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Antioxidant activity of kolaviron, complex of biflavonoids from *Garcinia kola*

BACHELOR'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled Antioxidant activity of kolaviron, complex of biflavonoids from *Garcinia kola* independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague 14th of April

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Barbora Šírová

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Abstract

The *Garcinia kola* tree is the original tree from Central and Western Africa. this tree is known mainly to the locals, who use it primarily in folk medicine. The locals call this tree a wonder tree because every part of this plant is used to treat various ailments. Unfortunately, outside Africa, this tree is not well known or widely used. This work is focused on kolaviron which is a complex of bifalvinoids found in the seeds of the *Garcinia cola* tree. Biflavonoids are known for their beneficial properties on the human body. One of these properties is antioxidant activity. Therefore, they are able as antioxidants to prevent oxidative stress, which causes an excess of free radicals. Oxidative stress can trigger various diseases and accelerates the signs of aging. Therefore, the intake of falvonoids in our diet is beneficial for us. This work investigated the values of antioxidant activity of kolaviron, a complex of biflavonoids contained in the seeds of the *Garcinia kola* tree. This research was carried out using three methods. The first value was measured by the DPPH method. Antioxidant values of 69,451 μg/ml were measured by this method. The second method was performed with Folin-Ciocalteul phenol reagent. This method measured a total phenol content (TPC) of 17,3 mg GAE / ml extract. And the last method was ORAC. In this method we measured the value 15,329 μg/ml. From these results, we found that the extract shows a higher antioxidant value.

Key words: Antioxidants, DPPH, ORAC, oxidative stress, free radical, multipurpose tree, TPC

Abstrakt

Strom *Garcinia kola* je původní strom ze střední a západní Afriky. Tento strom je známý především u místních obyvatel, kteří jej využívají především v lidovém léčitelství. Místní tomuto stromu říkají zázračný strom, protože každá jeho část se dá využít. Ve většině případech jsou části tohoto stromu využívány v lidovém léčitelství. Bohužel mimo Afriku není tento strom příliš známý, tím pádem není ani využíván. Tato práce je zaměřena na kolaviron, což je komplex bifalvonoidů vyskytujících se v semenech stromu *Garcinia kola*. Biflavonoidy jsou známé pro své pozitivní účinky na lidský organismus. Jednou z těchto vlastností je antioxidační aktivita. Díky tomu je kolaviron, jako antioxidant schopen předcházet oxidativnímu stresu, který způsoben nadbytkem volných radikálů. Oxidativní stres může být spouštěčem různých onemocnění a také urychluje projevy stárnutí. Proto je pro nás příjem falvonoidů důležitou součástí naší potravy. Tato práce se zabývala výzkumem hodnot antioxidační aktivity kolavironu, komplexu biflavonoidů obsažených v semenech stromu *Garcinia kola*. Tento výzkum byl uskutečněn pomocí tří metod. První hodnota byla naměřena metodou DPPH. Touto metodou byla změřena antioxidační hodnota 69,451 μg/ml. Druhá metoda byla provedena Folin-Ciocalteulovým fenolovým činidlem. Tato metoda nám změřila celkový obsah fenolů v hodnotě 17,3 mg GAE/ml extraktu. A poslední metodou byl ORAC. V této metodě jsme naměřili antioxidační hodnotu 15,329 μg/ml. Díky těmto výsledkům jsme zjistili, že extrakt vykazuje vyšší antioxidační hodnotu.

Klíčová slova: Antioxidanty, DPPH, ORAC, oxidativní stres, volný radikál, víceúčelový strom, TPC

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List of the abbreviations used in the thesis

TPC – total phenolic content

DPPH – 2,2-Diphenil-1picrylhydrazyl

G. kola – Garcinia *kola*

pH – potentional of hydrogen

ROS – reactive oxygen species

RNS – reactive nitrogen species

RSS – rective sulphur species

DNA – deoxyribonucleic acid

CO2 – carbon dioxide

Na_2CO_3 – sodium carbonate

AAPH – 2,2'-Azobisisobutyramidinium chloride

OH – hydroxide

H – hydrogen

OMe – methoxy group

UV – ultraviolet

ORAC – oxygen radical absorbance capacity

HPLC – high-performance liquid chromatography

FRAP –fluorescence recovery after photobleaching

TEAC – Ferric Reducing Ability of Plasma

TRAP – Total Radical-trapping Antioxidant Parameter

1. Introduction

Plants have been used for a long time to treat a variety of ailments and injuries. At present, they are still very important and widely used. The plants we use for healing are called medicinal plants. Unfortunately, the benefits of some of these plants have not yet been studied (Caffini 1999). Therefore, some plants are used only in folk medicine. But in recent years, interest in these plants has been rising (Manourova et al. 2019). The tropical and subtropical climate of Africa is one of the largest sources of plant diversity. An evaluation of the use of vast natural resources is needed as well as a plan for its subsequent use or more professional processing (Atawodi 2005). One of these medicinal plants is *Garcinia kola* also known as bitter kola. This tree is considered as a very important and indispensable component in folk medicine, but it is also an important social element. The locals use all the parts of this tree in various forms for many ailments. Therefore, the medicinal properties of this tree can be classified as antimicrobial, antiparasitic and purgative. *G. kola* is a medium-tall tree growing in West and Central Africa where it occurs in moist forests (Adesuyi et al. 2012). Ripe fruits are orange, and they contain seeds (Indabawa & Arzai 2011). This tree is often felled due to the possibility of using the whole plant but planting of new trees is difficult due to low seed germination. As a result of this problem this plant is not so popular with local farmers. So, we may face a decline in population in future (Agyili et al. 2007).

Garcinia kola gained its healing effects thanks to flavonoids and biflavonoids, which are among the antioxidants. The major biflavonoid complex in this plant is kolaviron (Erukainure et al. 2020). Which, as an antioxidant, it can reduce the activity of free radicals. Antioxidants are also capable of repairing damage caused by free radicals (Chalupová 2013). Excessive numbers of free radicals in the body cause oxidative stress, which causes many serious diseases (Kolečkář et al. 2007). Therefore, antioxidants should be an important part of our diet. But above all, our food intake should be varied and balanced (Gabrovská et al. 2017). Therefore, the antioxidant value of the *Garcinia kola* tree seed extract is investigated in this work.

2. Literature Review

2.1. Garcinia kola

2.1.1. Taxonomy of *Garcinia kola*

Garcinia kola is a tree that occurs in Western, Central Africa (Figure 1.) and also in Asia (Manourova et al. 2019). However, it was also introduced to South and Central America where seeds became a popular stimulant in the 17th century (Niemenak et al. 2008). *G. kola* belongs to the Clusiaceae family which contains 600 species and 16 of those are growing in Africa (Agyili et al. 2007). These individuals of Garcinia genus occur in moist forests (Agyili et al. 2007). These trees grow at low altitudes with annual temperatures from 32°C to 21°C and annual rainfall from 2 500 mm to 2 000mm (Fd & Bo 2010). This tree prefers neutral and slightly acidic pH soil which is moist or wet ("Garcinia kola Bitter Kola PFAF Plant Database" n.d.). In the genus Garcinia, there are also many others popular species for example *Garcinia mangostana* due to its edible fruits and *Garcinia hanburyi* due to its dyeing abilities (Valíček 1989).

Figure 1. Occurence of trees *Garcinia kola* Source:Manourova et al. 2019

2.1.1.1. Scientific classification

Table 1. Scientific classification of *Garcinia kola*

Taxonomy	Name
Kingdom	Plantae
Order	Malpighiales
Family	Clusiaceae
Genus	Garcinia
Species	G. kola
Binominal name	*Garcinia kola*

Ekene & Erhihe 2014

2.1.1.2. Botanical description of *Garcinia kola*

Garcinia kola is an evergreen tree growing originally in West and central Africa. It can grow to 15–17 meters. The tree has a smaller but dense crown with simple shiny leaves (Figure 2.) which are 2–6 cm wide and 6–14 cm long (Ekene & Erhihe 2014). The taste of the leaves is bitter ("Garcinia kola Bitter Kola PFAF Plant Database" n.d.). This dioecious tree blooms once a year with Greenish-white flowers covered with short hairs. Ripe fruits have an orange, velvety exocarp and the flesh is reddish-yellow (figure 3). These berries are round or slightly flattened (Manourova et al. 2017). These round fruits have a diameter of 5–1 cm and a weight of 30–50 grams (Ekene & Erhihe 2014). Harvest time varies according to environmental conditions. The ripe fruits are harvested once they fall from the tree or by tools (Manourova et al. 2017). One tree produces 85–1 717 fruits per one year (Fd & Bo 2010). Fruits of this tree have a very sour tase ("Garcinia kola Bitter Kola PFAF Plant Database" n.d.). Each fruit contains 2–4 seeds with an average length 3,2 cm, width 1,7 cm and weight 5,4 (Manourova et al. 2017). The raw seed has a bitter and aromatic taste ("Garcinia kola Bitter Kola PFAF Plant Database" n.d.).

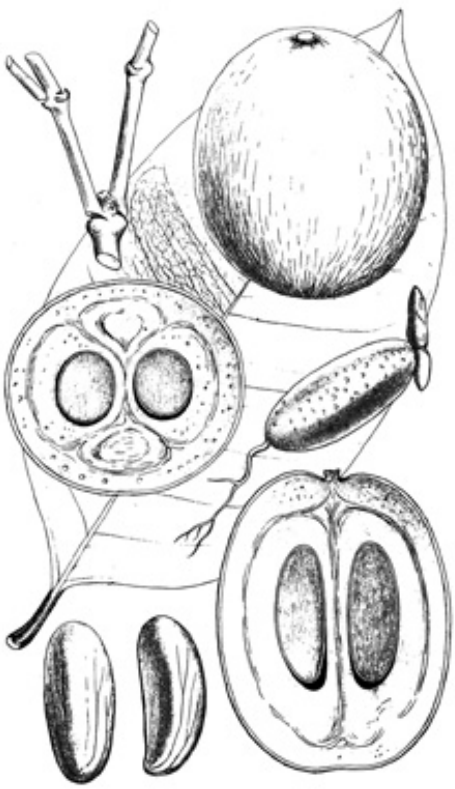


Figure 2. Leaf, fruit, cut fruit, seeds, twig and germinating seed of *G. kola*

Source: "Garcinia kola Bitter Kola PFAF Plant Database" n.d.

Figure 3. Twigs with fruits and leaves of *Garcinia kola*

Source: Scamperdale 2009

2.1.2. Germination and propagation

Although there is a great demand for *Garcinia kola* products, farmers grow this tree in very limited quantities due to low seed germination (Kanmegne & Omokolo 2008). The problem is due to uneven and slow germination or drying of the seeds (Agyili et al. 2007). The seed (figure 2.) is not covered by an impregnable coat, but the endosperm is surrounded by a water permeable testa. As a result of this case, we can assume that seed dormancy occurs due to embryo dormancy. Various hormones such as auxins and cytokinins can be used to disrupt dormancy and subsequent germination (Kanmegne & Omokolo 2008). This also depends on the storage of the seeds. It depends on the time, heat and humidity of the stored seeds (Dadjo et al. 2019).

However, propagation of the *Garcinia kola* tree can be done by other methods. One of these propagation methods is stem cuttings. Cuttings are cut off from a healthy plant. The form of the cutting is soft to semi-hard wood with a few shortened leaves. The stems should be treated with a rooting hormone. Subsequently, they are placed in containers, pots or special bags with a suitable substrate (Laurent Kouakou et al. 2016).

Another method is by using root cuttings. Each cutting should be 10,2–2,2 cm long and 1,3–0,1 cm thick. The cuttings should be a used parts from healthy plants, which are placed in containers with a suitable substrate (Laurent Kouakou et al. 2016).

The last method is coppicing of the tree stump, in which we cut 12-month-old seedings into 2–3 nodes. New shoots are subsequently observed on these stumps, and the remaining cut parts are used as cuttings (Laurent Kouakou et al. 2016).

2.1.3. Use of *Garcinia kola*

The use of plants in medicine has been important since the beginning of medicine. Even in this modern age we can come across medicinal plants in popular pharmaceutical products. These plant medicines are more affordable and less toxic (safer). There are many popular and very effective medicinal plants in the world. One of the lesser known of these is *Garcinia kola* also known as Bitter kola.

This plant is mainly used by indigenous people in their traditional healing methods and it is also a part of their customs (Ekene & Erhihe 2014). For example, its seeds are a major feature of hospitality in West Africa (Niemenak et al. 2008). Garcinia is also very popular because every part of this tree has a healing effect (Ekene & Erhihe 2014).

Garcinia kola is usually used in folk medicine in the form of decoctions or by chewing whole parts of the tree. Latex is also used to treat fresh wounds (Ekene & Erhihe 2014). Furthermore, latex is also known for its waterproof properties. The bitter leaves are used as a flea repellent.("Garcinia kola Bitter Kola PFAF Plant Database" n.d.). Chewing its seeds helps with cough, diarrhea, gonorrhea and many other illnesses. Barb decoction is used to treat fever, inflammation and burns. Both the seeds and the bark are used as an aphrodisiac and can be used to treat men's sexual dysfunction
(Ekene & Erhihe 2014). The branches (Figure 4.) and roots are chewed for their positive effects in dental hygiene. An infusion from the leaves cures fever and the wood can be processed to make tools (Manourova et al. 2019). This tree is also used by farmers who use it to create shade for growing coffee and cocoa (Niemenak et al. 2008).

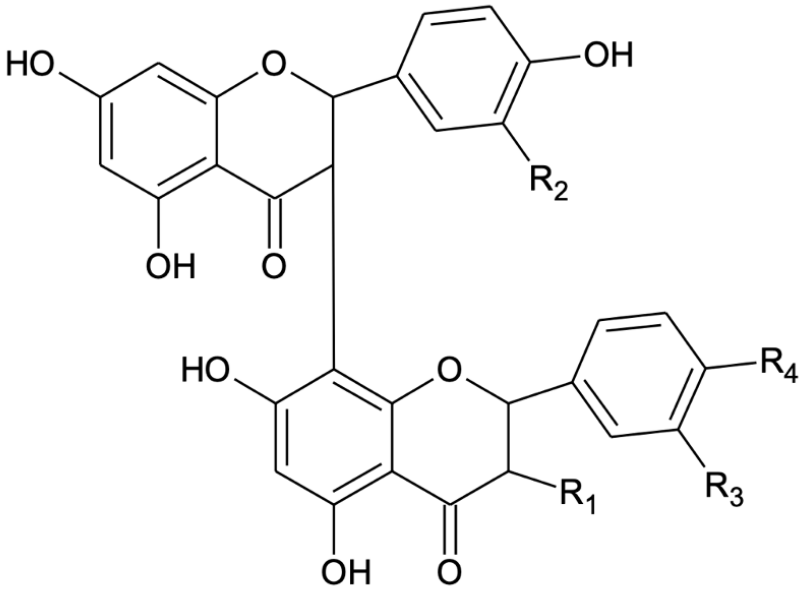
Unfortunately, these trees are on the verge of extinction, mainly due to commercial clearance for the purpose of collecting bark. The restoration of this species is difficult due to the slow-growing seeds, so we may encounter small diversity of this kind (Agyili et al. 2007).

Figure 4. Chew sticks and seeds of *G. kola*

Source: Naliaka 2021

2.1.4. Kolaviron

The healing effects of the *Garcinia kola* tree are influenced by the presence of flavonoids and biflavonoids (Okoko 2009). In recent years, there has been a growing interest in natural resources that have not yet been used. Many plants can be used in medicine, where they can provide a more affordable source of drugs. Therefore, more globally unknown plants, which are currently used only in folk medicine, are still being studied. The active ingredients in these plants are called phytochemicals. Phytochemicals are secondary plant metabolites that are healthy. We classify many classes as secondary metabolites, but the best known are phenols, which include flavonoids. Flavonoids can be found in all parts of plant (flower, fruit, stemp, leaf and root). Flavonoids are described as a phenylbenzopyran functional group which is a class of low molecular weight phenolic substances with a carbon skeleton (Erukainure et al. 2020). One of these biflavonoids is kolaviron which is extracted from the seeds (Farombi et al. 2013). In this tree we can also find other biflavonoids such as kolanone, garciniflavone and amentoflavone (Erukainure et al. 2020). Kolaviron is described as an anti-inflammatory, antigenic and antioxidant therefore has beneficial effects on human health (Farombi et al. 2013). Kolavirone is a biflavonoid complex composed of *Garcinia* biflavonoids 1 (GB 1), *Garcinia* biflavonoids 2 (GB 2) and kolaflavone (Erukainure et al. 2020).



	R1	R2	R3	R4
GB1	OH	H	OH	H
GB2	OH	H	OH	OH
Kolaflavone	OH	H	OMe	OH

Figure 5. Struucture of kolaviron

Source: Owoeye et al. 2014

2.2. Flavonoids

Flavonoids are substances belonging to secondary metabolites. They are low molecular weight and therefore are not required for plant viability.

Flavonoids are a large group of plant phenols, of which about 5 000 are currently discovered. They serve the plant as protection against negative external environmental influences. We can find these substances mainly in higher plants, especially in their leaves, flowers, bark, fruits and seeds. The content of flavonoids in plants is influenced by external factors such as sunlight. It also depends on the species and variety of the

plant. Flavonoids are important due to their varied properties, so they play a big role in our diet. Positive effects of flavonoids can include their function against free radicals, tumor cells or viruses. They can also act as a prevention against inflammation and heart disease. The most famous flavonoids include pelargonidine, rutin, catechin, cyanidine and malvidin (Říhová n.d., Valešová n.d.).

2.3. Antioxidants

Antioxidants are substances that reduce the effect of free radicals. They are also able to convert free radicals to non-reactive or less reactive. This process reduces oxidation. Antioxidants are also able to repair damaged molecules from free radicals. It follows that antioxidants form a complex system against the activities of free radicals (Chalupová 2013). These occur naturally in food. We can divide them according to their origin into natural and synthetic (Čegan & Korecká 2010).

Antioxidants can be found in carotenoids and vitamins A, C and E. They are also found in trace elements such as cooper, zinc, manganese and iron, antioxidant substrates (lipoic acid), enzymes (catalase) and hormones (melatonin) or metabolites (Q10). Many common foods such as spices, herbs (sage, thyme and rosemary), teas, onion and oatmeal also have antioxidant effects (Chalupová 2013).

Gaining antioxidants is just one of the important parts of our food intake. Therefore, our food should be balanced and varied. Many important antioxidants such as vitamins can be found in natural food sources. Usually, food contains a combination of different antioxidants, which is good for health ("turek-sima.pdf" n.d.). Antioxidants can also be taken in the form of food supplements. These supplements should be seen as secondary measure though, the primary one being a healthy and balanced diet (Kráľová n.d.).

2.3.1.1. Antioxidant classification

Natural antioxidants are substances that we can find in food, or ones that our body can create on its own. These include vitamins, flavonoids, phenols, trace elements, lignans glucosides, hormones and antioxidant substrates (Šípek 2000).

Antioxidants can be divided according to many criteria, the first is polarity.

- Lipophilic antioxidants – Those that are fat soluble (estrogen and vitamin E).
- Hydrophilic antioxidants – Those are water soluble, and they are divided into two groups. The first one is intracellular which contains non-enzyme antioxidants (Catalase and peroxidase) and enzyme antioxidants (glutathione). The second group is extracellular which contains high molecular weight (transferrin and hemopexin) and low molecular weight (ascorbic acid and uric acid) groups.
- Amphophilic antioxidants – They have both hydrophilic and lipophilic parts of the molecule (melatonin and lipoic acid) (Šulganová 2016).

We can also divide antioxidants according to the size of the molecules.

- Enzymatic antioxidants (glutathionetransferase and catalase)
- High molecular weight antioxidants (transferrin and albuminin)
- Low molecular weight antioxidants (Carotenoids, thiols and vitamins A, C and E)
- Flavonoids – They are anti-carcinogenic and anti-inflammatory (Šípek 2000).

Antioxidants can also be divided according to their origin.

- Natural antioxidants
- Synthetic antioxidants (Velíšek 1999)

Antioxidants have a complex mechanism that protects our body from oxidative reactions that can be mediated in various ways.

- Preventive – They prevent the formation of free radicals. For example, antioxidants which mediate the nonradical decomposition of hydrogenperoxides and dyhydrogenperoxides-catapllasse and aperoxidase.
- Reducing – They reduce the formation of free radicals-ascorbatereductase and GHS-reductase.
- Chelating proteins – They limit the availability of transition metals-albumin and hemopexin.
- Substances capable of quenching oxygen-carotenoids and superoxidedismutase

- Inactivating – Antioxidants that prevent chain reactions. This group is divided into lipophilic and hydrophilic. Lipophilic-rtinoids, ubiquinone and vitamin E. Hydrophilic-albumin, uric acid and vitamin C.
- Enzymes – These are repairing damaged membranes-lipase, transferase and proteases.
- Adaptation mechanisms – They have the ability to regulate and distribute antioxidants at the required time and place (Faltusová 2020, Pradedova et al. 2011).

2.3.2. Antioxidant activity

Antioxidant activity is the ability of substances to reduce oxidation. We can distinguish two different concepts of oxidative reactivity and capacity. Oxidative reactivity describes the initial dynamics of the antioxidant course based on the antioxidant concentration. Antioxidant capacity describes the time duration of the antioxidant effect (Šulc et al. 2007).

Measurement of antioxidant activity can be determined by many methods. The relationship between the antioxidation of the sample and the ascorbic acid or Trolox solution plays an important role in results (Šulc et al. 2007). In this laboratory research we used DPPH antioxidant activity bioassay and Folin-Ciocalteu reagent method.

2.3.2.1. Free radicals

Free radicals are an important part of biological processes. This does not mean that they are beneficial – on the contrary, they are primarily responsible for harmful processes such as aging (Kohen & Nyska 2002).

Free radicals are atoms and molecules with an unpaired number of electrons. However, electrons tend to occur in a pair state, so they direct electrons from other atoms and molecules. By this act a free radical neutralizes itself. The molecule or atom from which the electron was taken becomes a free radical. This process triggers a chain reaction. The reaction ends when two radicals are connected.

Free radicals are formed endogenously and exogenously. Endogenous radicals are produced by four methods. In mitochondria in which the cellular respiration process takes place. In leukocytes that destroy bacteria, viruses and parasites. In peroxisomes which, as a by-product of the degeneration of fatty acids and other molecules, produce hydrogen

peroxide. They can be formed by an enzyme in the metabolism of foreign substances. Exogenous sources are industrial waste, air pollution, cigarette smoke, certain drugs, radiation, UV radiation and many others.

Names are indicated by the elements from which they come. The Abbreviation ROS is used to denote oxygen-derived forms. RNS is a form of nitrogen and RSS is a form of sulphur (Brtnická 2009).

Table 2. Systematics names and chemical formulas of ROS group

Systematic name	Chemical formula
Superoxid	O_2
Hydroxyl radical	OH
Peroxyl	ROO
Alkoxyl	RO
Hydroperoxyl	HO_2

Source: Šípek 2000

Table 3. Systematics names and chemical formulas of RNS group

Systematic name	Chemical formula
Nitric oxide	NO
Nitrogen oxide	NO_2

Source: Šípek 2000

RSS groups are formed by reactions of oxygen-derived forms (ROS) with thiols (Šípek 2000).

2.3.3. Oxidative stress

An adequate number of free radicals do not harm the body, but a high amount can cause antioxidant stress (Čegan & Korecká 2010) The presence of free radicals and antioxidants in the body should be in balance. When this this balance is disturbed,

antioxidant stress occurs. This stress occurs not only with a large amount of free radicals but also with low activity of the antioxidant system (Boušová n.d.).

Damage occurs during oxidative stress. During damage, the structure and biological function of lipids, proteins and nucleic acids changes (Komrsková 2006). DNA is also affected by oxidative stress which can cause base changes or strand breakage. As a result of these changes, the synthesis of damaged fibers begins, and this affects the functionality of the cell. Oxidation of proteins causes a complete loss of their catalytic abilities. The phospholipid cell membrane is also damaged due to peroxidation of the polyunsaturated fatty acid. When the plasma membrane is damaged, the cell dead ensues. Damage to other membranes can cause the chloroplast to lose the ability to fix CO_2 and respiratory activity in mitochondria (Kyseláková 2012).

Some diseases are caused directly by oxidative stress, such as cancer caused by radiation. In other diseases, oxidative stress is not the primary cause, but it contributes to development of diseases. These diseases include rheumatoid arthritis and atherosclerosis. Free radicals can also affect aging, cancer, diabetes of type two or cardiovascular disorders (Kolečkář et al. 2007).

2.4. Methods of determining antioxidant activity

We can neutralize harmful free radicals with antioxidants. That is why there has been an interest in research on how to obtain antioxidants naturally. For example, we can get them in the form of antioxidant vitamins. But we can also take them in the form of natural substances such as polyphenolic compounds. We can find these substances in fruits, vegetables, herbs, spices and many others. Thanks to this, there is increasing interest in determining the antioxidant value of plant origin substances (Paulová et al. 2004).

These tests are mostly based on exposure of the tested substance to a free radical. As the composition and reaction of the substance can vary, we have multiple techniques based on different principles(Paulová et al. 2004). These tests can be combined. And in many researches they complement each other.

Table 4. Methods of determining antioxidant activity

Techniques	Antioxidant Capacity Assay
Spectrometry	ORAC
	HORAC
	TRAP
	CUPRAC
	FRAP
	PFRAP
	ABTS
	Fluorimetric Analysis
Electrochemical Techniques	Voltammetry
	Amperometry
	Biamperometry
Chromatography	Gas chromatography
	High performance liquid chromatography

Source: Munteanu & Apetrei 2021

3. Aims of the thesis

The aim of this bachelor thesis was to determine the antioxidant values of kolaviron-rich *Garcinia kola* seed extract by using DPPH antioxidant bioassay, ORAC assay and Folin-Ciocalteu reagent method.

4. Hypothesis

The *Garcinia kola* seeds contains kolaviron, complex of biflavinoids antioxidant source.

5. Material and methods

The entire research was carried at the Czech University of Agriculture in Prague, Faculty of Agrobiology and Faculty of tropical agrisciences in the laboratory. Folin-Ciocalteu reagent method was done under the supervision of Ing. Ivo Doskočil, Ph.D. DPPH antioxidant bioassay and ORAC assay was done under the supervision of Ing. Agnes Aba Abakah.

The antioxidant activity of substances can be measured by physical methods or chemical methods. Chemical methods are performed by using reagents that in combination with free radicals stain, the intensity of this color is then spectrometrically measured (Fidler & Kolá n.d.). Because the measurement of the antioxidant value of samples in vivo varies, we cannot use one method to determine the value. Therefore, we combine the methods. We can use luminometric, fluorescence and spectrophotometric methods ("12_Metody_stanoveni_antioxidantu.pdf" n.d.). In this laboratory research we used DPPH antioxidant activity bioassay, ORAC assay and Folin-Ciocalteu reagent method.

5.1. Material

5.1.1. Plant material

Plant material (seeds) from *Garcinia kola* was imported from Cameroon. These seeds were collected at two different locations.

5.1.2. Laboratory material

DPPH (2,2-Diphenil-1picrylhydrazyl), methanol, gallic acid, pure Folin-ciocalteu reagent, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) sodium carbonate 12%, AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride), fluorescein, phosphate buffer, solution, 96well plates, automatic pipettes and tips, breakers, eppendorf tubes, test tubes 10 ml, multiplate reader (Tecan), orbital shaker (GFL) and incubator (medline), transsonic ultrasonic cleaning unit (Elma), liquid handling station (Tecan), magnetic stirrer.

5.2. Methods

5.2.1. Preparation of *Garcinia kola* extract

Fresh seeds were purchased In Yaoundé, Cameroon (3°51' 58.52" N; 11°31'28.87" E). The identification and authenticity was verified by Anna Maňourová *G. kola* expert (Manourova et al. 2019).

The seeds were pre-dried at 40 °C before transport to the Czech Republic and further processed in the Laboratory of Ethnobotany and Ethnopharmacology of FTA and in the laboratories of the Department of Microbiology, Nutrition and Dietetics, a part of the Faculty of Agrobiology, Food and Natural Resources CZU. Then the seeds with peels were homogenized in a laboratory blander Grindomix GM 100 (Retsch, Germany) and microfine grinder MF 10 Basic (IKA, Germany). They were then defatted with petroleum ether extract separation by using the Soxhlet method with the Soxhlet-like extractor SER 148 (Mezos s.r.o., Hradec Králové, Czech Republic). The temperature was set at 70 °C for 120 minutes. The defatted seeds were then dried at 103 °C and weighed until they reached the desired weight. Then the samples were extracted according to modified methods (Adaramoye & Lawal 2014,Iwu et al. 1990, Iwu 1985). They were extracted first with mathanol and then with chloroform. The methanol extract was then separated into chloroform and methanol fractions. So, the methanolic extract was partitioned between chloroform and water. The dark yellow-brown chloroform extract was evaporated on a laboratory evaporator be used as kolaviron-rich extract from *G. kola*. This extract was then stored in a freezer.

5.2.2. DPPH antioxidant activity bioassay

This method is considered as one of best-known methods for determining antioxidant activity (Paulová et al. n.d.). DPPH antioxidant activity bioassay method is very often used in biological research and in the food industry (Jančová 2013). This method is based on the reaction of antioxidants in samples with free radical DPPH. In this method due to reaction the absorbance and color are changed (Čapková 2013). Trolox and methanol were used for the results as a control (Tauchen et al. 2016). Trolox is a water soluble analog of vitamin E. Therefore, it has very high antioxidant activity (Forrest et al. 1994). It is commonly used in the measurement of antioxidant values as an equivalent of antioxidant capacity.

This whole experiment was adapted to a 96-well plate. And then we proceeded with the help of liquid handling station. In robot EVO we add destiled water, methanol, *G. kola* sample, trolox. Into whole plate we added 75 µl methanol and 25 µl of DPPH by multichannel pipet. Subsequently, the plate was placed in an incubator where was left in the dark at 37 °C for 30 minutes. At this stage, color changes could be seen due to the antioxidant value. Subsequently, the values were measured by using reader.

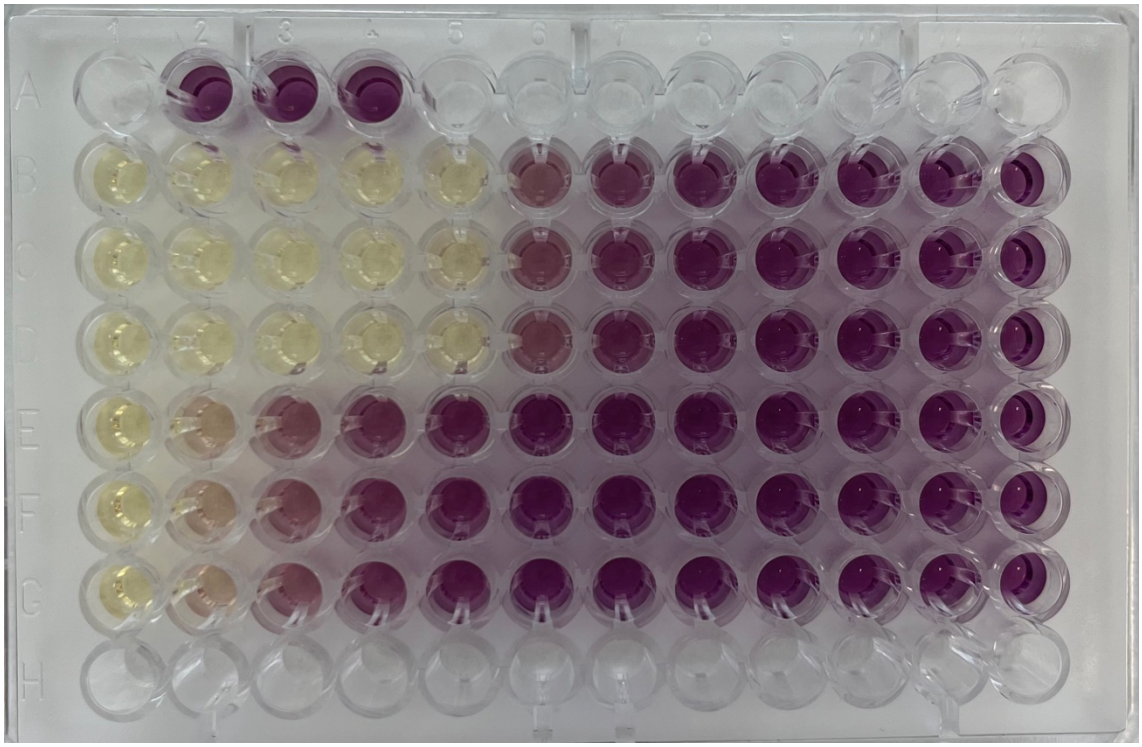


Figure 6. Color changes due to antioxidant activity

Source: author

5.2.3. ORAC assay

The orac method works on the principle of inhibiting free radicals that are created by the azo-compound AAPH. Free radicals are mixed with the fluorescent sample and losed their fluorescent properties in the presence of antioxidant activity. So, in this case, the antioxidant value is manifested by the loss of fluorescence. When the antioxidant value is stronger, the fluorescence is smaller (Munteanu & Apetrei 2021). Trolox is used as a positive control in this experiment. Trolox is a water-soluble analog of vitamin E. Therefore, it has very high antioxidant activity (Forrest et al. 1994).

This whole experiment was adapted to a 96 – well plate. And then we proceeded with the help of liquid handling station. In robot EVO we add destiled water, buffer, *G. kola* sample, trolox. Make dilution and take out 75 µl. Than we added 150 µl (by pipette) of fluorescein to the entire plate. Subsequently, the plate was placed in an incubator where was left in the dark at 37 °C for 10 minutes. After incubation we added 25 µl of AAPH except control. And to control we added 25 µl of phosphate buffer.

Then we continued the incubation for 1 hour and 30 minutes. Subsequently, the values were measured by using reader.

5.2.4. Folin-Ciocalteu reagent method

One of the common methods for the determination of felolitics is the Folin-Ciocalteu reagent method. We can also know this method as the spectrophotometer-cuvette method (Attard 2013). This method is based on the oxidation of phenols with a Folin-Ciocalteu reagent (Rover & Brown 2013). This reagent is reduced in the presence of a phenolic antioxidant (Munteanu & Apetrei 2021).

This whole experiment was adapted to a 96 – well plate. The samples in 96-well plate were diluted with a pipette in distilled water. Then we added 25 µl Folin-Ciocalteu reagent in each well and placed on a shaker for 10 minutes. After 10 minutes on an orbital shaker, we added 75 µl of Na_2CO_3. Subsequently, the plates were placed in an incubator where the plates were left in the dark at 37 °C for 50 minutes. The absorbance was determined from the plate by the reader. Gallic acid and distilled water were used for the results as a control substance (Rangel et al. 2013). The acid was used as a positive control because it is an antioxidant (Badhani et al. 2015).

6. Results

6.1. DPPH antioxidant activity bioassay

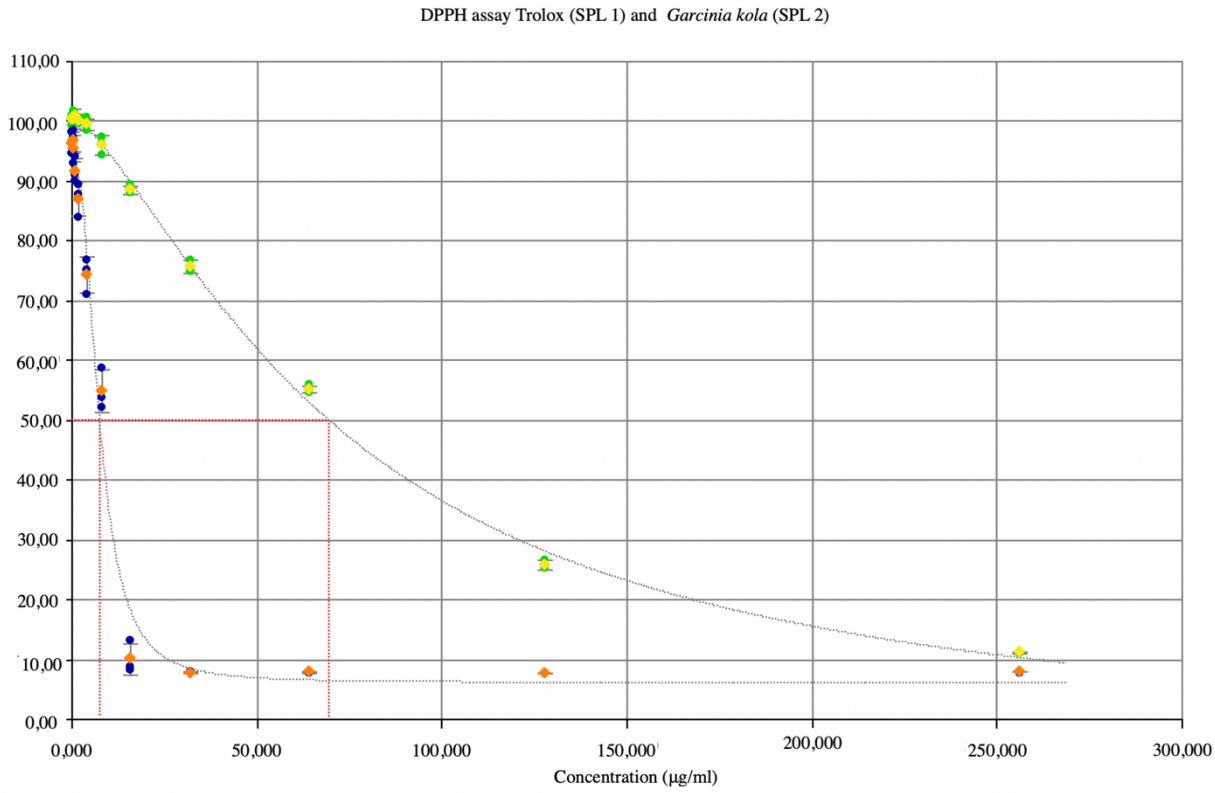
All calculations were performed by using reader. Antioxidant value was proved in *Garcinia kola* extract sample.

In this method a *Garcinia kola* seed sample and Trolox was added in one plate in which was experiment repeated three times.

By using graph of trolox and samples, was determinate the IC_{50} value. Whit this value, we further calculated the average of antioxidant value of *G. kola* sample which was 69,451 μg/ml and the average of antioxidant value of trolox 7,67 μg/ml as an equivalent of antioxidant capacity.

Based on the color change of the samples (figure 6.), we can confirm the antioxidant effect of *G. kola*. But by comparing *G. kola* sample with trolox, we find that the antioxidant values of *G. kola* are significantly lower.

Chart 1. DPPH results



Source: author

Table 5. DPPH results

Curve Name	Name	Y Formula	Y	X
Curve SPL1	SPL1	50	50	7,67
Curve SPL2	SPL2	50	50	69,451

Source: author

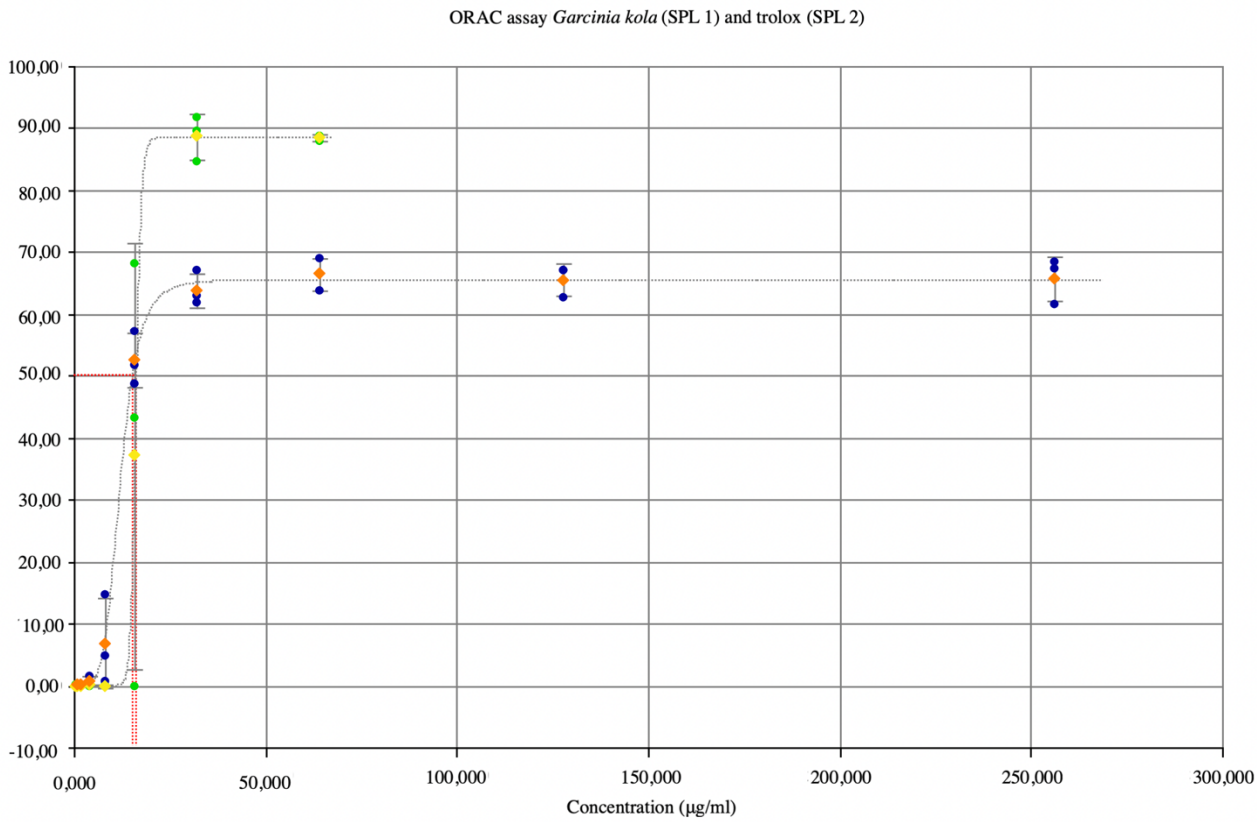
6.2. ORAC assay

All calculations were performed by using reader. Antioxidant value was proved in *Garcinia kola* extract sample.

In this method a *Garcinia kola* seed sample and Trolox was added in one plate in which was experiment repeated three times.

Using the graph of trolox and samples, we can see that their values are almost the same. Which indicates a high antioxidant value of the *G. kola* sample. We further calculated the average of antioxidant value of *G. kola* sample which was 15,329 µg/ml and the average of antioxidant value of trolox 16,412 µg/ml as an equivalent of antioxidant capacity.

Chart 2. ORAC results



Source: author

Table 6. ORAC results

Curve Name	Name	Y Formula	Y	X
Curve SPL1	SPL1	50	50	15,329
Curve SPL2	SPL2	50	50	16,412

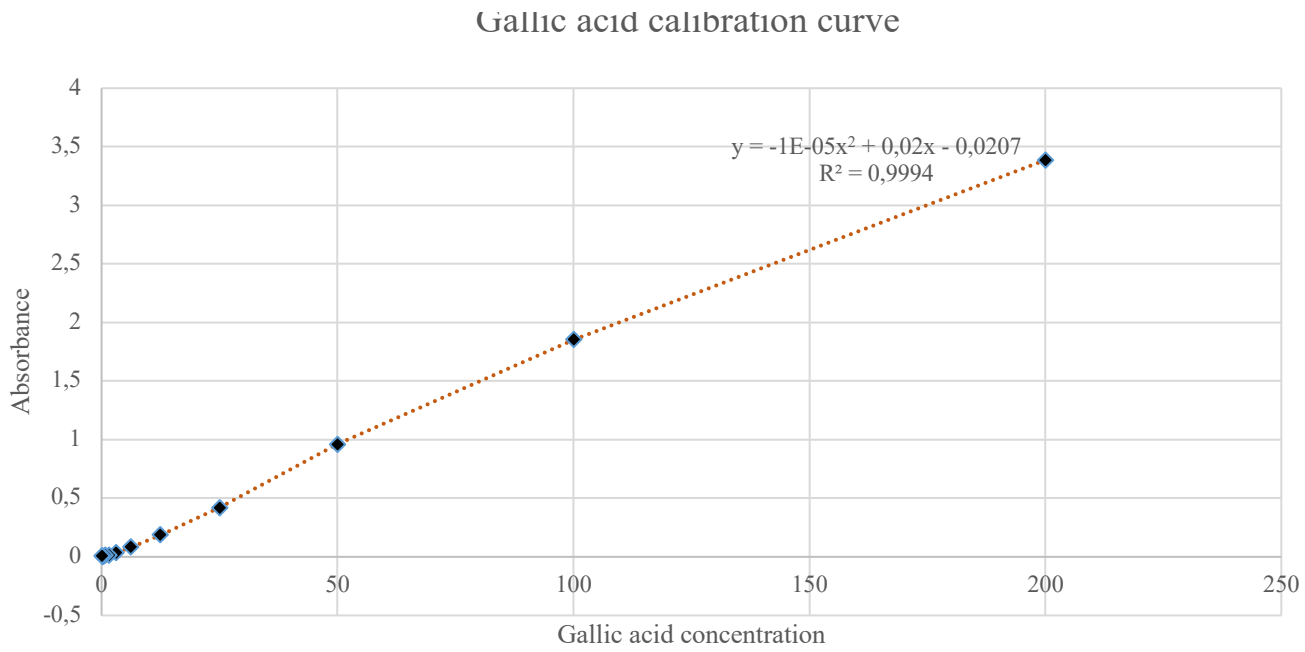
Source: author

6.3. Folin-Ciocalteu reagent method

All calculations were performed by using MS excel. The presence of phenolic substances in the *Garcinia kola* extract sample was proved.

A total of three plates were used, from which all samples were subsequently averaged. Samples and gallic acid were loaded into 96-well plate. Subsequently, the results were calculated of the gallic acid calibration curve (Chart 2.). Result was expressed in milligrams of gallic acid per millilitres of extract. The average measured value of *Garcina kola* seeds extract was 17,3 mg GAE/ml extract.

Chart 3. TPC results



Source: author

7. Discussion

The seeds of the *Garcinia kola* tree are a source of kolaviron, which is one of the lesser-known bioflavonoids (Ekene & Erhihe 2014). Bioflavonoids are substances that are healthy, so their consumption helps against various diseases (Havsteen 2002). As a result, parts of this tree are widely used in folk medicine, where they have been used against malaria, cough or gonorrhea. Due to the content of kolaviron, it is possible to use this plant in medicine or as a beneficial dietary supplement (Ekene & Erhihe 2014).

One of the beneficial properties of bioflavonoids is their antioxidant properties, so they are antioxidants. Antioxidants are substances that cause the reduction or inhibition of free radicals. Therefore, they prevent oxidative stress, which is perceived as an excess of free radicals in the body. Oxidative stress can trigger various diseases, so it is perceived as negative. Examples of these diseases are type two diabetes, cancer and accelerated aging. Therefore, antioxidants should be an important element of our balanced diet (Šípek 2000). Antioxidants can be replaced in the diet by various nutritional supplements, but these ingredients should be primarily supplemented in the form of a plant-based diet, which is part of our daily diet (Pláteník n.d.). Unfortunately, *G. kola* is not publicly known. Therefore, it is not used as a food supplement. But we have many other plants that are used even if they are less beneficial like Garcinia kola.

Garcinia Mangostana (mangosteen) is botanically close to the *G. kola* plant. But unlike *G. kola*, it is better known and used more often. It is a slow growing medium-tall tree with glabrous leaves. It bears a popular tropical fruit with a purple skin in which is a white juicy pulp. This pulp makes the mangosteen well known. Because the pulp is very delicious and therefore it is a favorite delicacy for many people (José Pedraza-Chaverri et al. 2008). This tree is widely used, for example, as animal feed, dye for textiles and many others (Aizat et al. 2019). It is also widely used as a medicinal plant. It is used in the treatment of dysentery, cholera, inflammation, diarrhea and treating infected wounds. Mangosteen is popular in Ayurvedic medicine (José Pedraza-Chaverri et al. 2008). There are also studies investigating the anti-cancer effects of Mangosteen (Akao et al. 2008). The extract of mangosteen is used as a dietary supplement, mainly due to anti-inflammatory, antimicrobial and antioxidant activity (Chin & Kinghorn 2008). Regular us of food supplements increases immunity (Tang et al. 2009).

Due to the botanical similarity of the plants and the different popularity among the general public, I decided to compare the antioxidant values of these two trees. In both cases, three assay were compared – DPPH, ORAC and TPC.

In the ORAC test, we got a very positive result for the *Garcinia kola* sample because it was better than Trolox. And that is a water-soluble analogue of vitamin E and therefore has very high antioxidant activity (Forrest et al. 1994). Therefore, I can say that *Garcinia kola* has a very high antioxidant value. Which was measured at 15,33 µg/ml in this assay. When we compared this result with *Garcinia Mangostana* 95,16 µg/ml (Xie et al. 2015). We concluded that the sample of *G. kola* has higher antioxidant values *than G. mangostana*.

In the DPPH test, we confirmed the antioxidant activity thanks to the color change on the 96-well plate. In this case, trolox was also used as an equivalent of the antioxidant capacity. But the comparison of our sample with trolox was not as positive as with the ORAC method. But it has been proven to have a medium high antioxidant value. In this case, the measured value of the *Garcinia kola* tree was 69,5 µg/ml. This value is also very positively different from mangosteen. Whose measured value by using the DPPH assay is 153,2 µg/ml (Rohman et al. 2019).

In the last TPC test. We determined the total number of phenols, which are together with vitamins, falvonoids and other natural antioxidants (Šípek 2000). In this case, the result of Garcinia kola was worth 17,3 mg GAE/ml extract and *Garcinia Mangostana* 18,7 mg GAE/ml extract. In this case, mangosteen has a slightly higher antioxidant value, but the difference in values is very small, so we can say that in this case the antioxidant value is almost the same.

8. Conclusion and recommendation

Kolaviron is a complex of bioflavonoids that are generally known for their antioxidant value. Therefore, this work is focused on determing the antioxidant value of kolaviron, a complex of biflavonoids from the seeds of *Garcinia kola* tree seeds extract. The antioxidant value was assessed for this work by three different methods. The first two methods were the DPPH assay and ORAC assay which are based on the method of generating synthetic free radicals that respond to the presence of antioxidants in a seed extract sample. The third method was the TPC method is based on the oxidation of phenols with a Folin-Ciocalteu reagent.

All these methods came out positive for the presence of antioxidant activity. The highest antioxidant value was measured by ORAC assay with the result 15,329 μg/ml. This value expresses a high antioxidant value. But the other two methods, DPPH with the result 69,451 μg/ml and TPC with the result 17,3 mg GAE/ ml extract, demonstrate rather medium high antioxidant values. Because of these results, I confirmed the positive properties of kolaviron. Therefore, it could be used in the future in pharmacy, food industry, or in many other sectors.

In the next phase of the research, it is necessary to carry out more methods of measuring the antioxidant value. For example- ABTS, TEAC, TRAP, HPLC and FRAP. I would also use methods that are not only spectrometric, for example – High performance liquid chromatography, Voltammetry and Biamperometry. These methods should be performed several times and then compared with each other.

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