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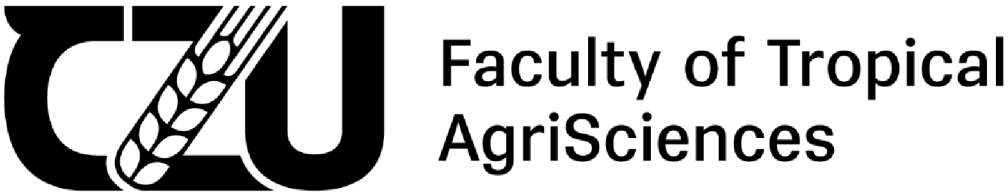

Toxicity assessment of kolaviron, biflavonoid from *Garcinia kola*

BACHELOR'S THESIS

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Declaration

I hereby declare that I have done this thesis entitled .... 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.

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Abstract

Garcinia kola (Clusiaceae) is a fruit tree species indigenous to Western and Central Africa. The tree is frequently called 'wonder plant' because all its parts have medicinal properties. *Garcinia kola* is a plant that has been used in traditional African medicine to treat a variety of infectious disorders. The seed of *Garcinia kola* are commonly chewed as a snack and at the same time there are used for various medicinal purposes and health benefits and that is why the aim of this study was to determined the cytotoxicity of *Garcinia kola* seeds. In this work, the inhibitory concentration IC_{50} of human colorectal carcinoma cell lines Caco-2 and HT-29 was determined by the MTT method in toxicity tests of kolaviron-rich *G. kola* seed extract. As a result, none of the extracts had an inhibitory IC_{50} concentration against any cell line less than 200 μg/ml. *Garcinia kola* seeds do not show cytotoxic activity.

Key words: African flora, multipurpose tree, bitter kola, Southern Nigeria

Abstrakt

Garcinia kola (Clusiaceae) je ovocný strom pocházející ze západní a střední Afriky. Stromu se často říká „zázračná rostlina“, protože všechny jeho části mají léčivé vlastnosti. *Garcinia kola* je rostlina, která se v tradiční africké medicíně používá k léčbě různých infekčních poruch. Semena *Garcinia kola* se běžně žvýkají jako svačina a zároveň se používají pro různé léčebné účely a zdravotní přínosy, a proto bylo cílem této studie zjistit cytotoxicitu semen *Garcinia kola*. V této práci byla inhibiční koncentrace IC_{50} buněčných linií lidského kolorektálního karcinomu Caco-2 a HT-29 stanovena metodou MTT v testech toxicity extraktu semen *G. kola* bohatého na kolaviron. Výsledkem bylo, že žádný z extraktů neměl inhibiční koncentraci IC_{50} proti buněčné linii nižší než 200 ug/ml. Semena *Garcinia kola* nevykazují cytotoxickou aktivitu.

Klíčová slova: africká flóra, víceúčelový strom, hořká kola, jižní Nigérie

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G. kola – Garcinia kola

MTT - 3-(4,5-dimethylthiazol-2yl)-2,5-difenyltetrazolium bromid

Caco-2 - human epithelial colorectal adenocarcinoma cells

HT-29 - human colon cancer cell line

IC_{50} – the half maximal inhibitory concentration

1. Introduction

Garcinia kola Heckel known as bitter kola in the Clusiaceae (formerly Guttiferae) family occurs in Western and Central Africa forests and its products have many uses due to their medicinal and nutritional properties. It is a meaningful medicinal tree, which locals chew dried its seeds mainly as a remedy to treat several diseases such as liver disorders, hepatitis, diarrhoea, malaria and gonorrhoea. *Garcinia kola* contains a wide range of biochemically active molecules, including kolaviron. Kolaviron is the main constituent isolated from *G. kola* seeds and contains biflavanones. Recent studies have shown that kolaviron has anti-cancer, anti-inflammatory, antimalarial, antimicrobial and hepatoprotective effects on human health. This species of plant is very popular among local communities and its use is only significant locally, also it has potential for pharmacological research.

2. Literature Review

2.1. Garcinia kola

Garcinia kola (bitter kola) is introduced to as a "miracle plant" because every part of it has been found to have medicinal significance. The plant is used as an antidiabetic, antioxidant and for the activities of chemoprevention of aflatoxin B1 and antihepatotoxic (Farombi et al. 2005). *G. kola*, sometimes known as bitter kola or male kola, is a plant that has been used in traditional African medicine to treat a variety of infectious disorders. *G. kola* seed has a bitter, astringent, resinous flavour when chewed, similar to raw coffee, followed by a faint sweetness (Iwu et al. 2002). *G. kola* belongs to the family of plants called Clusiaceae and it is a dicotyledonous plant (Earnest 2014). It is a perennial crop growing in the forest, widespread throughout Western and Central Africa (Iwu 2013). It is also occurring in the forest zone of Sierra Leone, Cameroon, Ghana and other countries in Western African. It is common in the southwestern states in Nigeria and the state of Edo (Uko et al. 2001). It is an evergreen tree of a medium sized about 15-17 m high with a fairly, narrow crown. It has simple leaves about 6-14 cm long and 2-6 cm across. The leaves are shiny on both sides and spotted with resin glands. The small flowers are covered with short red hairs (Iwu 2013). The fruit is a drupe about 5-10 cm in diameter and its weighs 30-50g. Usually it contains yellow-red pulp, and it is smooth. During maturarion the fruit changes its color from green to orange and each fruit contains 1-4 smooth elliptically shaped seeds (Earnest 2014). The seed has a bitter astringent taste when consumed (Akintonwa & Essien 1990).

The most prized commodity are the seeds, often chewed by both rural and urban communities to avoid and treat gastrointestinal problems or just for their unique astringent flavour. The kernel includes a variety of beneficial phytochemicals, such as high tannins and flavonoid content. The biflavonoid kolaviron complex is the most widely discussed of these substances. This compound is said to contain neuroprotective, anti-inflammatory, antimicrobial, and other health-promoting properties (Usunomena 2012). Kolaviron has anti-malarial and wound-healing effects as well (Nwaehujor et al. 2015). Kolaviron's therapeutic potential has been demonstrated in the treatment of benign prostatic hyperplasia (Kalu et al. 2016), neurodegenerative diseases such as multiple sclerosis

(Omotoso et al. 2018) and acquired immunodeficiency syndrome (AIDS) (Nworu et al. 2008), and in laboratory trials, the seed extract was able to stop Ebola virus growth (Iwu 2013).

2.1.1. Taxonomy

Garcina kola Heckel known as bitter kola, is a medium-sized tree with coarsely hairy blooms and huge fruits that are the size and color of an orange (Nielsen 1991). It is found in subtropical and tropical lowland woods and belongs to the *Garcinia* genus of the Clusiaceae/Guttiferae family (Okoye et al. 2014). The Clusiaceae family is composed of 18 genera that include woody perennials, trees, shrubs, or even lianas. *Calophyllum*, Clusia, and *Garcinia* are the most popular pantropically (Engel et al. 2016). Exudation of white-yellow coloured latex from diverse plant sections is a common morphological characteristic shared by all family members (Buba Chukkol & Okhale 2016). *Garcinia* is a large genus of dioecious woody plants including over 250 species that are a frequent understory element of lowland tropical forests (Guedje et al. 2002). Swiss botanist Laurent Garcin (1683–1757), working for the Dutch Indies Company, after whom the genus was named, presented the first description of mangosteen (*Garcinia mangostana*), the most famous fruit species from the *Garcinia* genus (Corley 2007). The genus may be classified into two groups: Asian and African, with certain species were also brought to South America. There are roughly 21 species of *Garcinia* in the West and Central African area (Guedje & Fankap 2001).

Clusiaceae, the garcinia family (order Malpighiales), is a tropical tree and shrub family with roughly 14 genera and 800 species. A number of species are planted as ornamentals, and others are valuable for their fruits, resins, or timbers (Cabral et al. 2017). It is defined by the slow rate of growth as a tropical fruit tree species. It may reach a height of 12 meters and a width of 1.5 meters as a medium-sized tree (Adegoke et al. 1998). Clusiaceae leaves are generally broad-ended rectangular in shape, leathery, and contain a strong central vein from which several delicate horizontal veins branch. The plants feature a sticky resinous sap, blooms with several stamens that are typically bundled together, and separate petals and sepals. Male and female organs are frequently seen in different flowers (Cabral et al. 2017). The leaves are simple, measuring 6-14 cm long and 2-6 cm across, with a glossy surface and resin glands scattered on both sides.

The little blossoms have short, red hairs covering them (Iwu 2013). *Garcina kola* fruit is a drupe with a diameter of 5-10 cm and a weight of 30 to 50 g (Fig. 1). The fruit turns from green to orange as it ripens (Fig. 2), and each one contains 1-4 smooth elliptically shaped seeds (Earnest 2014).

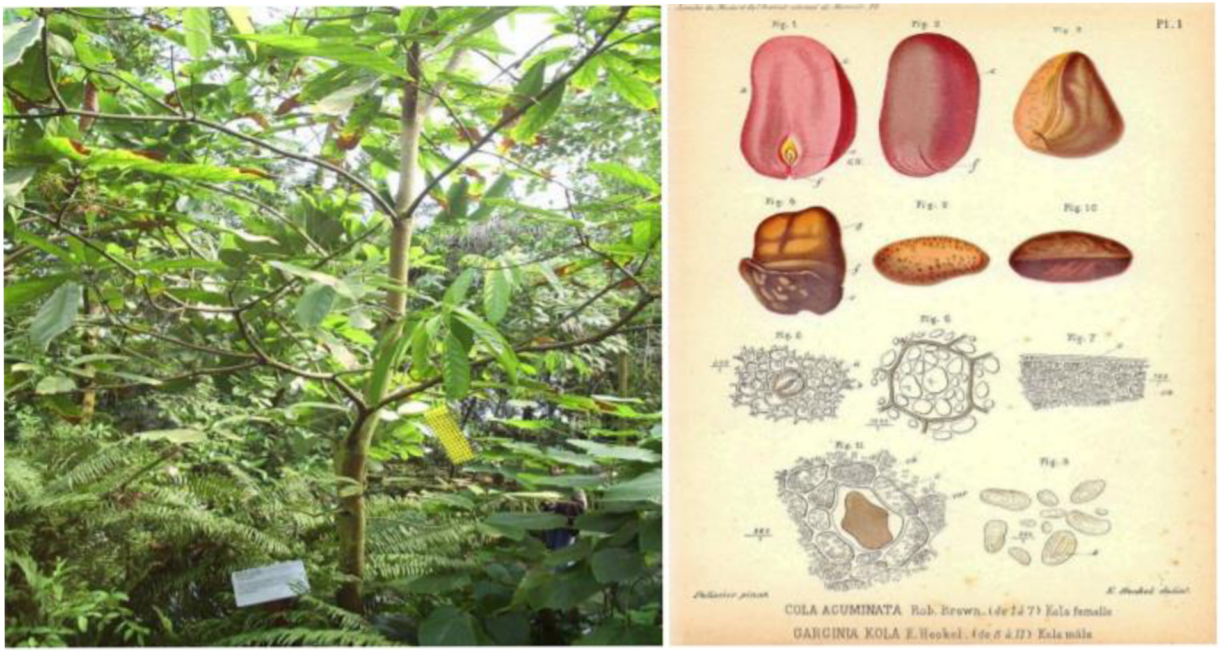


Figure 1 Showing pictures of *Garcinia kola* (bitter kola) plant and fruits. Source: (Earnest 2014)

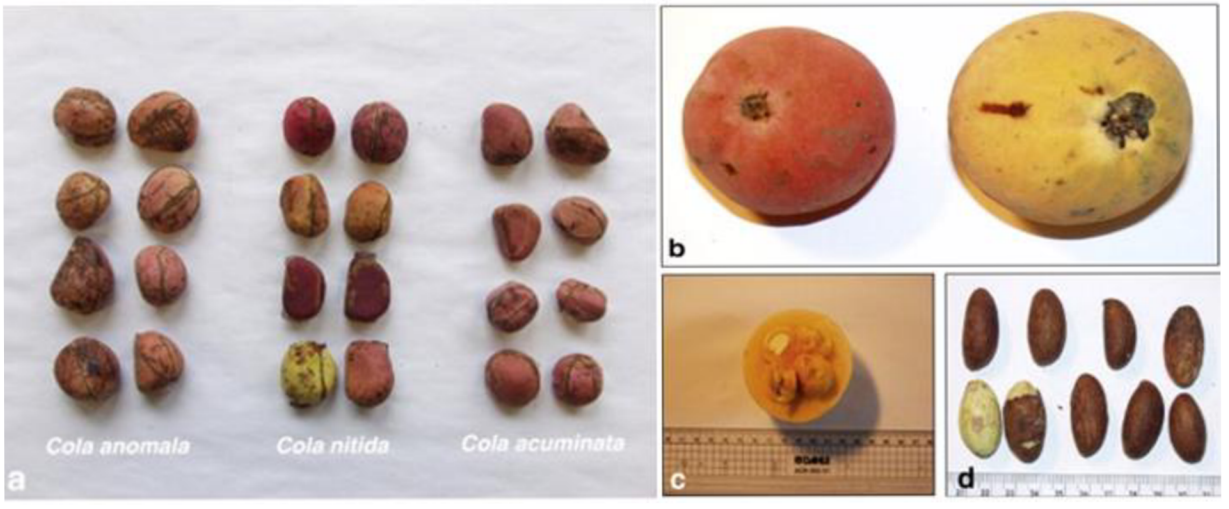


Figure 2 Seeds of Cola sp. (a), fruits of Garcinia kola (b), transversal section of G. kola showing the disposition of seeds in the fruits (c) and seeds of G. kola (d). Source: (Niemenak et al. 2008)

2.1.2. Botanical Description of *Garcinia kola*

G. kola is a polygamous tree with more than 180 species that belongs to the botanical family Guttiferae. It is a member of the genus *Garcinia* and the botanical family Guttiferae. *G. afzelli*, *G. handburii*, *G. indica*, *G. mangostana*, *G. mannii*, and *G. morella* are some additional prominent members of the family (Jena et al. 2002). It is frequently referred as bitter kola (English), Orogbo (Yoruba), Aka ilu (Igbo) and Namijin goro (Hausa) (Fapohunda et al. 2017). It is a well-branched, evergreen, medium-sized tree that may reach a total height of around 12 meters in 12 years (Fig. 3). *Garcinia kola* has been dubbed the "wonder plant" since nearly every component of the tree has been shown to have medicinal use (Fondoun & Manga 2000).

Garcinia kola creates brown, nut-like seeds that are utilized in Nigeria and other African nations during cultural festivities such as weddings and traditional rites. This plant is revered by many African tribes, who employ it in their rituals and ceremonial rites. Across cultural, educational, economic, social, and religious lines, the edible seed is valued and devoured. *Garcinia kola* seed has a bitter, astringent, resinous flavour when chewed, similar to raw coffee, followed by a faint sweetness (Adesuyi et al. 2012). *Garcinia kola* is an evergreen tree species native to Western and Central Africa's tropical rainforests (Lewis 1986), and it is extensively dispersed throughout these regions. Nigeria, Benin Republic, Ghana, Cameroon, Sierra Leone, Togo, Congo Democratic Republic, Central African Republic, Angola, Liberia, Gambia, Ivory Coast, Gabon, Congo Brazzaville, and Senegal are among the nations where the tree is found in cities and villages (Adebisi 1997). The leaves are large, elongated, and leathery, with 10 pairs of lateral veins running parallel to the border and conspicuous resinous canals (Fig. 4). Around December-March and May-August, the tree bears male, and female blooms individually. Male flowers are smaller and have more pronounced stamens than female blossoms, which are yellow and meaty (Fig. 5). The tree produces fruits every year, and *Garcinia kola* has a predictable fruiting cycle (Farombi 2011).

Figure 3 Morphological features of *Garcinia kola*. (a) bark with a fresh cut; (b) seedlings; (c) leaf collection from one tree; (d) seeds obtained in Yaoundé market; (e) branches with ripening fruits; (f) trunk with typical irregular branching pattern. Source: (Maňourová et al. 2019)

Figure 4 Leaves of different ages. Source: (Onyekwelu & Stimm 2019)

Figure 5 Flowers in various stages. Source: (Onyekwelu & Stimm 2019)

Figure 6 A = leafless semi-hardwood cuttings; B = leafy softwood **c**uttings of *G. kola*. Source: (Laurent Kouakou et al. 2016)

2.1.3. Use of *Garcinia kola*

Garcinia kola has a wide range of applications. For a long time, people in Western and Central Africa have used this tree species in their daily life. Many African tribes regard this plant as sacred and utilize it in their rituals and celebrations. Bitter kola is revered as a sacred plant by all Nigerian tribes, notably the Yoruba and Igbo. Bitter kola is a major component of the materials used in traditional Yoruba marriage and naming ceremonies, and it is also utilized in Igbo 'fetish' recipes (Adebisi 1997). *Garcinia kola* has several ceremonial purposes in Ghana (Adu-Tutu et al. 1979), while the seeds are widely given as presents to guests in Cameroon and Nigeria (among the Igbos) to display the host's hospitality, a sign of peace and acceptance, and an indicator that the visitor is welcome (Ayuk et al. 1999).

As a result, *Garcinia kola* seeds serve an essential social role. The seed (bitter kola) is commonly used as a stimulant (Atawodi et al. 1995); when chewed, it has a bitter, astringent, and resinous flavour, followed by a small sweetness. Bitter kola is a well-known aphrodisiac. Many Africans consider this species to be a powerful bug and vermin repellant, as well as a deterrent to ghosts and malevolent spirits. The seed is said to drive snakes out of places where they are housed (Aremu 2009). Bitter kola causes a modest reduction of appetite via increasing serotonin levels in the brain, and is hence utilized for dieting purposes (Joseph & Adeyemi 2011). The most major source of chewing sticks in West Africa is *Garcinia kola* (Adu-Tutu et al. 1979), which provides natural tooth care (Fig. 6). It has also been claimed that *Garcinia kola* might be used as a hop replacement in lager beer production; it is particularly beneficial in reducing beer spoiling (Dosunmu & Johnson 1995). The pulp of the fruit is used to make ethanol (Nzelibe & Okafoagu 2007). The tanning and dyeing industries make use of the stem's bark. The dead branches are utilized as firewood, while the wood is used as yam lumber, poles, and stakes. The species is also utilized in traditional soft drink production. *Garcinia kola* seed extract and dry powder have been manufactured into a variety of products, including creams, vials, and tooth paste (Iwu 1985).

The most major application of *Garcinia kola* is in healthcare. *Garcinia kola* is known as a wonder plant since practically every component of the plant has been proven to have therapeutic potential (seed, bark, leaves, root, wood, etc.). The tree species has been utilized in African culture for medicinal purposes for generations. *Garcinia kola* is

widely used in traditional medicine to treat a variety of ailments. The seeds are employed in many herbal preparations in traditional medicine and have potential therapeutic effects owing to the activity of flavonoids and other bioactive chemicals in the seeds (Farombi et al. 2000). Bitter kola is used by traditional herbalists in a variety of pharmacopoeia formulations for a variety of diseases. The bark of the *Garcinia kola* tree is used to cure diarrhoea. The species is used by traditional African medicine men to cure coughs and for its purgative, anti-parasitic, and antibacterial properties (Madubunyi 1995).

According to reports in the literature, *Garcinia kola* is used as an antidiabetic, antiparasitic, antiviral, anti-hepatotoxic, antidiabetic, antimicrobial, anti-inflammatory, purgative, antidote to the effects of Strophanthus gratus, remedy for guinea worm infection, and for the treatment of asthma, gastroenteritis, rheumatism, bronchitis, throat infections, menstrual cramps, cure of head or chest colds, cough and liver disorders (Kay 1978). *Garcinia kola* seed is thought to contain a wide range of organic substances, including flavonoids, that have antibacterial and antifungal properties against Gram-negative and Gram-positive microorganisms. Flavonoids have biological actions such as fighting allergies, inflammation, free radicals, and hepatotoxins (Terashima et al. 2002).

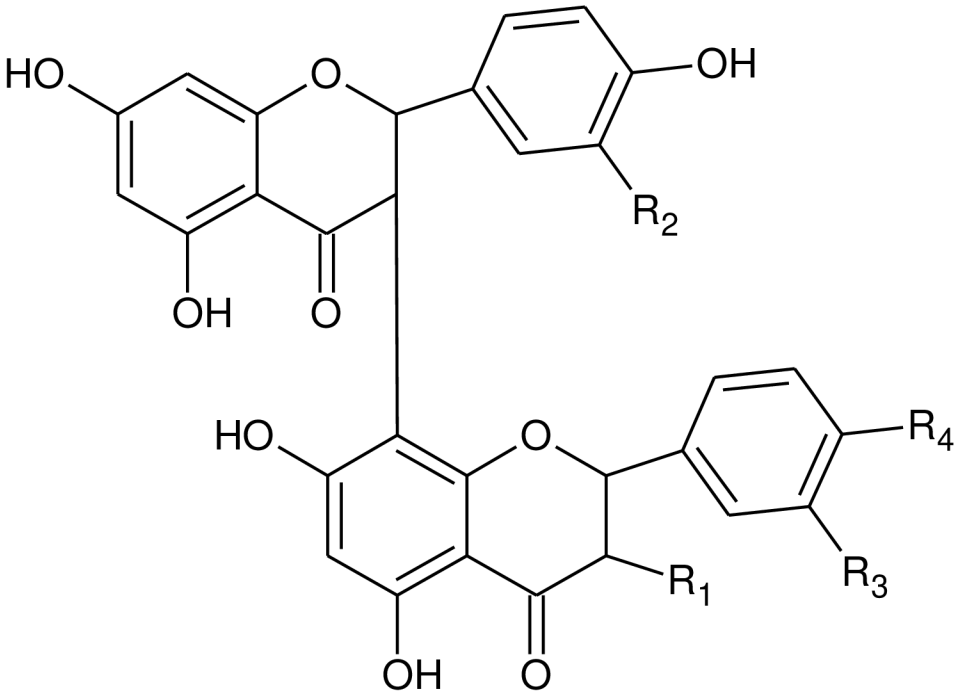
2.1.4. Biochemical characterization

Carbohydrates, also known as nitrogen-free extracts (NFE), make up the majority of the seed's proximate composition, accounting for around 65 percent. On the other hand, ash content, which is the consequence of the entire sample being burned to inorganic substituents, is extremely low, averaging only 1.5 percent. Crude protein has a mean value of 3.5 percent, crude fat is approximately 6.2 percent, and crude fiber is around 9.4 percent (Maňourová et al. 2019).

Flavonoids, oleoresins, tannins, saponins, alkaloids, and cardiac glycosides have been found in the seeds, according to chemical analysis (Buba Chukkol & Okhale 2016). The primary components of *Garcinia kola* seeds are *Garcinia* biflavonoid 2 (GB-2), *Garcinia* biflavonoid 1 (GB-1), kolaflavanone (KF), and *Garcinia* biflavonoid 1a (GB-1a), which were discovered in earlier study by characterisation and isolation of *Garcinia kola* seeds. Furthermore, African scientists discovered that kolaviron (Fig. 7) (Iwu 1985), the main constituent of *Garcinia kola* seeds, is a biflavonoid complex containing GB-1, GB-2, and KF, and that it has a variety of pharmacological properties,

including anti-hepatotoxicity (Alabi et al. 2017), radioprotective (Adaramoye 2010), vasodilation, hypoglycemic, hypolipidemic, and gastroprotective properties (Adaramoye 2012), among others. Garcinoic acid (GA) and its derivatives, which are intriguing tocotrienol compounds, have anti-inflammatory (Wallert et al. 2019), antioxidant (Terashima et al. 2002), and DNA polymerase inhibitory properties (Maloney & Hecht 2005).

Kolanone, one of the main components of *Garcinia kola's* petroleum spirit extract, has antibacterial activity against both Gram-positive and Gram-negative organisms. The inclusion of biflavonoids, GB1, GB2, kolaflavonone, and garciniflavonone is thought to be responsible for its immune-boosting properties. Anti-inflammatory, antibacterial, antiviral, and antidiabetic properties have also been claimed to biflavonoids. They have demonstrated exceptional antiviral effectiveness against a variety of viruses including Punta Toro, Pichinde, Sandfly fever, Influenza A, Venezuelan Equine Encephalomyelitis and Ebola. IC_{50} values vary from 7.2 to 32 μg/ml, with magnetization transfer contrast (MTC) of more than 320 μg/ml (Iwu et al. 2002). Overall, the studies concur that the seeds contain relatively large levels of moisture, about 70%, which is critical for kernel preservation.



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

Figure 7 Chemical structure of kolaviron (KVN). Source: Author

2.2. The human gastrointestinal tract

In the human gastrointestinal system, food digestion is a continual and dynamic process. It all begins in the mouth, with the two complimentary oral systems of mastication and salivation. Oral processing of solid meals results in the decrease of food particle size and the development of food bolus (Hiiemae 2004). Liquids, on the other hand, are practically ready to swallow and require very minor processing beyond equilibration to body temperature and diluting with saliva (Engelen et al. 2003). The central nervous system (CNS) is thought to interpret final changes in bolus characteristics

as a signal that the bolus is ready to be swallowed without pain, discomfort, or danger of dysphagia (Peyron et al. 2004).

Saliva is a viscous aqueous medium made up of 99.5 percent water, 0.3 percent proteins/enzymes, and numerous electrolytes such as sodium, potassium, calcium, magnesium, phosphate, and bicarbonate generated by salivary glands. Enzymes have the ability to change the structure and content of food to some extent. Around 30 distinct enzymes have been discovered in human saliva based on their catalytic capabilities (Salles et al. 2011). Immunoglobulin A (IgA), lysozyme, lactoferrin, and mucosal glycoproteins are among the other proteins present in saliva (mucins) (Minekus et al. 2014). Furthermore, physical and chemical aspects of consumed meals, as well as psychological and physiological factors, influence the composition and flow rate of saliva (Salles et al. 2011).

Mastication is a complicated oral motor action in which the CNS and various peripheral sensory inputs from epithelium mechanoreceptors, joints, and muscles modulate jaw motions. During mastication, the mandible moves not only vertically, but also anteroposteriorly and laterally, and the teeth are the grinding implements that break food into pieces by mechanical cutting, crushing, grinding, compressing, and shearing. Fragments generally reach a crucial particle size (0.8–3 mm) before bolus production, depending on mechanical features of the meals, and this size is rather consistent among participants (van der Bilt & Fontijn-Tekamp 2004).

The food bolus is then conveyed into the stomach by esophageal peristalsis, which has four basic motor functions: storage, mixing, grinding, and emptying. The human stomach is split into four primary divisions (fundus, body, antrum, and pylorus), with a resting (fasted state) capacity of around 25 ml that can extend to 1.5–4 l to handle huge amounts of food (Norton et al. 2014). The fundus and body of the proximal stomach serve as a repository for undigested material, whilst the distal stomach (antrum) serves as a grinder, mixer, and siever of solid foods. By forcing tiny particles through the pyloric sphincter and into the duodenum, the antrum also works as a pump for stomach emptying of solids (Kong & Singh 2008).

Gastric motility significantly affects the rate of food breakdown in the stomach by changing the forces, pressures, and specific flow profiles encountered by food particles. It is characterized during the fasting state by a cyclic contractive pattern, while the

contractions become continuous during the fed state, moving food from the top to the pylorus at an averaged propagation velocity of 2.5 mm/s and frequency of 2.6–3 waves/min. The peristaltic waves cause the chyme to be propelled back into the stomach's main body via retropulsion (Fig. 8). Retropulsion is responsible for drastic mixing and emulsifying the food with gastric juices. The contraction forces are reported ranging from 0.2 N to 1.89 N, depending on the stomach's fasting or fed state (Norton et al. 2014). The ingested food bolus is subsequently redistributed to the distal stomach, where irregular antral, tonic, and phasic pyloric contractions grind it into particles with a diameter of 1–2 mm. Within these contractions, there may be a lot of variation, which has a big impact on the stomach emptying and digesting patterns of various meals (Houghton et al. 1988).

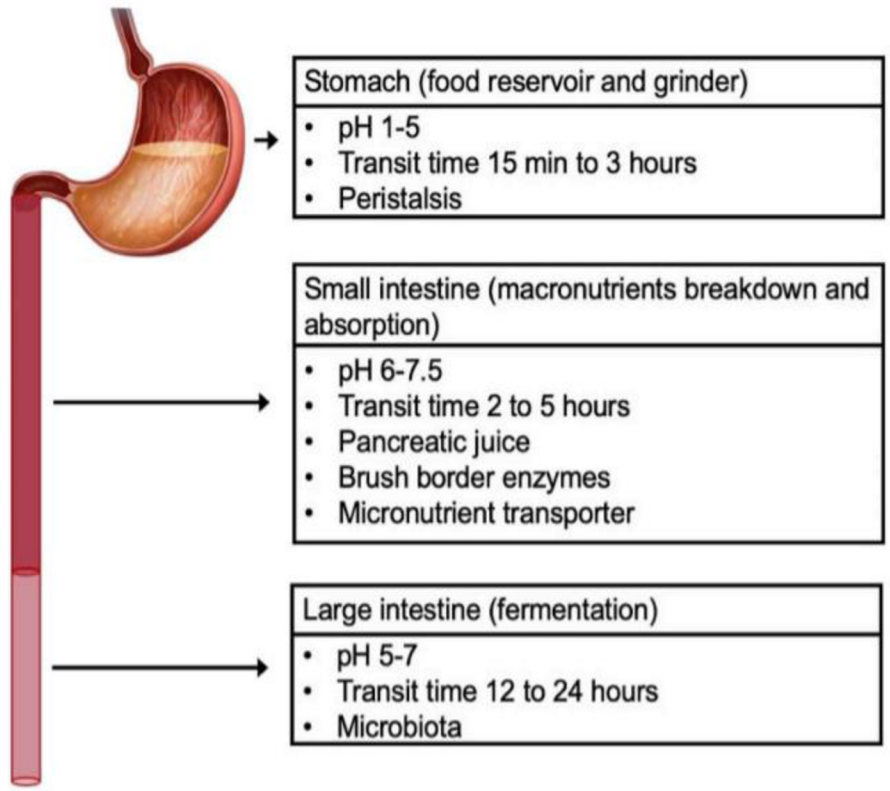


Figure 8 Region specificity and functionality of the human gastrointestinal tract. Source: (Li et al. 2020)

2.3. Toxicity

With the role of digesting and absorbing nutrients, the gastrointestinal tract (GI), which acts as a barrier against undesired materials and as a main site of biotransformation and excretion, is crucial for normal body homeostasis. Many medicines have been demonstrated to cause gastrointestinal toxicity, including gastrointestinal damage, gastrointestinal perforation, and gastrointestinal bleeding, according to studies. As a result, determining the medication candidate's potential gastrointestinal toxicity prior to clinical trials is critical. Toxicities in the gastrointestinal tract, particularly the stomach, small intestine, and colon, are difficult to measure. This is due in part to the intricate nature of the tissue that makes up the gastrointestinal tract, as well as functional changes in these components in reaction to a toxic insult (Fig. 9) (Gerson et al. 2018). The presence of food components and endogenous substances, as well as the gastrointestinal epithelium and microbial flora, are important factors not only in the digestion and absorption processes, but also in transformation processes that can result in significant changes in the chemical form of these elements. The chemical alterations eventually lead to changes in the absorption and toxicity of these toxic trace elements (Vázquez et al. 2015).

Some toxic compounds cause only one significant pathophysiological outcome, whereas others cause a wide range of alterations (Gelberg 2018). Probiotics have been shown in several trials to be effective in lowering oral exposure to harmful trace elements. The binding of diverse strains of lactic bacteria to Pb is quick (Halttunen et al. 2007) and long-lasting (48 h), making these bacteria a viable technique for lowering Pb absorption in the intestine. Some food ingredients may impact the solubilization of Hg in foods during digestion, hence affecting its absorption. Several in vivo studies have shown that certain food components can lessen the toxicity of orally administered Hg species; however, few investigations have looked into whether this positive effect is related to changes in Hg bioavailability (Chapman & Chan 2000).

Probiotics are another dietary component that has been suggested as a possible protector against Hg toxicity (Brudnak 2002). The degree of toxicity of Hg and the locations where it is most toxic are determined by the chemical form in which it is found. Hg, like the other hazardous trace elements, has effects on the GI tract when exposed acutely. The inorganic form has the greatest impact on the digestive tract, which is one of its target organs, after extended exposure (Fig. 10) (Abadin et al. 2007). The GI tract

tissues are significantly irritated by acute consumption of Hg (II) and CH_3Hg. After accidentally ingesting HgCl, humans have experienced significant precipitation of intestinal mucosal proteins, diarrhea, colicky stomach discomfort, oropharyngeal pain, ulceration, and hemorrhages throughout the GI tract (Faroon et al. 2012).

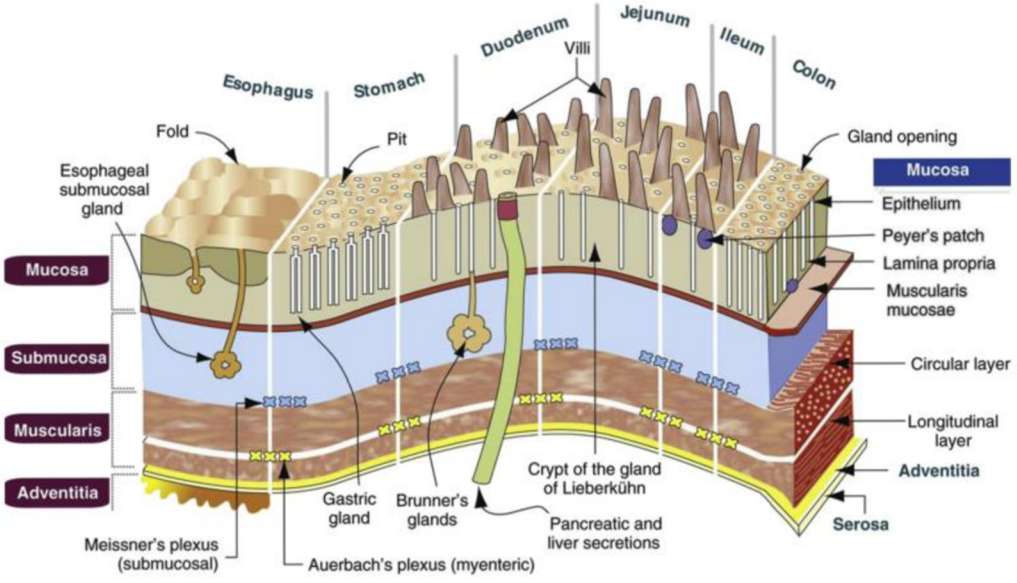


Figure 9 Schematic diagram of the anatomic and histologic organization of the digestive tube. Source: (Gelberg 2018)

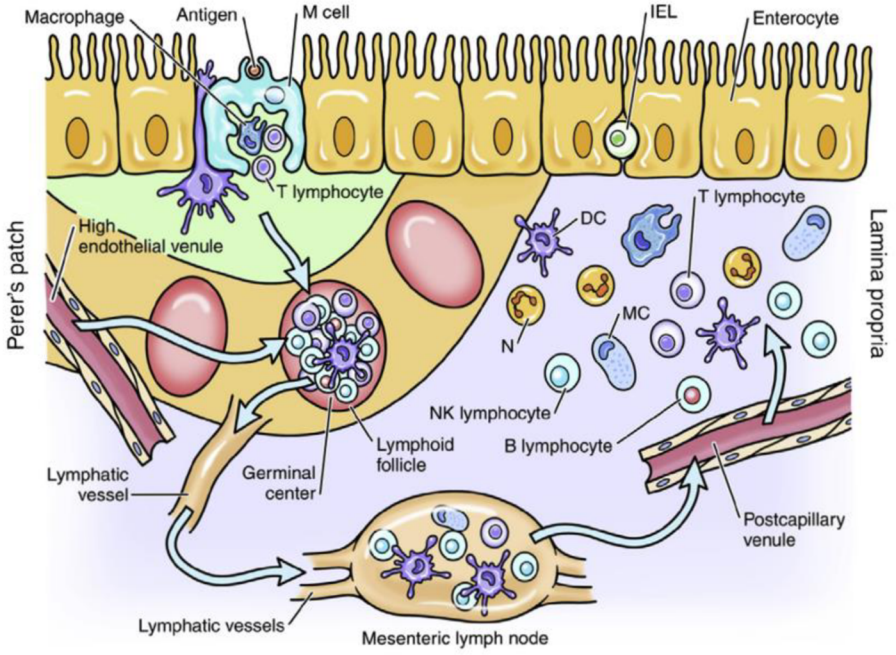


Figure 10 Schematic illustration of GALT. Dendritic cell (DC), intraepithelial lymphocyte (IEL), microfold cell (M), mast cell (MC), and neutrophil (N). Source: (Gelberg 2018)

2.3.1. Toxicity testing methods

The functions of 3D tissues, which include substantial cell–cell and cell–matrix interactions, as well as markedly variable diffusion/transport conditions, are not properly represented by common 2D cell cultures. As a result, cytotoxicity testing in two-dimensional cultures may not correctly reflect the toxicity of nanoparticles (NPs) and other nanostructures in the human body. Both 2D and 3D spheroid cultures are used to investigate the toxicity of CdTe (Cadmium telluride) and Au NPs. When comparing the spheroid culture data to the 2D culture data, the results show that NP harmful effects are greatly reduced in the spheroid culture (Fig. 11). The key factors in reducing toxicity have been recognized as tissue-like morphology and phenotypic modification (Lee et al. 2009).

Almost every cell in the body exists in a 3D environment, which is essential for their growth and metabolism. Individual cells' phenotypes and functions are largely reliant on complex interactions with 3D-organized extracellular matrix (ECM) proteins and nearby cells (Abbott 2003). However, in 2D cell culture on a flat substrate, these cell–cell and cell–matrix interactions are greatly decreased, severely limiting their ability to recapitulate the required degree of in vivo biological responses (Lee et al. 2008). Due to the lack of critical physiological processes such as the transport of nanoparticles (NPs) through cell layers when they come into contact with tissues, experiments based on in vitro 2D-cell-culture models do not reliably predict in vivo toxicity and other biological impacts (Griffith & Swartz 2006). Furthermore, critical impacts of NPs (and other substances) on cellular activities that rely heavily on 3D organization are overlooked. Granular epithelial cells, for example, have an improved particular protein secreting activity only when they form a 3D-organized acinus structure (Bissell et al. 2003). As further proof of the need of extending cell toxicity experiments from 2D to 3D cultures, it should be noted that there is a significant difference in toxicity outcomes depending on whether in vitro 2D-cell-culture or animal models were utilized (Sayes et al. 2007).

To bridge the gap between in vitro 2D-cell culture and in vivo models, in vitro 3D-cell-culture models have been developed (Yamada & Cukierman 2007). The regeneration of native tissue form and function is aided by extensive cell–cell interactions in a 3D-assembled sphere-shaped cell colony. The structure of human tumors and their spheroid equivalents, for example, is essentially identical under histological and electron microscopic examination (Lazar et al. 1995). Hepatocytes of spheroid culture has shown

exceptional survivability while sustaining metabolic activities in the liver (Dvir-Ginzberg et al. 2003). As a result, toxicity testing using physiologically realistic spheroid models is predicted to extend present cellular cytotoxicity to the tissue level (Griffith & Swartz 2006).

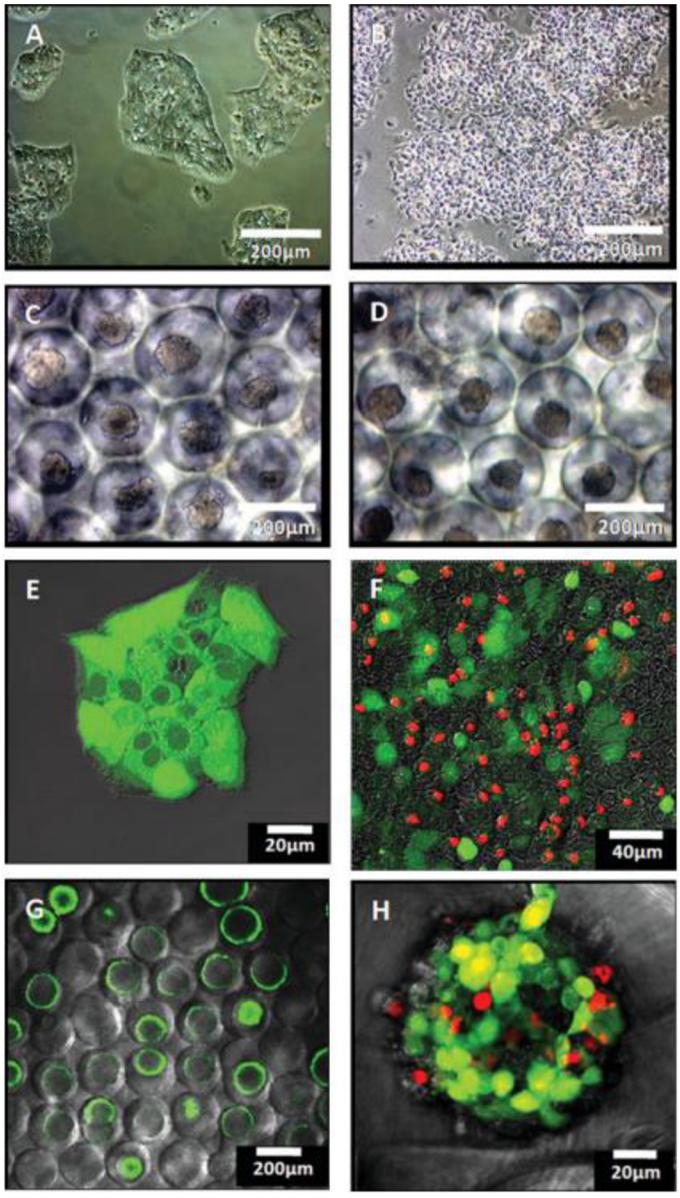


Figure 11 Comparison of 2D and 3D culture of HepG2 cells after 12 h of CdTe NP exposure. A–D) Optical images of normal A) 2D and C) 3D spheroid cultures. After CdTe NP introduction, the 2D culture showed a dramatically different morphology (B), while it was hard to distinguish any change in the 3D culture under an optical microscope (D). E–H) Confocal images of live/dead-stained normal E) 2D and G) 3D spheroid cultures; live cells are green and dead cells are red. Most cells in both cultures showed excellent viability. F) Again, after CdTe NP exposure, 2D culture revealed that a significant number of cells were dead. H) Although a few cells located on the surface of spheroids were dead, overall, the number is much smaller than the 2D culture. Source: (Lee et al. 2009)

3. Aims of the Thesis

The aim of this work was to determine cytotoxicity of kolaviron-rich *G. kola* extract on colorectal cancer cell lines using standard methods expressed by inhibitory concentration IC_{50} value.

4. Materials and methods

4.1. Plant material

Fresh *Garcinia kola* seeds were obtained on Mfoundi market in Yaoundé, Cameroon (3°51' 58.52" N; 11°31'28.87" E). They were identified and authenticated by Anna Maňourová, *G. kola* expert (Maňourová et al…). Seeds were pre-dried at 40 °C, transported 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. Seeds with peels were homogenized using laboratory blender Grindomix GM 100 (Retsch, Germany) and microfine grinder MF 10 Basic (IKA, Germany). Subsequently, they were defatted by separating the petroleum ether extract 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 samples were then dried at 103 °C and weighted until a constant sample weight was achieved. Afterwards, the samples were extracted with methanol and then with chloroform using modified methods described elsewhere (Iwu 1985, Iwu et al. 1990, Adaramoye & Lawal 2014). In short, the defatted methanolic extract was partitioned between chloroform and water. The dark yellow-brown chloroform extract was evaporated to be used as kolaviron-rich extract from *G. kola*.

All laboratory analyses were performed at least in duplicates based on Commission Regulation (EC) No 152/2009 (European Commission, 2009). The final result is then arithmetic average of those measurements (per tree) in complying with standard deviation.

4.2. Cytotoxicity assay

Human intestinal epithelial cell lines HT-29 (American Type Culture Collection, USA) and Caco-2 (European Collection of Animal Cell Cultures, UK) were employed between passages 30–50. McCoy's 5a media with 2 mM l-glutamine, 100 U/ml penicillin, 100 g/ml streptomycin, and 10% (v/v) fetal bovine serum were used to culture HT-29

cells. Caco-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM l-glutamine, 1% (v/v) non-essential amino acids, 100 U/ml penicillin, 100 g/ml streptomycin, and 10% (v/v) fetal bovine serum (Sigma–Aldrich, Ireland). Both cell lines were cultivated at 37 degrees Celsius in a humidified environment with 5% CO_2. Prior to the cytotoxicity testing, the viability of the test cells was greater than 99 percent, as assessed by the absence of the vital dye trypan blue.

Caco-2 and HT-29 colorectal cancer cell lines were grown in DMEM medium with 10% FBS (fetal bovine serum), 1% penicillin and streptomycin solution, 1% new line hydrogen, 1% pyruvate and 1% non-essential amino acid. The cells were grown in culture flasks with 15 ml of the appropriate medium, which were placed in a controlled atmosphere incubator containing 5% CO_2 and 37 ° C. The medium was changed every two days. After culturing for 7 days, the cells were washed with PBS (phosphate buffer, phosphate buffer) to remove the old medium. Finally, 5 ml of trypsin was added over 3 minutes. After 3 minutes, trypsin was neutralized with 1 ml of medium. Subsequently, the cell monolayer was scraped with a cell scraper and pipetted into a 15 ml Falcon tube. Thus, the samples were centrifuged for 10 minutes at 200 × g. Finally, the old medium was removed, and the cells were diluted in the new medium. 0.5 ml of cell medium was taken from the suspension thus prepared, which was then added to 15 ml of new medium in a culture flask, and further cultivation was performed. The rest of the cells were counted using a Bürker chamber and diluted to a concentration of 2.5 × 103 cells/ml suspension.

Mosmann's modified thiazolyl blue tetrazolium bromide (MTT) cytotoxicity assay was used to further evaluate the antiproliferative activities of the extracts that exhibited some inhibitory effect against the tested cells (Mosmann 1983). A 96-well microtiter plate were seeded for 24 hours with cancer (2.5 103) and normal intestinal (2.5 105) cells. Plant extracts (0.25–512 g/ml) were diluted twice serially and incubated for 72 hours in cells. The cells were then cultured in EMEM or Hybri-Care media for further 2 hours at 37°C and 5% CO_2 with MTT reagent (1 mg/ml) (Sigma-Aldrich, Prague, Czechia). The intracellular formazan product was dissolved in 100 l DMSO after the MTT-containing media was removed. The optical density of the cultures was measured at 405 nm ($OD_{450\ nm}$) using a Cytation 3 Imaging Reader (BioTek, Winooski, VT, United States) before and after the growth period.

The half-maximal inhibitory concentration (IC_{50}; g/ml) of the analyzed plant extracts was used to indicate their antiproliferative activity. As a positive control, the colon cancer chemotherapy medication 5-fluorouracil (Sigma-Aldrich, Prague, Czechia) was employed (Fuente et al. 2020). For each test, three separate experiments (two replicates each) were conducted. The mean and standard deviation are used to present the data. Cytotoxic (IC_{50} values 100 g/ml), moderately cytotoxic (IC_{50} values = 100–400 g/ml), and weakly cytotoxic (IC_{50} values = 401–512 g/ml) antiproliferative activity was assessed (Srisawat et al. 2013). At the studied concentration (1%), the solvents had no effect on the viability of normal and cancer intestinal cell lines. Results are expressed as mean ± standard deviation (SD).

5. Results and discussion

In this work, the inhibitory concentration IC_{50} of human colorectal carcinoma cell lines Caco-2 and HT-29 was determined by the MTT method in toxicity tests of kolaviron-rich *G. kola* seed extract. Extract 5 inhibited Caco-2 cells at an inhibitory concentration IC_{50} of 316.3 µg/ml, the inhibitory IC_{50} concentration against HT-29 cells was greater than 512 µg/ml. In the LME sample, the inhibitory concentration IC_{50} against Caco-2 cells was 234.6 µg/ml and against HT-29 cells 341.6 µg/ml. The results (shown in the Table 1.) are the average of three independent measurements.

Table 1 Values of IC_{50} of tested samples for individual cell lines

	Caco-2	**HT-29**
	µg/ml	
	mean ± SD	
5	316.3 ± 58.1	>512
LME	234.6 ± 49.5	341.6 ± 32.2

IC_{50}: half-maximal inhibitory concentration of proliferation in µg/ml

SD: standard deviation

The Tropical Diseases (WHO) research and training program lists the distribution of substances according to the degree of cytotoxicity. If the IC_{50} is >90 µg/ml, the compound is classified as non-cytotoxic, if the IC_{50} is in the range of 2-89 µg/ml, the compound is classified as mildly cytotoxic and if the IC_{50} is <2 µg/ml, the compound is classified as cytotoxic. According to this division, it can be said that none of the tested plants did not show significant cytotoxic activity against the examined cell lines. Resulting values for extract 5 with IC_{50}= 316.3 ± 58.1 µg/ml against colorectal cancer cell line Caco-2, against colorectal cancer cell line HT-29 with IC_{50}= 512 µg/ml, extract LME with IC_{50}= 234.6 ± 49.5 µg/ml against colorectal cancer cell line Caco-2, against colorectal cancer cell line HT-29 with IC_{50}= 341.6 ± 32.2 µg/ml can be classified as mild cytotoxic only.

These two graphs show the effect of the amount of extract on cell viability. The graphs below describe how the ability of the cells changes, depending on how much extract is added there. Treatment concentration-response curves are presented in Fig. 12 and Fig. 13. The kolaviron-rich extract reduced cell viability of the tested cell lines in a treatment-dependent manner.

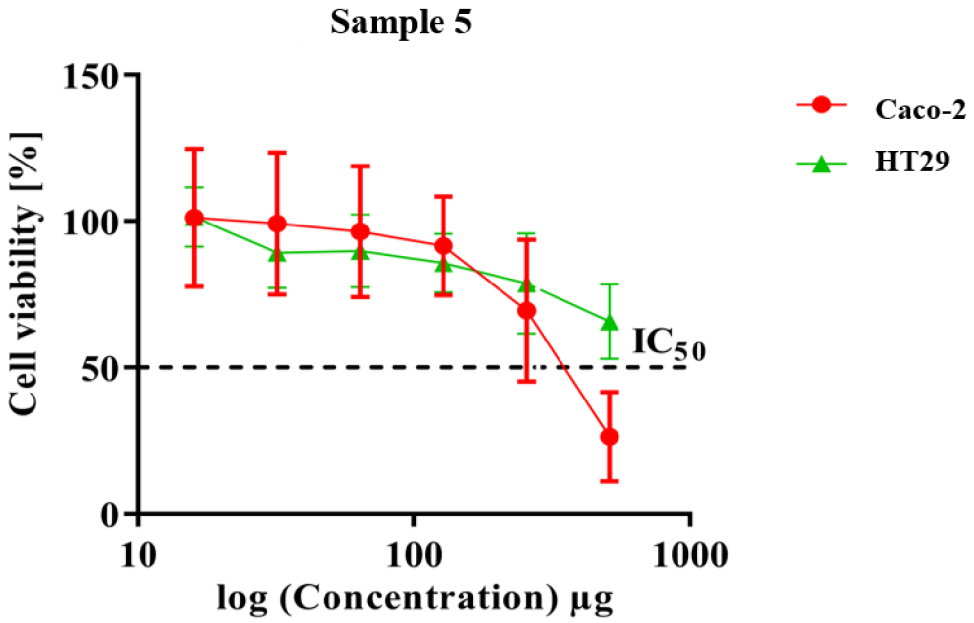


Figure 12 The influence of the amount of extract 5 on cell viability.

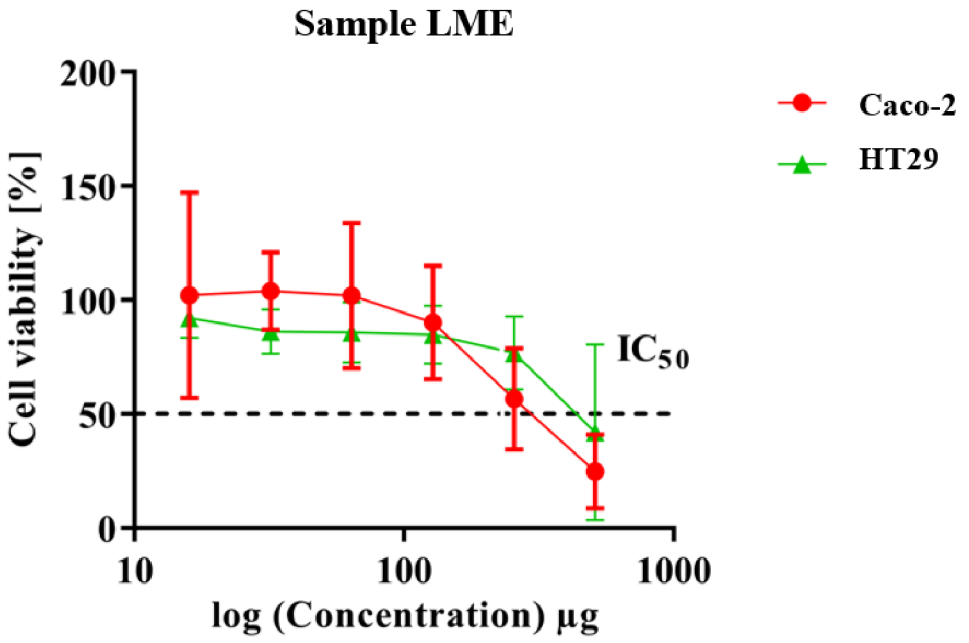


Figure 13 The influence of the amount of extract LME on cell viability.

Cancer is a complex disease that is a global public health problem. Many anticancer drugs currently in clinical use have been isolated from or based on medicinal plant species. Therefore, in the last few decades, crude extracts from medicinal plants have been studied in order to discover and isolate new compounds that exhibit biological activity. Medicinal plants are a valuable source of biologically active substances that can be effective in the treatment of many diseases. For this reason, research is currently focused on them. Among the main advantages and benefits of using herbal medicines are low cost, affordability and usually fewer side effects. Research studies on medicinal plants are necessary to confirm their efficacy and safety.

In this bachelor thesis, the cytotoxic activity of two kolaviron-rich extracts of *G. kola* called 5 and LME on two human colorectal carcinoma cell lines Caco-2 and HT-29 was investigated. The results are such that the cytotoxicity of both extracts was very low. Extract 5 showed cytotoxic activity against the Caco-2 cell line with IC_{50}= 316.3 ± 58.1 μg/ml and showed cytotoxic activity with IC_{50}= >512 μg/ml against the HT-29 cell line. The LME extract showed cytotoxic activity against the Caco-2 cell line with IC_{50}= 234.6 ± 49.5 μg/ml and the cytotoxic activity against the HT-29 cell line IC_{50}= 341.6 ± 32.2 μg/ml. According to Červenková (2018), extracts from *D. ambrosioides* and *P. alliacea* (*Mucura macho*) plants showed in comparison with our investigated kolaviron-rich higher cytoxicity on the HT-29 cell line with IC_{50}= 69.90 ± 9.00 μg/ml for *D. ambrosioides* and with IC_{50}= 81.1 ± 2.8 μg/ml for *P. alliacea* (*Mucura macho*). In contrast, there were no major changes to the Caco-2 cell line, where the extract from *D. ambrosioides* showed IC_{50}= 129.2 ± 6.9 μg/ml and the extract from *P. alliacea* (*Mucura macho*) showed mild cytotoxicity with IC_{50}= 251, 7 ± 2.0 μg/ml.

Extracts of *E. bulbosa*, *P. alliacea* (*Mucura hembra*) and *P. niruri* were among the samples with very low cytotoxicity compared to our examined cell lines, colorectal carcinoma cell lines Caco-2 and HT-29 with results for *E. bulbosa* with IC_{50}= >512.0 μg/ml against Caco-2 cells and with IC_{50}= 289.4 ± 11.7 μg/ml against HT-29 cells, for *P. alliacea* (*Mucura hembra*) with IC_{50}= 277.0 ± 9.0 μg/ml for Caco-2 cells and with IC_{50}= 136.0 ± 7.4 μg/ml for HT-29 cells and for *P. niruri* with IC_{50}= 103.5 ± 5.9 μg/ml for Caco-2 cells and with IC_{50}= 322.0 ± 5.1 μg/ml against HT-29 cells.

Momtaz et al. (2013) demonstrated in his study using the MTT method a high cytotoxic effect of *Maytenus procumbens* extract in comparison with our examined samples on the colorectal carcinoma cell line Caco-2 with IC_{50}= 68.796 ± 0.012 μg/ml and against the HT-29 cell line with IC_{50}= 78.491 ± 0.011 μg/ml. According to the above results, the extract from the plant *Maytenus procumbens* appears to have the highest cytotoxic activity.

The aim of comparing the samples we studied with samples similar to our plant, was to make it clear that the cytotoxicity would have to be significantly higher for the substance to be in anti-cancer remedies. On the other hand, relatively high concentrations of the *G. kola* extract is needed to inhibit even cancer cells, which are usually more sensitive than normal cells; therefore it can be concluded that it is not toxic to normal cells. The results of this study cannot support the use of kolaviron as a cancer drug due to such a high inhibitory IC_{50} concentration. This would only be possible if it did not directly target the destruction of cancer cells, but some other effects such as Popoola et al. (2016), who also identified *G. kola* as a potential plant that can be indicated to combat cancer; the proposed virtue was as analgesic and anti-inflammatory drug rather than directly inhibiting cancer cells.

6. Conclusions

The aim of this work was to determine cytotoxicity of kolaviron-rich *G. kola* seed extract on human colorectal cancer cell lines Caco-2 and HT-29 using standard methods expressed by IC_{50} value. None of the samples showed significant cytotoxicity to the tested cell lines. No justification for its use against cancer has been demonstrated, kolaviron-rich may be widely used for its other biological effects due to its low cytotoxicity.

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