



(Research Article.)

HPLC Analysis of Two Major Flavonoids and Antimicrobial Effectiveness against Multi-Drug Resistant Bacteria of Different Parts of *Khaya senegalensis*

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Abstract: Flavonoids are an important group of plant secondary metabolites that are widely used as powerful antioxidants in the management of a variety of illnesses, including inflammatory joint diseases, atherosclerosis, diabetes, in addition to free radical-induced degenerative eye disorders, cancer, dementia, and stroke. The main flavonoids of *Khaya senegalensis* plant are quercitrin and rutin. This work aims to determine the quercitrin and rutin content in methanolic extracts of *K. senegalensis* leaves, stems and fruits in order to determine the most productive part of these bioflavonoids by utilizing a precise HPLC quantitative method. HPLC analysis showed that leaf had the highest yield of quercitrin and rutin among the three parts (2.454 and 0.776 g/kg respectively), the stem had 1.812 and 0.286 g/kg, while fruit had a small amount of quercitrin and had no rutin. The antibacterial activity of methanolic extracts of leaves, fruits, stems and mixture of quercitrin and rutin was investigated by determining the MIC values against Gram positive and Gram negative standard and multidrug resistant bacteria (Methicillin-resistant *Staphylococcus aureus* and *Acinetobacter baumannii*) and revealed that the mixture of quercitrin and rutin as the major flavonoids of *K. senegalensis* in the three different parts showed the highest antibacterial activity against all tested bacteria MIC= 1.25mg/mL, followed by the leaf and stem extracts, however the fruit extract showed no antibacterial action.

Keywords: *Khaya senegalensis*; Meliaceae; quercitrin; rutin; HPLC; antibiotic-resistant bacteria.

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1. INTRODUCTION

Large deciduous tree *Khaya senegalensis* (Desr.) (*K. senegalensis*), [Family Meliaceae], generally referred to as African mahogany, endemic to the savannahs of sub-Saharan Africa¹. It is simple to recognize the tree due to its height of fifteen to thirty metres in addition to the dark-tinted, circular crown with lustrous leaves throughout. It produces fruit-like woody capsules. About Nigeria and other nations in West Africa, it is frequently used to treat a variety of illnesses¹. The leaves are employed in headaches and fevers treatment, extract of the bark stem is utilized to treat dermatoses, hookworm, malaria, and jaundice². Adults can use the oil to treat ulcers, syphilis, and leprosy. *K. senegalensis*'s ethanolic

stem bark extract has been discovered to possess anti-free radical capabilities². Fever and headaches can also be treated with the seeds and leaves, and leprosy, syphilis, and mental sickness were treated with the root extract.³

Studies conducted in both *in vitro* and *in vivo* showed that the bark ethanolic extract had antioxidant activity⁴ and the stem bark's aqueous extract had anti-inflammatory action², anti-diarrheal properties of leaf aqueous extract⁵, aqueous stem-bark extract's antimicrobial properties, roots and the ethanolic leaf extract⁶, antifungal activity of defatted acetone extract of flowers⁷, anti-parasitic activity of proanthocyanidins isolated from bark⁸, hypoglycemic effect of aqueous extracts of stem bark and ethanolic extracts of roots^{9,10}, stem bark aqueous

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extract with hepatoprotective activity¹¹, neuro-protective effect of limonoids from seeds¹² and anti-cancer activities of methanolic extract of stem¹³. According to reports, *K. senegalensis* has abundant limonoids¹⁴⁻¹⁷, besides that to phenolic substances like quercitrin catechin and rutin¹⁸.

It has repeatedly been discovered that infectious diseases are one of the main threats to world health. In 2013, the World Health Organisation (WHO) stated that 5.9 million of the 9.6 million deaths in the sub-Saharan African region were caused by infectious illnesses, or 61.7% of all deaths. Plants continue to be the most prevalent major natural source of active medications and are crucial in the ethnomedical treatment of a variety of diseases. Medicinal plants typically contain a variety of phytochemicals, some of which are responsible for their biological activity¹⁹.

Although *K. senegalensis* plant has great medicinal value, there is insufficient study concerning its phytonutrients and biological activity. Infectious diseases have been consistently found to be among the leading causes of threat to global health. The World Health Organization (WHO) in 2013 reported that infectious diseases accounted for 61.7% (5.9 million) of the 9.6 million deaths in the sub-Saharan African region. Thus, this research objective was to investigate the levels of rutin and quercitrin, the two major flavonoids in *K. senegalensis* in three parts (leaves, fruits and stems) as well as to evaluate their antimicrobial activity. The findings of this study can help pharmaceutical companies make the best choices in terms of functionality, performance, and cost-effectiveness.

2. METHODS

2.1. Plant material

K. senegalensis leaves, stems, and fruits were gathered at the Giza Zoo., Egypt, in June 2019. The plant was identified and compared to a reference herbarium by Mrs. Trease Labib, Senior Botanist, the Orman Garden in Giza, Egypt, moreover, in comparison to the reference specimen. Voucher specimens for leaves, stems and fruits (code: KSL19, KSS19, KSF19 respectively) have been placed in the herbarium of the Faculty of Pharmacy (Girls), Department of Pharmacognosy and Medicinal Plants, University of Al-Azhar in Cairo, Egypt. The samples were stored individually and dried in a ventilated shaded place, pulverized and stored in tightly closed containers away from sources of heat and moisture.

2.2. Extraction

Each dried plant part's air-dried powder (40g) was macerated with 70% methanol to extract it individually. (3x500ml) at 25±2 °C each for one day. To get a viscous extract of each part separately, the solvent was eliminated after straining the extract using a rotary evaporator at 60°C (Buchi Co., Switzerland); 5.28 gm, 8.83 gm and 1.49 gm leaves, stems and fruits extracts respectively.

2.3. Quantitative analysis of the active substances

Three plant samples from three parts extracts (leaves, stems and fruits) and the mixture of quercitrin and rutin were subjected to quantification of rutin and quercitrin Using high performance liquid chromatography (HPLC), Extensive freeze-drying of semi-solid extracts was done for 24 hours to remove interfering components prior to HPLC analysis, including solvent and water, during quantification. HPLC technique was applied according to Nugroho et al.; 2017²⁰ method, with some improvements to get the finding efficient.

2.4. Antimicrobial activity evaluation

The antibacterial activity of the of *k. senegalensis* leaves, stems, fruits extracts and the mixture were evaluated by microbroth dilution test designed to determine the Minimum Inhibitory Concentration (MIC) according to the CLSI reference standards 2018 (CLSI Document M100-S28, Wayne PA).²¹⁻²³ All the tests were run in duplicate. The antibacterial activity for all natural samples had been screened against two Gram-positive bacterial isolates; Methicillin-resistant *Staphylococcus aureus* (MRSA) (ATCC 43300) and one clinical isolate of MRSA in addition to two Gram-negative bacterial isolates; *Acinetobacter baumannii* (ATCC 19606) and multidrug resistant (MDR) *Acinetobacter baumannii* clinical isolate.

3. RESULTS

3.1. Quantitative analysis of the active substances

The level of quercitrin and rutin in the three tested extracts of *K. senegalensis* were displayed in Table 1 and Figure 2. Table 2 and figure 3 displayed their level in the mixture. It was concluded that leaves showed the highest yield of quercitrin and rutin among the three parts (2.454 and 0.776 g/kg, respectively), stem exhibited 1.812 and 0.286 g/kg while fruit showed little amount of quercitrin and no rutin. While the mixture contains 38.184 and 6.295 g/kg quercitrin and rutin, respectively (Figure 3, Table 2).

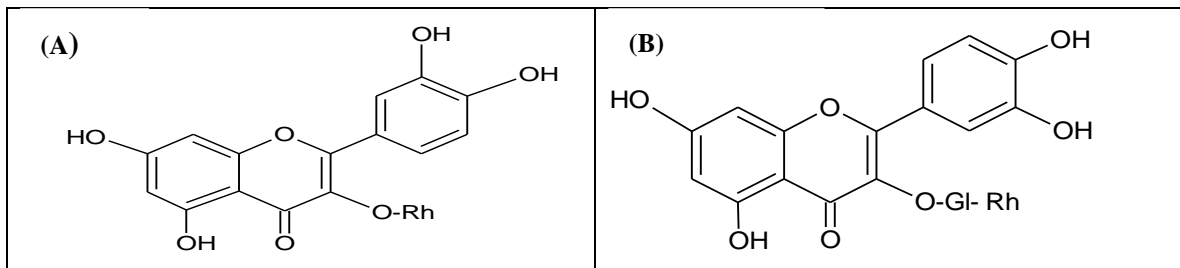


Figure 1. Structures of compounds; A: quercitrin, B: rutin

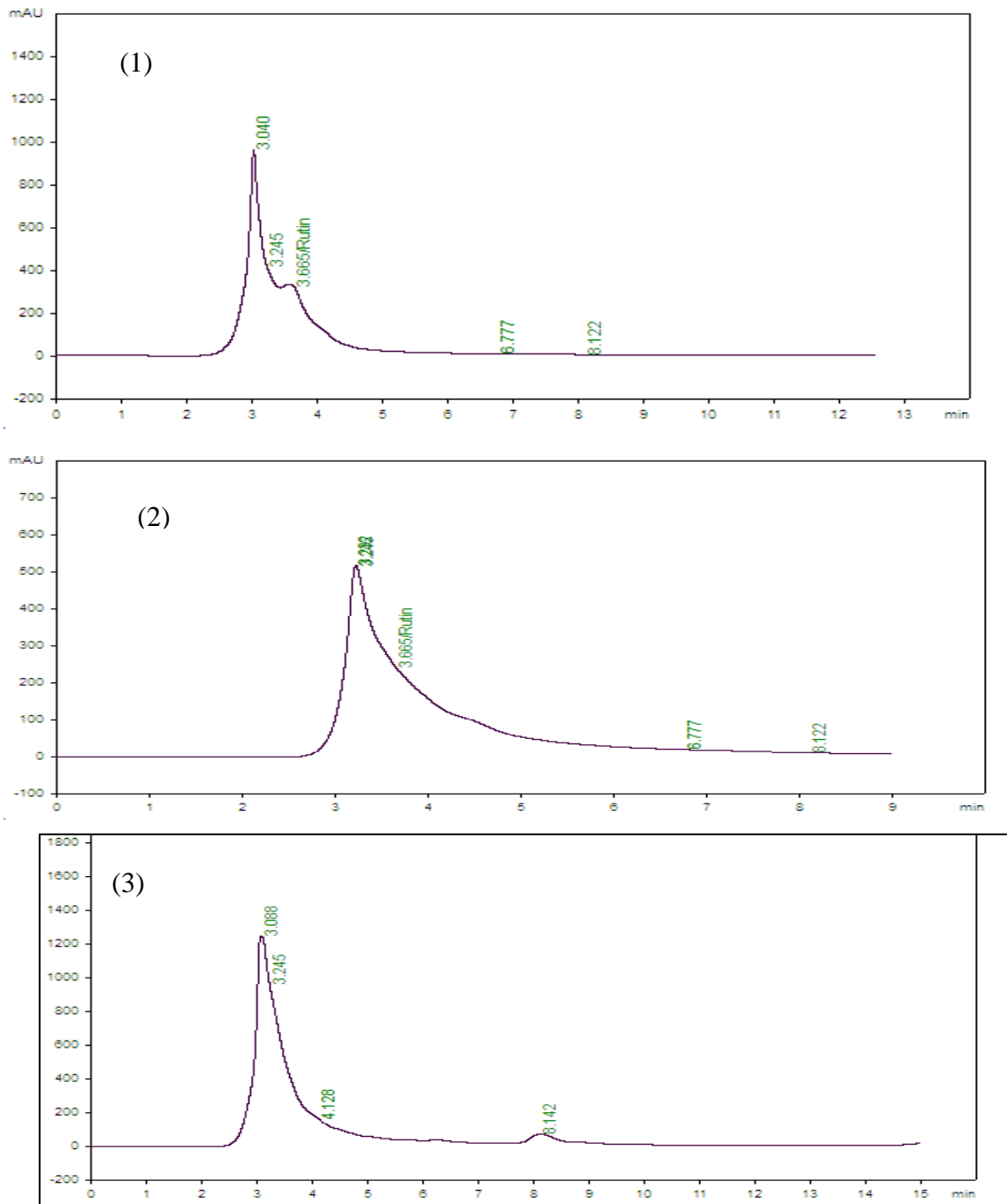


Figure 2. HPLC chromatograms (1: leaf, 2: stem, 3: fruit)

Table 1. Quercetin and rutin levels in leaves, stems, and fruit extracts

Plant part	Quercitrin (g/kg)	Rutin (g/kg)	Total
Leaves	2.454	0.776	3.23
Stem	1.812	0.286	2.098
Fruit	-----	-----	-----

Table 2. Quercetin and rutin levels in the mixture

Plant part	Quercitrin (g/kg)	Rutin (g/kg)	Total
mix.	38.184	6.295	44.479

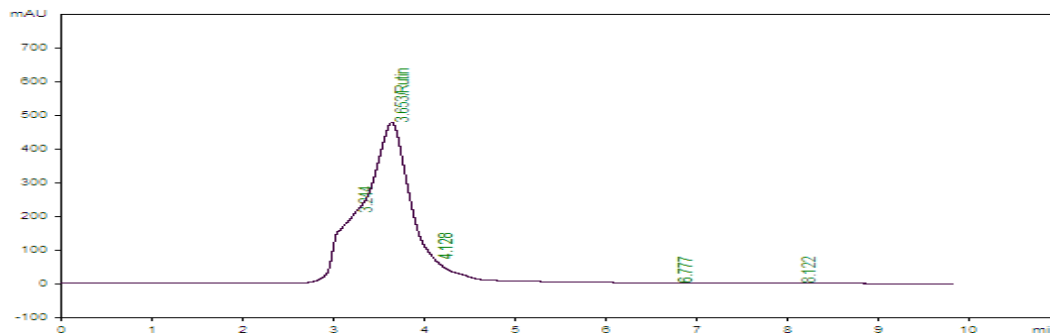


Figure 3. HPLC chromatogram of the mixture

Table 3. MIC (mg/mL) of methanolic extracts of *K. senegalensis* stem, leaf and fruit extracts against Gram-negative bacteria and Gram- positive bacteria.

Sample	Gram- Negative bacteria		Gram-Positive bacteria	
	<i>Acinetobacter baumannii</i> (ATCC 19606)	<i>Acinetobacter baumannii clinical isolate</i>	<i>MRSA</i> (ATCC 43300)	<i>MRSA clinical isolate</i>
Stem	0.625	1.25	2.5	2.5
Leaf	0.625	1.25	2.5	2.5
Fruit	>10	>10	>10	>10
Amax.	0.625	>0.5	>0.5	>0.5

Amax.: amoxicillin; ATCC: American type culture collection, MRSA: Methicillin- resistant *Staphylococcus aureus*

Table 4. MIC (mg/mL) of mixture of quercitrin and rutin against Gram-negative bacteria and Gram- positive bacteria

Sample	Gram- Negative bacteria		Gram -Positive bacteria	
	<i>Acinetobacter baumannii</i> (ATCC 19606)	<i>Acinetobacter baumannii clinical isolate</i>	<i>MRSA</i> (ATCC 43300)	<i>MRSA clinical isolate</i>
Mix.	1.25	1.25	1.25	1.25
Amax.	0.625	>0.5	>0.5	>0.5

Amax.: amoxicillin; ATCC: American type culture collection, MRSA: Methicillin- resistant *Staphylococcus aureus*

3.2. Antimicrobial activity evaluation

3.2.1. Gram positive bacteria:

Antimicrobial activity of extracts was investigated against Gram positive bacteria: MRSA (ATCC 43300) and one MRSA clinical isolate by determination of MIC. Leaves and stem total extracts showed good antibacterial activity (MIC = 2.5 and 2.5 mg/mL, against both bacteria respectively. However, total fruit extract demonstrated weak antibacterial activity with MIC > 10 mg/mL compared to amoxicillin with MIC > 0.5 mg/mL (Table 3). While, the mixture of quercitrin and rutin showed good antibacterial activity (MIC = 1.25 mg/mL) against both bacteria (Table 4).

3.2.2. Gram negative bacteria

Leaves and stem total extracts showed good antibacterial activity against *Acinetobacter baumannii* (ATCC 19606) and *Acinetobacter baumannii* clinical isolate with MIC value of 0.625 mg/ml and 1.25 mg/mL for both extracts respectively. However, fruit extract showed weak antibacterial activity against both isolates with MIC > 10 mg/mL compared to amoxicillin (Table 3). While the mixture of quercitrin and rutin showed good antibacterial activity (MIC = 1.25 mg/mL) against both bacteria (Table 4).

4. DISCUSSION

The public's health is seriously threatened by the growth of infections brought on by bacteria that are multidrug resistant (MDR). A significant global threat to human health is posed by the emergence and rapid spread of antibiotic-resistant bacteria. In terms of clinical resistance to antibiotics, ESKAPE species which include *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterococcus faecium* are the most prevalent antibiotic-resistant strains. Gram-negative *Acinetobacter baumannii* and Gram-positive *S. aureus* are among them, and they have become significant bacteria in increasing death and morbidity rate due to their high antibiotic resistance²⁴. MRSA spread worldwide, causing bacterial infections in healthcare and in the community. Asymptomatic colonisation of mucosa of the nose, minor infection of skin and soft tissue, and an invasive fulminant sickness with a high fatality rate are all clinical symptoms of MRSA infection^{25,26}. *Acinetobacter* species have recently emerged as an important driver of essential healthcare-associated illnesses and infections.

Acinetobacter baumannii, an aerobic Gram-negative bacterium that causes infections of the skin, circulation, urinary tract, and other soft tissues, is mostly associated with hospital-acquired infections worldwide. Unfortunately, the global spread of *A. baumannii* resistance to different antibiotics especially carbapenem endangers human life and the current healthcare system^{27,28}.

Plants continue to be the most plentiful major natural source of active medications and are crucial in the ethnomedical treatment of a variety of illnesses. The majority of phytochemicals found in medicinal plants, some of which are typically responsible for their biological actions, are found in these species²⁹.

K. senegalensis contains a variety of secondary metabolites, including tannins, flavonoids, leucoanthocyanins, alkaloids, and mucilage. Its abundance in total polyphenols and flavonoids was also demonstrated by the earlier study³⁰. The most important phenolics are flavonoids, which can either be in a glycosidic or non-glycosylated form. Since flavonoids are made up of two phenyl rings (A and B) connected to a heterocyclic ring (C), their carbon skeleton is abbreviated as C6-C3-C6. These compounds exhibit great chemical diversity, mostly as a result of variations in the positions of the B- and C-rings, their degree of hydroxylation, oxidation, and ring C saturation³¹. Flavonols are a significant class of flavonoids and are distinguished by the presence of a hydroxyl group at the C3 position of B ring³⁰. According to this study, quercitrin and rutin are two of the principal metabolites in *K. senegalensis*. Each of them contains a quercetin aglycone. Quercetin has a wide range of pharmacological effects. Numerous studies examine its antibacterial effects. It has been demonstrated that quercetin inhibits the growth of several Gram-positive, Gram-negative bacteria, fungi, and viruses.^{32,33} It has been demonstrated that quercetin has antibacterial effects on a variety of Gram-positive bacteria, including MRSA, Methicillin-sensitive *S. aureus* (MSSA), and *Enterococcus* spp.³⁴.

In the current study the antimicrobial results, revealed that the antimicrobial properties is directly related to quercitrin and rutin levels, which encouraged us to try a mixture previously isolated from the plant total extract composed of mixture of quercitrin and rutin, the quantity of each flavonoid was done according to the HPLC method mentioned above which revealed that this mixture contains 38.184 and 6.295 g/kg quercitrin and rutin, respectively (Figure 3, Table 2).

The mixture of quercitrin and rutin showed the highest antimicrobial activity as evidenced by their low MIC values (1.25 mg/ mL) against all tested

bacterial isolates of MRSA and *Acinetobacter baumannii* as illustrated in table 4. Leaf and Stem extracts showed similar results to that of mixture of quercitrin and rutin as demonstrated in table 3 (MIC = 2.5 and 2.5 mg/mL, for both extracts against both MRSA isolates and MIC value of 0.625 mg/mL and 1.25 mg/ml against both *Acinetobacter baumannii* isolates.

5. CONCLUSIONS

An initial assessment of the MIC values for various standard and antibiotic-resistant bacteria using the whole extracts of *Khaya senegalensis* leaves, stems, fruits, and a mixture of two isolated chemicals (rutin and quercitrin) showed that, the mixture (rutin and quercitrin) showed the highest antimicrobial activity as evidenced by their low MIC values (1.25 mg/ mL) against all tested bacterial isolates. Leaf and stem methanolic extracts showed good antimicrobial activity against all tested bacterial isolates, MIC ranged from 0.625 to 2.5mg/mL, while the fruit extract showed no antibacterial activity against all tested bacterial isolates suggesting that the mixture has the highest antibacterial activity, ensuring that antimicrobial activity was related to rutin and quercitrin levels

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Ethical Statement: NA, neither animals nor human used in this study.

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List of Abbreviations:

HPLC: High performance liquid chromatography

MIC: Minimum Inhibitory Concentration

CLSI: Clinical and Laboratory Standards Institute

ATCC: American type culture collection

Gm/ kg: Gram per kilogram

WHO: The World Health Organization

Amox: Amoxicillin

Mix. Mixture

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