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An overview of natural plant antioxidants: analysis and evaluation

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Abstract: Antioxidative compounds play a vital role to inhibit the oxidation of oxidizable products and influences the body defense system against Reactive Oxygen Species (ROS), which are harmful byproducts engendered during normal aerobic cellular respiration. ROS are specialized class of highly reactive molecules that originates during oxygen metabolism, whereas extensive evidences indicate the involvement of ROS in the development of degenerative diseases in humans. Despite the fact that synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are threat to human's health; they are widely employed as chemical preservatives to avert potential deterioration of food products. Several toxicological findings reveal that natural antioxidants are capable of providing protection against free radical-related diseases, cancer and other cardiovascular infections. In cognizance with these facts, natural antioxidant attracts a great deal of research interest; therefore, this review provides a comprehensive and an up-to-date overview of the three most essential natural plant antioxidants; their antioxidative properties, methods of analysis and evaluation. These Polyphenols comprise: Phenolic acid, an essential polyphenol that are broadly dispersed in plants to exert positive influence on oxidative stress; Flavonoid, an ubiquitous antioxidant that functions in free radical scavenging, metal ion chelation and enzymes inhibition to suppress the synthesis of free radicals; and Tannic acid, a water soluble anti-nutrient that acts to precipitate alkaloids & proteins, reduce mineral & vitamin utilization and restrict the activities of digestive enzymes. In conclusion, a variety of plant species were characterized to indicate *in vitro* antioxidative properties.

Keywords: Antioxidant, Analysis, ROS, Polyphenols, Phenolic Acid, Flavonoid, Tannic Acid

1. Introduction

There is now upsurge interest in antioxidant activity of phytochemicals present in the diet [1]. Antioxidants are compounds that can prevent or delay the oxidation of oxidizable products by scavenging free radicals and reducing oxidative stress. Oxidative stress is an imbalanced state where excessive quantities of reactive oxygen and/or nitrogen species overshadow the endogenous antioxidative capability of the cells which stimulates the oxidation of macromolecules, such as proteins, enzymes, lipids and DNA [2].

Oxidative stress signifies the presence of ROS and free radicals. The complex synthesis of these free radical from oxygen i.e. ROS induce significant damage to the intermediate metabolite via electron loss in order to remain stable. These free radicals possess a single or more unpaired electrons spinning on the peripheral layer around the nucleus.

These are oxygen-centered free radicals consisting of superoxide anion (O_2), alkoxyl (RO^-), peroxyl (ROO^-), hydroxyl radical (OH^-) and nitric oxide. They are formed under typical physiological conditions and then become detrimental if not being eliminated by the endogenous metabolizing systems [3, 4]. ROS are not only associated with lipid peroxidation that causes the food deterioration, but also entangled in the development of human diseases such as cellular aging, carcinogenesis, coronary heart disease, diabetes, mutagenesis and neurodegenerative infections.

Antioxidant compounds play a vital role to the body defense system against Reactive Oxygen Species (ROS), which are the harmful byproducts engendered during normal aerobic cellular respiration [1]. [5] reports that the influence of antioxidants are predominantly determined by their ability to establish anti-aging effects and free radical damage. The functional role of antioxidant is to prevent oxidation of highly reactive free radicals which is a chemical process that are known to cause premature aging, cancer and other cardiovascular infections [4]. Antioxidants from natural source increase the antioxidative capability of the plasma and decrease the risk of many diseases. The increase in dietary antioxidant intakes may help to support the limiting antioxidant concentrate and also promote the normal functioning of physiological systems [1].

While unknown to many, the synthetic antioxidants; butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are most extensively used as alternative preservatives to prevent unwanted deterioration of food products, which poses threat to human's health [6]; thus, there is necessity for less toxic, cost effective and more efficient antioxidants [4].

The fact that a variety of natural antioxidants occur in plants has been proven [6]; these comprise of phenolic acid, flavonoids/bioflavonoid, tannic acid (tannins) and commonly less lignans and stilbenes which are collectively referred to as polyphenols (phenolics). They are predominantly found in medicinal plant parts such as fruits, leaves and vegetables plants (*see* Table 1) [1, 7]. Therefore, the overall goal of this review is to provide an insight into the antioxidative analysis of products from a natural sources and evaluating their properties based on the scientific perspective, most of them encompassing polyphenols and already considered to be valuable phytomedicines.

2. Phenolics and their Antioxidative Properties

Phenolics are organic acids composed of one or more hydroxyl groups linked to a single or multiple aromatic rings. The versatility of phenolics in the organs of different plant species necessitated it as functional secondary metabolites for human biological systems. It is a predominant constituent of plant nutrients found in vegetables & fruits and primarily accounts for the organoleptic properties of phyto-foods [2]. It has also been reported that these acidic compounds found in plant sources exists at varying concentrates.

Recently, phenolics antioxidative properties have been considered active *in vitro* and confirmed to be more reactive than carotenoids, Vitamin C and E [9]. The metabolic activities of fruit and vegetable intakes in preventing the risk of oxidative stress and other associated syndromes such as cancer and cardiovascular diseases has been partially ascribed to phenolics [10, 11]. The antioxidative properties of phenolic compounds have been analysed and achieved via three main mechanisms:

I. Scavenging radical species (for removing impurities) such as reactive oxygen species (ROS) and reactive nitrogen species (RNS).
II. Suppression of ROS/RNS synthesis by inhibiting specific enzymes or chelating trace metals involved in free radical generation.
III. Up-controlling or inhibition of antioxidant defense system [2].

2.1. Phenolics Sample Preparation and Characterization

2.1.1. Extraction

The bioactive compounds are first extracted from plant materials for the analysis of phytochemicals to standardize the preparation technique of dietary supplements, pharmaceutical & cosmetic products and food ingredients. Extraction of polyphenols can be from fresh/dried/frozen samples [2]. Grinding, milling and homogenization treatment are mostly carried out on the samples before the extraction process (*see* Figure 1) and may be preceded by freeze drying or air drying [2, 6].

2.1.2. Purification and Fractionation

Extracts from plant crude materials usually contain high quantity of carbohydrate and lipid components, which influences the phenolics concentration in the crude extract. The precipitation and determination of polyphenols-rich fractions prior to analysis include solid phase extraction (SPE) based on acidity & polarity and/or column chromatography (*see* Figure 1) [2].

2.1.3. Analysis

Polyphenol assays are usually grouped either in the quantification or classification of specific phenolic compounds. The quantification of phenolics in plant extract is controlled by the chemical nature of the analyte & assay procedure, standards selection and presence of interfering constituents. Because of the heterogenic natural of phenolics and its limitation to other readily oxidizable substances in plant materials, several methods for the determination of total polyphenols have been established. These include Folin-Ciocalteu (F-C) method, Folin-Denis (F-D) method [2], colorimetry (using iron salts), ultraviolet absorbance and permanganate titration as shown in Figure 1. Mostly, the F-C method is more preferred to other quantification methods due to its accuracy [2, 12, 13, 14].

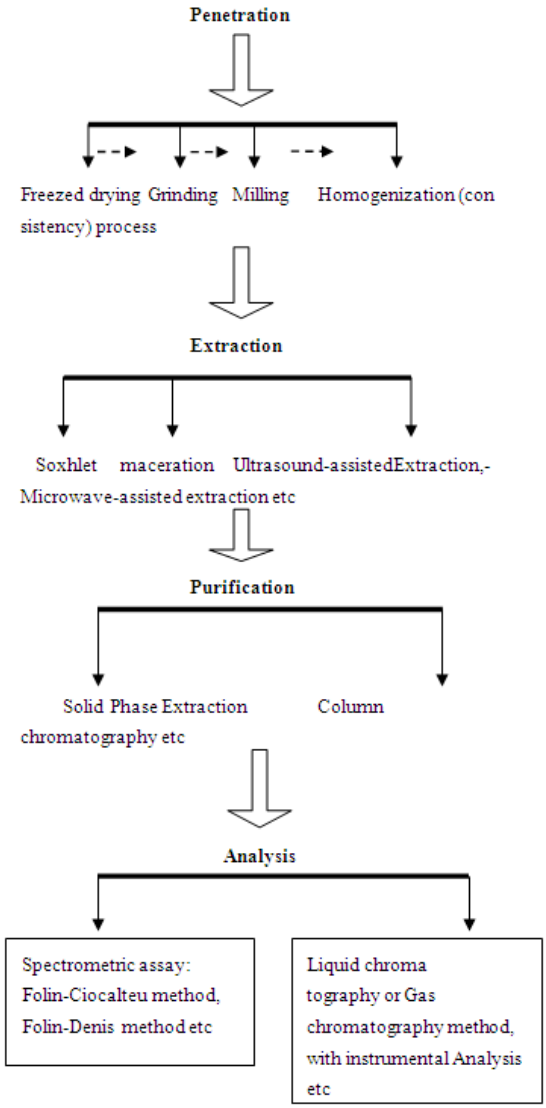

***Figure 1**; Preparation strategies and characterization of plant phenolics samples [2, 8].*

3. Polyphenols Antioxidant Analysis

For chemical analysis of polyphenols, appropriate dilution of sample powder of plant organ is usually measured and used as aliquot. Phenolics compound are extracted from 0.5 g plant sample with 40 mL of 80 % $^{v}/_{v}$ acetone or aqueous methanol under sonication bath for 20 minutes. The residual solution is subsequently centrifuged at 4000 rpm for 10 min and collects the supernatant for the analysis [6, 13, 15].

3.1. Phenolic acid

Phenolic acids belong to a group of natural products which originate from several cereals and fruits. Higher concentrations of this compound are found at the superficial layers of the kernel which set-up the bran [16]. As a group, phenolic acid have been found as strong antioxidants against the negative influence of free radicals and ROS, which is the basis of several chronic human diseases [17, 18].

Phenolic acid is categorized into 2-classes

I. Derivatives of cinnamic acid (caffeic acid and ferulic acid).
II. Derivatives of benzoic acid (gallic acid) [19].

The most common phenolic acid in fruits & vegetables is caffeic acid which is esterified with quinic acid. While ferulic acid is abundant in cereals and esterified to hemicelluloses, an essential component of the cell wall [2, 20]. The structural diversity of the different phenolic acids are based on their variation in hydroxylation pattern and stereochemistry which are determined by various analysis and characterization as illustrated in Figure 2,.

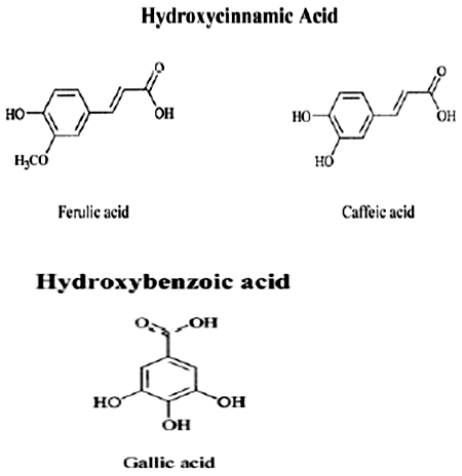

***Figure 2**; Structure onfiguration of the different phenolic acids [2].*

3.2. Determination of Total Phenolic Acid Content

Total amount of phenolic can be determined by Folin-Ciocateu assay as proposed by [21, 22]. 0.2 - 0.5 mL of aliquot extract is mixed with 0.1 ml of Folin-Ciocalteu reagent (mixture of molybdenum oxides and tungsten) in a volumetric flask containing deionized distilled water and incubates at room temperature. 5–10 mL of sodium carbonate (Na_2CO_3) is added to the mixture and the solution is diluted with distilled water, prior to incubation at room temperature for an hour or more. The absorbance is read at wavelength of 765 nm using UV-VIS spectrophotometer with blank [4, 8, 13]. Gallic acid solution is prepared as standard at 100 mg/L [6, 23] and total phenolic content is expressed as a standard equivalent of the extracted compound [4].

3.3. Flavonoids

Flavonoids are polyphenolic compounds that are ubiquitous in nature, comprising a number of hydroxyl groups attached to aromatic ring structures which determines its antioxidant activity. The compounds exhibit a diphenylpropane (C_6-C_3-C_6) skeleton and classified according to their chemical structure as:

a- Monomeric flavanols
b- Flavonols
c- Flavones

d- Flavanones and

e- Anthocyanidins (anthocyanin) (refer to Figure 3)

In addition to hydroxycinnamic acid derivatives (C6-C3), phenylpropanoids, flavonols and flavones exist in lesser quantity in many plant species. Flavones and flavanones are mostly found in citrus fruits; flavonol is abundant in apple, tea and onion; while anthocyanin are responsible for the red colouration of several fruits (e.g. strawberry, raspberry etc.) which are all attached by peculiar enzymes. The flavonoid metabolic interconnections are illustrated in Figure 4. Individual variations within these groups are as a result of the differences in the nature & structural organization of its hydroxyl groups, degree of glycosylation and/or alkylation.

Flavonoids are of beneficial advantages to humans due to its capacity to function as antioxidant, anti-allergic, anti-viral, anti-inflammatory, anti-carcinogenic, anti-bacterial, anti-tumor, immune-stimulating, vasodilatory and estrogenic drives. [2, 9, 11, 24]. The antioxidant potency of flavonoids has been evaluated in vitro by measuring their ability to trap free radicals and demote more chemicals. This ability depends upon the molecular structure of the compounds [10, 25]. Flavonoids' antioxidative capacity influences different metabolic mechanisms that includes free radical scavenging, metal ions chelation and enzymes inhibition responsible for free radical generation [4, 24]. As earlier reported, the chemical structure and hydroxyl group positioning in polyphenols are important features of flavonoids as scavengers of free radical/superoxide while exerting antioxidant activities, inhibitory effects on lipid peroxidation, inhibition of low density lipoproteins (LDL) oxidation induced by copper ions and macrophages [24].

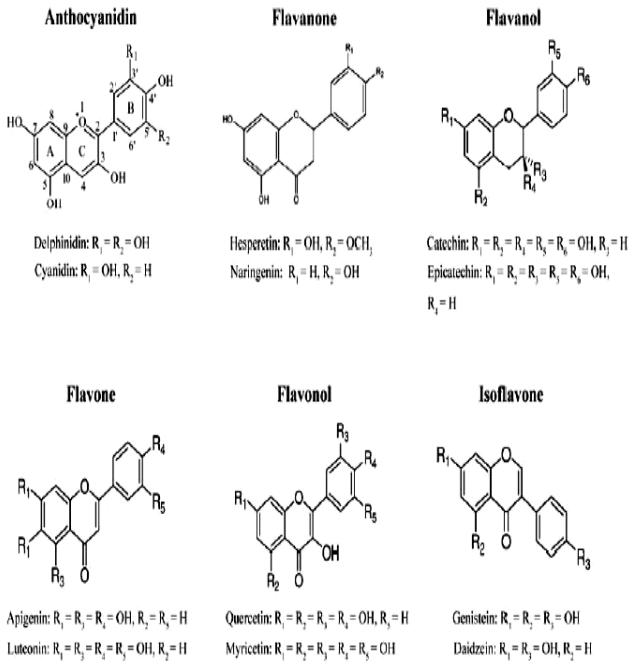

***Figure 3**; Chemical structure of the different sub-classes of flavonoid based on variations in heterocyclic C-ring [2, 11].*

The pharmacological, medicinal and biological characterization of flavonoids has been extensively reviewed. The antioxidant properties is hypothesized to protect tissues against ROS and lipid peroxidation which are linked to many many pathological states such as atherosclerosis and chronc inflammation. Thus, flavonoids contribute immensely towards deterrence of such diseases. They also act as inhibitors of cyclooxygenase, phospholipase A2 & lipoxygenase, xanthine oxidase and glutathione reductase.

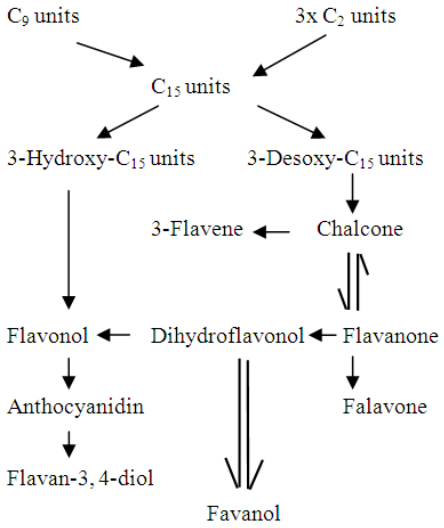

***Figure 4**; Metabolic chains for the synthesis of different sub-classes of flavonoids*

3.4. Determination of Total Flavonoid Content

The total amount of flavonoid can be determined by modified colorimetric assay [4] or aluminium chloride colorimetric method [13, 26]. 0.1-0.5 mL of an aliquot extract is mixed with 2-4 mL of distilled water in volumetric flask. Subsequently, 0.1-0.3 mL of $NaNO_2$ is added and well mixed. After 5 min of incubation, 0.15-0.3mL of $AlCl_3$ solution should be added and preceded for 5min incubation at room temperature and 2 mL of 1M NaOH solution is added to the test solution. This is followed by mixing and incubation for 20minutes at room temperature. Absorbance is read to quantify the total flavonoid content by using UV-visible spectrophotometer/HACH spectrophotometer programme, DR 5000 at 510 nm versus prepared blank with distilled water. Rutin (E. Merck) as standard compound or quercetin is used for calibration curve [4, 6, 13].

3.5. Tannic Acid (Tannins)

Tannic acid is a water-soluble polyphenol with molecular weight of 500–3000 g/mol. Apart from its phenolic reactions; tannins possess specific properties that cannot be found in other natural antioxidants. These include the ability to precipitate alkaloids and other proteins. They are responsible for the bitter taste of certain beverages and foods such as tea, wine and unripe fruits. This sensation apparently results from the molecular interaction between tannic acid

constituents and salivary proteins secreted from the mouth mucous tissue [27, 28]. As a result, tannins are defined as anti-nutrients' from plant origin that can precipitate proteins, reduce mineral ions & vitamins utilization and prevent the activities of digestive enzymes. Likewise, tannins are considered as health-promoting components in plants and beverages [27]. For instance, it possesses anti-mutagenic and anti-carcinogenic potentials in addition to its anti-microbial properties as reported by [29].

Moreover, the consumption of this polyphenol has been in line to establish preventive and inhibitory effects in human heart related diseases. In addition, tannins inhibit lung, skin and fore-stomach tumor infections which are primarily caused by the degenerative activity of N-methyl-N-nitrosourea and polycyclic aromatic hydrocarbon carcinogens [30].

The phenolic nature of tannins which is embedded within its comparatively hydrophilic "shell" and hydrophobic "core" are the features responsible for its antioxidant action [30, 31]. The properties of tannin-rich extracts was observed in meat model system studies on bearberry leaf (*Arctostaphylos uva-ursi*) extract and characterized by the existence of hydrolysable and condensed tannins [32]. Tannins act as secondary antioxidants and does not function independently as primary antioxidants (i.e. it donates electrons or hydrogen atom). This compound possesses the power to chelate metal ions such as Fe^{2+} and interferes with metabolic phase of Fenton reaction to retard oxidation [33].

Structurally, tannins are made up of a central glucose molecule and 10 galloyl groups. The molecular derivatives of tannic acid with one or more galloyl residues at its hydroxyl positions are illustarted in figure 5. This represents the form of polyphenols present in fruits and plant barks (e.g. grapes, bananas, sorghum, coffee, red wine, persimmons and tea) [34].

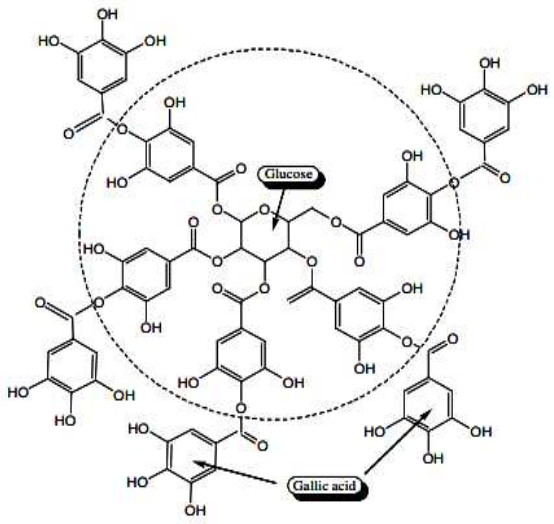

Figure 5. *Chemical structure of tannic acid, deca-galloyl glucose containing the central glucose molecule and two gallic acid molecules. The shaded circle highlights the core structure of tannic acid and pentagalloyl glucose [30].*

3.6. Determination of Tannic Acid

Tannic acid content in vegetables and fruits are analyzed according to AOAC and Pharmacopoeia methods. 25 mL of infusion is provided in 1 L conical flask containing 2 mL of indigo solution and 750 mL of deionised distilled water. Aqueous solution of $KMnO_4$ is used in burette titration until the blue coloration of the mixture turns green. Chemical indicator is continuously dropped until the solution becomes golden yellow. However, a standard solution of *Indigo camine* is prepared by dissolving 6 g of *Indigo camine* into 50 mL deionized distilled water while heating and allowed to cool before 50 mL of 95-97 % $^{v}/_{v}$ H_2SO_4 is added to dilute the solution to 1 L prior to filtration [35].

3.7. Total Condensed Tannic Acid determination

For total condensed tannins determination in gambir extract samples. 100 mg of sample is dissolved in 10 mL of distilled water and 2 mL of 37 % $^{v}/_{v}$ formaldehyde (Merck) is added. Thereafter, 2 mL of 5 M HCl are added and the mixture is heated under reflux for an hour before filtration is established via vacuum suction while it is still hot. The reddish precipitate observed is washed 5 times with 10 mL warm water and dried using silica gel desiccators and total condensed tannic acid is measured [36].

3.8. Antioxidant Activity Evaluation

Several analytical procedures have been employed to determine the effectiveness of natural antioxidants, as either pure compounds or extract from plant. *In vitro* techniques are classified into two main classes:

I. Hydrogen atom transfer reactions; includes Total Radical Trapping Antioxidant potential (TRAP), β carotene bleaching and Oxygen Radical Absorbance Capacity (ORAC).
II. Electron transfer reactions; includes Ferric Reducing Antioxidant Power (FRAP), Trolox Equivalent Antioxidant Capacity (TEAC), Superoxide anion radical scavenging assay, Hydroxyl radical scavenging assay etc [37].

The above system techniques are the most popular due to their sensitivity and accuracy. Though, it is important to use more than a single method to evaluate plant material antioxidant power, because of the complex nature of phytochemicals. Antioxidants evaluation assay commonly used with variety of standards as positive control.

Extraction of Antioxidant for Evaluation; The plant material is subjected to extractions with water and acetone. For acetone extraction, approximately 10 g of the plant material is powdered using ordinary blender or laboratory mill. 0.5 g of the powdered is taking to extraction tubes and mixed with 20 mL of the extragent (i.e. 80 % acetone in 0.2 % formic acid). This extraction is conducted on an orbital shaker at room temperature for an hour. After then, the sample is centrifuged and collects the supernatant and re-extracts the solids residue under the same conditions. Both the supernatants from first and second extraction are

combined and evaluate for antioxidant activity [38].

For water extraction; water infusions is prepared in compliance with the traditional preparation which is close to home conditions. 5 g of powdered material is added to 200 mL of 90 ^{0}C water and collects the supernatant after centrifugation [38]. These are the extraction procedures, while the next is evaluation analysis. In this review single evaluation method was reviewed that is ORAC assay.

ORAC assay: ORAC is measured according to the method of [39, 40]. This method measures the antioxidant scavenging activity against of peroxyl radical generated by thermal decomposition of AAPH at 37 ^{0}C. Fluorescein (FL) is used as the fluorescent probe. The loss of fluorescence of FL is an indication of the extent of damage from its reaction with the peroxyl radical. The antioxidant protective effect is measured by evaluating the fluorescence decay curve (AUC) relative to that of a blank without the presence of antioxidant. AAPH solution, Trolox and fluorescein are prepared with phosphate buffer (75 mmol/l, pH 7.4) as dilution liquid [38].

Reaction mixture (about 200 µL) contained 170 µL of FL, 20 µL of AAPH, and 10 µL of sample. The sample and FL are incubated at 37 ^{0}C for 10 - 30 min in a microplate reader with AAPH that dissolved in buffer at 37 °C is added and read the initial fluorescence after incubation for 30 sec. The readings are taken after 1 min shaking cycle. Blank is prepared with 10 µl of phosphate buffer. The antioxidant activity is expressed in micromole Trolox equivalents per gram of dry weight. Trolox solutions in different µmol/L are used for defining the standard curve [38].

Table 1.0:- *List of some plant species with their family and part(s) examined for antioxidant compounds studies*

Plant species	Family	Part examined	Reference
Solanum lycopersicum	Solanaceae	friut	[41, 42]
Mangifera indica L	Anacardiaceae	Fruit	[43]
Moringa oleifera	Moringaceae	fruit and leaves	[44]
Piper nigrum Linn	Piperaceae	Fruit and seed	[45]
Azadirachta indica	Meliaceae	Leaves	[46]
Amaranthus lividus L	Amaranthaceae	leaves flower and stem	[47]
Allanblackia floribunda Oliv	Guttiferae	leaves and fruits	[48]
Aristotelia chilensis	Elaeocarpaceae	fruit	[49]
Careya arborea Roxb	Barringtoniaceae	bark	[50]
Cassia siamea Lam	Caesalpiniaceae	flower	[51, 52]
Citrullus colocynthis L	Cucurbitaceae	fruit	[53]
Cydonia vulgaris	Rosaceae	Leaves, fruit	[54, 55]
Dimocarpus Longan Lour	Sapindaceae	leaves	[56, 57]
Dipsacus asper Wall	Dipsacaceae	root	[58]
Cassia tora L	Caesalpiniaceae	seeds	[59]
Pyrrosia petiolosa Ching	Polypodiaceae	whole plant	[5]
Phyllanthus emblica L	Euphorbiaceae	fruit	[60, 61]
Lecaniodiscus cupanioides Planch	Sapindaceae	leaves	[5]
Phoenix dactylifera L	Arecacea	fruit	[62, 63]
Phyllanthus niruri Linn	Euphorbiaceae	fruit and leaves	[64]
Uncaria tomentosa Willd.DC	Rubiaceae	back	[65]
Trigonella foenum-graecum L	Leguminosae	seed	[66]
Curcuma longa	Zingiberaceae	Rhizome	[67]
Cynamonum zeylanicum	Lauraceae	seed	[66]
Carum carvi	Umbelliferae	fruit	[66, 68]
Phytolacca Americana	Phytolaccaceae	leaves	[69]
Capparis spinosa	Capparaceae	leaves	[70]
Syzygium aromaticum	Myrtaceae	fruit	[66, 71]

4. Conclusion

Concisely, plant polyphenols have been found to play a vital role in human biological systems and influence major stages of disease development in addition to their antioxidative power. Solvents such as methanol are mostly used for the extraction of antioxidants (bioactive compounds) from it source according to the variations in the polarity of the solvent prior to water extraction techniques. The usage of non-polar solvents is relatively rare due to the characteristics of the active constituents with higher solubility in polar solvents. Usually, all plant parts can be used for antioxidant analysis and evaluation, but fruits/seeds and leaves are mostly been analyzed. This review achieves the aim of providing useful information on polyphenol analysis, its properties and evaluation. However, extensive studies of these natural plant antioxidants will provide significant insights to their potential pharmaceutical functions in the field of oncology.

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