl radicals. The ascorbate radical is poorly reactive and can be reduced to ascorbate by reductase dependent NADH and NADPH. Alternatively, ascorbate radical can undergo a disproportionation reaction that depends on h, and forms ascorbate and dehydroascorbic acid.

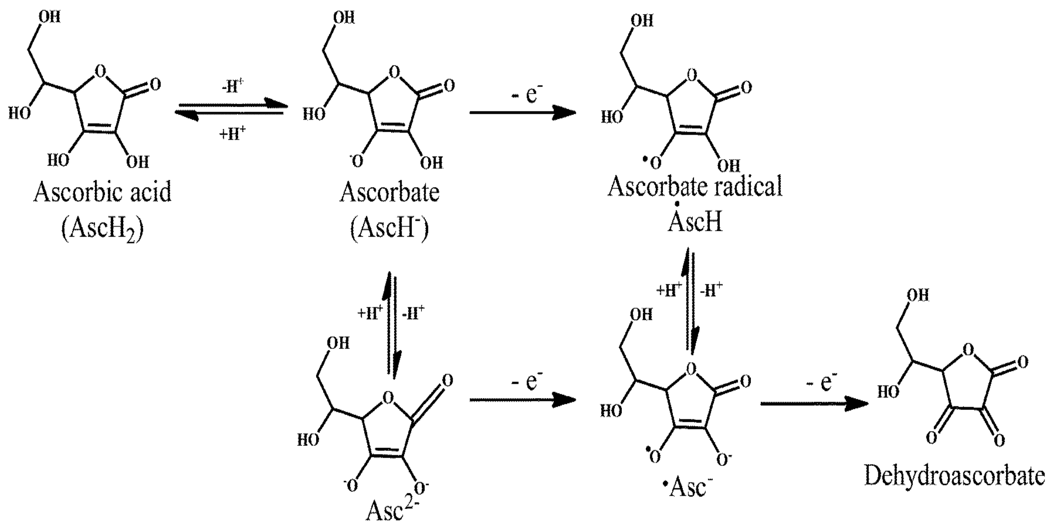


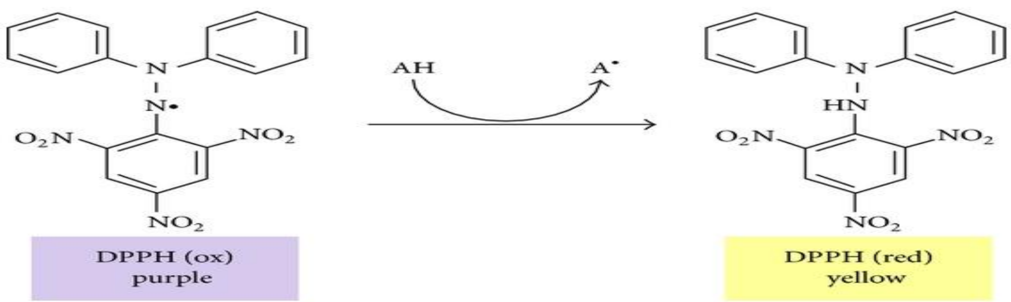
Figure 4: Mechanism of Ascorbic acid

Methods for Antioxidant Analysis

Desired plant material is collected from various medicinal plants and then microorganisms are isolated from it to check antioxidant activity. To assay antioxidant analysis, different types of assay methods are performed.

DPPH Free Radical Scavenging Assay

This method was developed by Blois (1958) by taking it in mind to determine the antioxidant activity by using a stable free radical ɑ, ɑ-diphenyl-β-picrylhydrazyl (DPPH). DPPH is stable free radical that display maximum absorbance at 517nm. When DPPH radicals come across proton (hydrogen) donating substrate such as antioxidant, free radical should be scavenged and absorbance would be reduced. The decreased in the absorbance is taken as measure for "Radical Scavenging action". After 30 minutes of incubation free radical scavenging property with sample is compared with Ascorbic acid as control. 95% scavenging activity of 0.02mg DPPH was achieved by 1mg/ml Ascorbic acid. Generally, 50µl of sample extract in methanol is mixed with 100µl of DPPH solution and 850µl methanol, by making final volume 1ml. Methanol is used as blank and distilled water + DPPH solution is used as control. Percentage of scavenged DPPH radical is calculated by using following formula:



$$\text{DPPH radical scavenging activity}(\%) = \left(\frac{A_0 - A_i}{A_0}\right) \times 100\%$$

Where, A_0 = Absorbance of control, A_1 = Absorbance of sample

Figure 5: DPPH Reaction Mechanism

Ferric Ion Reducing Assay

Ferric reducing ability of plasma (FRAP) assay is based on the principle of reduction of ferric tripyridyl triazine (Fe^{3+}-TPTZ) complex to ferrous tripyridyl triazine (Fe^{2+}-TPTZ) by the antioxidants of the sample at low pH. At absorption maximum 593 nm end product, Fe^{2+}-TPTZ gives blue color and the change in absorbance is related to the antioxidant capacity of the plasma.

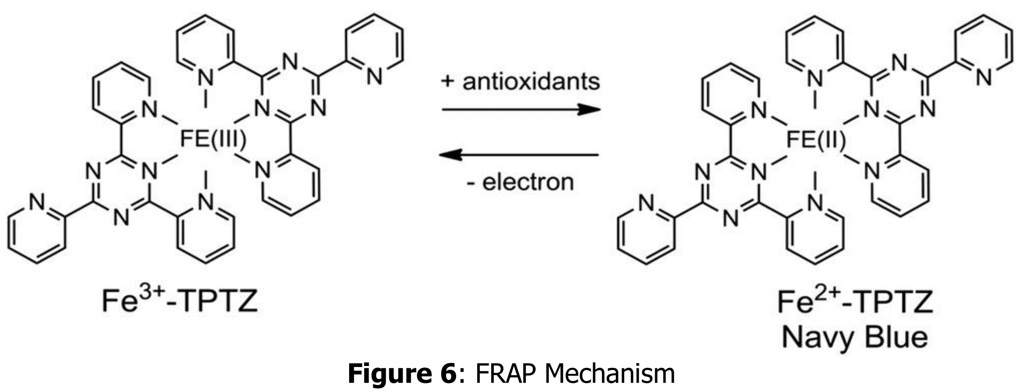


Figure 6: FRAP Mechanism

Here, mentioned method was reported by Jayanthi et al. (2011) using trichloro acetic acid and ferric chloride solution. Ascorbic acid is used as standard. Sample extract is mixed with 2.5 ml phosphate buffer (0.2M, pH-6.6) and 2.5 ml potassium ferricyanide (1%w/v).

Nitric Oxide Radical Scavenging Assay

Nitric oxide (NO^-) radicals are measured by the method of Marcocci et al. (1994). The basic principle behind this procedure is at physiological pH in aqueous solution sodium nitroprusside spontaneously generates nitic oxide which directly interacts with oxygen to produce nitrite ions which are measured by Griess reagent. To reduce production of nitrite ions, nitric oxide scavengers compete with oxygen. Higher amounts of NO^-may lead to tissue damage. 1ml sample extract with various concentrations mixed with 0.5 ml of 10mM sodium nitroprusside in phosphate buffer saline. Incubation is done at 25ºC for 180 minutes. After incubation, an equal amount of freshly prepared Griess reagent is added. Control is prepared using buffer in place of sample extract. As positive control, Ascorbic acid is used. Absorbance is measured at 546nm on UV visible spectrophotometer.

% Scavenging /reduction =
[Absorbance of control – Absorbance of test sample/Absorbance of control] × 100

Cupric Iron Reducing Antioxidant Capacity Assay (CUPRAC assay)

In this assay, Cu^{2+}-Neocuproine (Cu^{2+}-NC)is reduced into Cu^{+}-Neocuproine (Cu^{+}-NC)by the action of the non-enzymatic antioxidants presented in the sample. Cu^{+}- NC is a stable complex which has maximum absorbance at 450 nm. The assay used for CUPRAC in the present study was based on the method described by Apak et al. (2008) with some modifications.

In this procedure, 1ml of sample extract in ethanol is mixed with 1ml of 7.5×10^{-3}M Nc solution, 1ml of 1×10^{-2}M $CuCl_2$ solution, 1ml of ammonium acetate buffer and 1ml of water. Mixture is incubated at 25ºC for 30 minutes and absorbance is measured at 450nm. Ascorbic acid is used as standard.

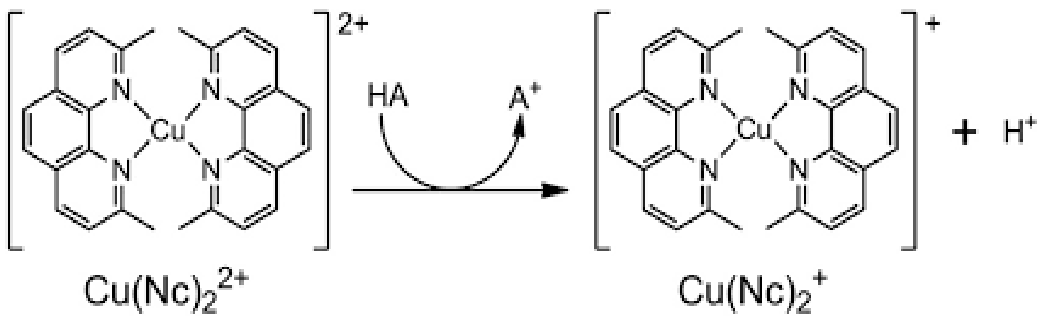


Figure 7: CUPRAC Assay Mechanism

GC-MS Analysis and Identification

Gas chromatography – Mass spectrometry analysis of sample extract is performed on GC system fitted with various types of columns (Rtx-5MS capillary column) accompanied with MS. As a carrier gas, a constant flow rate of 1ml/min of ultra-high purity helium gas (99.99%) is used. The ionizing energy at 70eV is maintained. The injection, transfer line and ion source temperatures are set at 290ºC. Initially, oven temperature is set at 60ºC for 2 minutes and then increased to 280ºC. The sample extract is diluted by using appropriate solvent and then filtered to prepare particle free diluted sample extract solution. After that, prepared sample is injected using a syringe into the injector with a split ratio 30:1. For collecting the full-scan mass spectra, 40-550 amu scan range is used. A percentage of peak area is calculated to evaluate percentage composition of sample extract constituents. Based on GC retention time, the identification and characterization of chemical compounds are expressed. The data is compared with thestandards available in mass spectrum libraries. The database of National Institute Standard and Technology (NIST) is utilized to interpret Mass-Spectra of GC-MS.

Statistical Analysis

All data of extracts which are analyzes by above methods are expressed as mean ± Standard Deviation of mean. The mean values are statistically analyzed using One-way Analysis of Variance (ANOVA) using the graph pad instant software package. Comparison among mean values is made by Least Significance Difference (LSD).

Potential Applications of Antioxidants
Medicinal applications of Antioxidants
Antioxidants as anti-cancerous agents

i. Flavonoids are considered as free radical scavengers. That's why they are used as anticarcinogenic agents. Almost 28 naturally occurring and synthetic flavonoids have been identified as novel antileukemic compounds. They are also beneficial as anti-inflammatory, anti-allergic and anti-viral agents.
ii. Lycopene have ability to prevent heart diseases and protect the skin against the sun damage and they are widely used in diverse types of cancers such as pancreas, colon, oral cavity, esophagus, ovaries etc. Lycopene significantly decreases the risk of prostate cancer in men.
iii. Glutathione is a tripeptide thiol compound which is a major intracellular antioxidant in the body.
iv. Selenium is present in very small amount in the body, but it is one of the most important mineral antioxidants for the endogenous enzymes. It controls the heart failure.

Antioxidant and Nervous system

Antioxidants maintain the wellness of cerebellum by curing oxidative damage and they are also considered as therapeutic agents for acute nervous system injury.

Antioxidant and Red Blood Cells

RBCs are damaged by Reactive Oxygen Species (ROS) as they are daily encountered with them. Antioxidant enzymes such as Cu Zn Superoxide Dismutase (CuZnSOD) and catalase get accumulated at RBC membrane as first line of defense against oxidative stress.

Role in Food

Some antioxidants are maintaining the texture, flavor and color of the food products during storage. Nutritional and dietary antioxidant supplements and functional foods possess antioxidants like ɑ-tocopherol, vitamin-C and some other plant derived phytochemicals such as lycopene, lutein, isoflavones, green tea extracts and grape seed extracts have a large impact on current market potential.

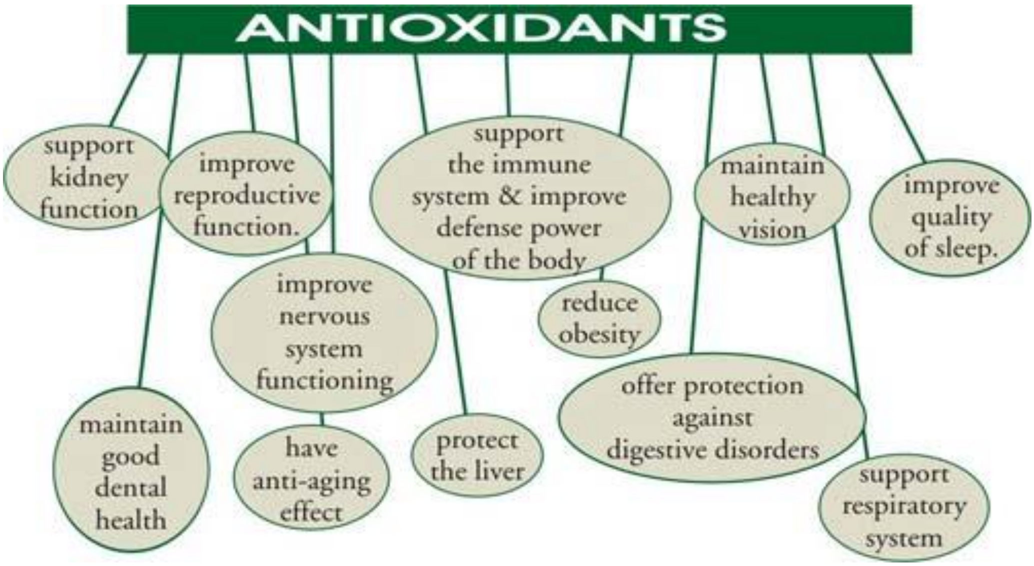


Figure 8: - Applications of Antioxidants

Role of Antioxidants in Diabetes

Concentration of free radicals are increased and antioxidant potential is decreased in Diabetes mellitus which cause oxidative damage of cell components such as proteins, lipids and nucleic acids. Glucose autooxidation is a widely responsible for production of free radicals and ultimately cause oxidative stress. Apart from that, higher amount of some prooxidants including ferritin and homocysteine is also responsible for oxidative stress. Deficiencies of glutathione, ascorbate and superoxide dismutase have been reported in diabetic people.

Role of Antioxidant in Premature Infants

Enzymatic and/or non-enzymatic antioxidants are useful for infants by reducing injury from excess production of ROS, significantly in disorders such as bronchopulmonary dysplasia, retinopathy of prematurity, periventricular leukomalacia and necrotizing enterocolitis.

Therapeutic Applications of Antioxidants

Fruits and vegetables are considered as a good source of antioxidants which are used to treat neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and amylotrophic lateral sclerosis. It can also reduce the risk of rheumatoid arthritis, cardiovascular disorders, ulcerogenesis and acquired immunodeficiency. It has been found that antioxidant supplement therapy as an adjuvant therapy is useful in the patients with stress induced psychiatric disorders and generalized anxiety disorders.

Future Prospects of Antioxidants

The use of antioxidants in various industries is progressively escalated and that's why great research is required to revolutionize current perspectives. In antioxidant therapy, biomarkers should be employed to measure the oxidative stress. Good biomarkers are required for that. Single type of antioxidants is not be able to completely neutralize the damage produced in different macromolecules and tissues. Thus,combination of antioxidants might have synergistic effect and may be able to cure the diseases, which are not curable earlier. Mitochondrial targeted delivery of antioxidants is a new electrifying field of research that seeks to concentrate antioxidants in the inner membrane of mitochondria to protect the body from mitochondrial oxidative stress and is also helpful for atherosclerosis. For the identification and modulation of disease specific redox sensitive signaling pathways, molecular targeted therapy might be used in near future. To overcome availability of antioxidant at its target sites antioxidant gene therapy has also recently been proposed as a treatment strategy. Some enzyme has a function to produce ROS, one of them is NADPH oxidase. Inhibition of such an enzyme may be reduced risk of many diseases. Antioxidant drugs possess advantage over other types of drugs due to their effectiveness against oxidative stress related diseases as well as other pathologies. As an example,edaravone is an antioxidant drug.

Conclusion

The researchers from all over the world are decidedto use of synthetic compounds should be reduced to safe level. By taking it in a mind, they extracted more natural compounds from the medicinal and herbal plants for beneficial implementation and more convenient use. Antioxidants being a one of them, reduce the level of free radicals from the body and act as anti-carcinogenic, anti- inflammatory, anti-ageing and reduce occurrence of harmful diseases. In future, antioxidants may be widely used as drugs and antioxidant gene therapy.

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