RESEARCH ARTICLE

Flavonoids extracts from *Psidium guajava, Vernonia amygdalina,* and *Azadiracta indica* exhibited synergetic inhibitory activities against some food spoilage microorganisms

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Received: 25 May 2021, Revised: 03 August 2021, Published: 17 August 2021

ABSTRACT

Background: Flavonoids from medicinal plants have been reported to be a possible alternative to synthetic drugs due to their antimicrobial activities. In the present study, flavonoids extracts from the leaves of *Psidium guajava, Vernonia amygdalina,* and *Azadiracta indica* were evaluated for antibacterial and anti-fungi activities against some microbial isolates from spoiled tomato fruit. **Methodology:** Food spoilage microorganisms were isolated from spoiled tomato fruit. Flavonoids were isolated from the leaves of *P. guajava, V. amygdalina,* and *A. indica*, and were evaluated for their inhibitory activities against the isolated microorganisms using standard protocols. **Results**: The flavonoids extract demonstrated dose-dependent antimicrobial activities. Flavonoids extracts from *V. amygdalina* exhibited an inhibitory effect *on C. tropicalis*, *K. pneumonaie, M. roseus, and P.* aeruginosa with maximum inhibition diameters (MID) of 17.00±0.05, 25.00±0.45, 15.00±0.05, and 16.00±0.34 mm while the flavonoids from *P. guajava* exhibited inhibitory effects on the same organism with MID of 15.00±0.05, 14.00±0.10, 16.00±0.55, 15.00±0.43 and 14.00±0.05 mm respectively, while the flavonoids extract from *A. indica* exhibited the least activity on the bacteria and fungi isolates. Results of the MICs and MBC revealed that the gram-positive bacteria tested (*M. roseus* & *S. faecalis*) were more susceptible to inhibition by the flavonoids extract of the plants. The combination of total flavonoids from leaves of *P. guajava* and *V. amygdalina* exhibited higher and synergetic antimicrobial activities against all the tested organisms when compared with individual flavonoids extracts. This combination could serve as a potential source of novel antimicrobial agents for food industry.

Keywords: Flavonoids, Antimicrobial, *A. indica; P. guajava; V.* amygdalina; food spoilage organism

Citation: Oluwajobi, I., Kabiru, Y. A., and Jigam, A. A. (2021). Flavonoids extracts from *Psidium guajava, Vernonia amygdalina,* and *Azadiracta indica* exhibited synergetic inhibitory activities against some food spoilage microorganism. AROC in Food and Nutritions, 1(1),15-21, https://doi.org/10.53858/arocfn01011521

1.0 Introduction

Flavonoids are a group of natural compounds in plants with variable phenolic structures [1]. They are present virtually in all parts of the plant and are responsible for the taste, color, protection of vitamins and enzymes, and prevention of fat oxidation [2]. The pharmacology importance of flavonoids, including, antioxidant, hepatoprotective, antibacterial, anti-inflammatory, anticancer, and antiviral activities are indispensable in the human body [2,3]. Flavonoids displayed strong antimicrobial activity and inhibit several microorganisms, as they form complexes with cell wall components and adhesins to prevent microbial growth [4].

The guava (*Psidium guajava*) is a phytotherapeutic plant used in folk medicine that is believed to have active components that help to treat and manage various diseases [5]. The many parts of the plant have been used in traditional medicine to manage conditions like malaria, gastroenteritis, vomiting, diarrhea, dysentery, wounds, ulcers, toothache, coughs, sore throat, and other infectious diseases [5].

Azadirachta indica, commonly known as the Neem tree belongs to the family *Meliaceae*. It is widely cultivated and well adapted in drier and humid ecological zones with an estimation of four million trees in the North-West Zone of Nigeria [6]. Traditionally, the neem is a very important medicine in Indian culture for the treatment of different ailments. It is one of the main sources of many therapeutic agents [7].

The bitter leaf (*Vernonia amygdalina*) has been ascertained to provide various culinary and medicinal properties, these medicinal properties exert bacteriostatic and bactericidal effects on some bacteria [8]. Antihelmitic, Antitumourigenic [9], and Antimalarial, [10] have also been reported for extracts from the plant. V. amygdalina possesses flavonoids including luteolin, luteolin 7-O-β-glucuronoside and

lutelin7-O-β-glucoside [11]. The present study evaluated the anti-bacterial and anti-fungal activities of flavonoids extracts from *P. guajava, A. indica, and V. amygdalina*.

2.0 Materials and Methods

2.1 Collection of plant

The leaves of *Veronia amygladina* (bitter leaf), *Azadirachta indica* (Neem plant), *Psidium Guajava* (Guava leaves) were collected from Green farms in Niger State, Nigeria. Spoilt tomato fruits (*Lycopersicum esculentum*) were gotten from Kure market in Minna. All sample species were identified by a plant taxonomist at the Federal University of Technology, Minna. The plants were brought to the laboratory and rinsed with water to remove the soil particles. Then air-dried at room temperature.

2.2 Isolation of food spoilage microorganism

Bacteria isolates including; *Klebsiella pneumonaie, Pseudomonas aeruginosa, Micrococcus roseus,* and *Streptococcus faecalis*, and fungi including; *Trichophyton tonsurans, Aspergillius niger,* and *Candida tropicalis* were cultured and isolated from spoilt tomatoes fruit according to the methods described in previous studies [4].

2.3 Isolation of Flavonoids:

The leave samples were washed and dried for 2 weeks at 37oC, and ground using a grinder mill. A 50g of the plant material was extracted with 200mL of methanol, using soxhlet apparatus and the resulting extract was concentrated in a rotary evaporator. The methanol extract was dissolved in distilled water and extracted with n-butanol mixed with distilled water. The butanol extract was subjected to column chromatography on silica gel, eluted with n-hexane and methanol according to the method described by Al-Jadidi and Hossain, [12].

2.4 Antibacterial and anti-fungal Activity

The plate-hole diffusion assay as described by Ieven et al. [13] was used to determine the zone of inhibition of bacteria (antibacterial activity) by the flavonoids extract of the plants. The selected microorganisms obtained were maintained at 4°C on nutrient agar plates before use. Using a sterile cork-borer of 5 mm diameter, five holes per plate were made into the set agar containing the bacteria culture. A total of 7 drops (40 mg/ml, 80 mg/ml, and 120 mg/ml) of the flavonoids were poured into the wells, and one contained distilled water and ampicillin (50mg/ml) as the negative and positive controls respectively. The plates were placed in the incubator at 37 °C for 12 hrs. Antibacterial activity was recorded in millimeters [14, 15]. This was also done for isolated fungi to know the antifungal activity of the extracts. Ampicillin was used as a standard for antibacterial assay, while vastatin was used as the standard for antifungal assay.

2.5 Statistical analysis

Values were analyzed using a statistical package for social science (SPSS) version 21 and presented as means ± SE of the mean. Comparisons between different groups were carried out by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). The level of significance was set at $P < 0.05$

3.0 Results

3.1 Antimicrobial activities of flavonoids extract from *V. amygdalina*

the flavonoids extract from *V. amygdalina* exhibited a dose-dependent inhibitory effect on *K. pneumonaie, M. roseus, and Pseudomonas aeruginosa* with maximum inhibition diameters of 25.00±0.45, 15.00±0.05 and 16.00±0.34mm respectively (table 1). Antifungal activity of the flavonoids from *V. amygdalina* was observed only against *Candida tropicalis* (15.00±0.15- 17.00±0.05mm).

3.2 Antimicrobial activities of flavonoids extract from *A. indica*

The total flavonoids of *A. indica* exhibited antifungal activities against *A. niger, T. tonsurans*, and *C. tropicalis* only at higher concentrations of 80 and 120 mg/ml. Similarly, the antibacterial activity of the flavonoids from *A. indica* was demonstrated against *M. roseus* (15.00±0.15 mm) and *S. faecalis* (17.00±0.05 mm) only at a higher dose of 120 mg/mL (Table 2).

Table 1: Antimicrobial activities of total flavonoids from leaves of *Vernonia amygdalina*

	Concentrations (mg/mL)			Ampicillin	
BACTERIA	**40**	**80**	**120**	**50 mg/ml**	**DMSO**
Klebsiella pneumonaie	10.00±0.05	12.00±0.10	15.00±0.36	25.00±0.45	-
Micrococcus roseus	13.00±0.05	14.00±0.87	15.00±0.55	15.00±0.25	-
Streptococcus faecalis	-	-	-	-	-
Pseudomonas aeruginosa	-	13.00±0.55	14.00±0.05	-	-
FUNGI	**40**	**80**	**120**	**Vastatin (67 mg/L)**	**DMSO**
Aspergillius niger	-	-	-	15.00±0.25	-
Trichophyton tonsurans	-	-	-	20.00±0.45	-
Candida tropicalis	15.00±0.15	15.00±0.11	17.00±0.05	-	-

Values are mean ± SEM of 3 determinations.

Table 2: antimicrobial activities of total flavonoids from leaves of *Azadirachta indica*

	Concentrations (mg/mL)			Ampicillin		
BACTERIA	**40**	**80**	**120**	**50 mg/ml**	**DMSO**	
Klebsiella pneumonaie	-	-	-	25.00±0.45	-	
Micrococcus roseus	-	-	15.00±0.15	15.00±0.25	-	
Streptococcus faecalis	-	-	17.00±0.05	-	-	
Pseudomonas aeruginosa	-	-	-	-	-	
FUNGI	**40**	**80**	**120**	**Vastatin (67 mg/L)**	**DMSO**	
Aspergillius niger	-	14.00±0.15	14.00±0.35	15.00±0.25	-	
Trichophyton tonsurans	-	15.00±0.25	7.00±0.15	20.00±0.45	-	
Candida tropicalis	-	-	19.00±0.25	-	-	

Values are mean ± SEM of 3 determinations.

3.3 Antimicrobial activities of flavonoids extract from *P. guajava*

The total flavonoids of *P. guajava* exhibited a dose-dependent inhibitory effect on *K. pneumonaie, M. roseus, S. faecalis,* and *P. aeruginosa* with maximum inhibition diameters of 14.00±0.10, 16.00±0.55, 15.00±0.43, and 14.00±0.05 mm respectively (table 3). Antifungal activity of the flavonoids from *P. guajava* was observed only against *Candida tropicalis* (13.00±0.05-15.00±0.05mm).

3.4 Synergetic antimicrobial activities of flavonoids from leaves of *P. guajava* and *V. amygdalina*

The combination of total flavonoids from leaves of *Psidium guajava* and *Vernonia amygdalina* at different ratios achieved synergetic antibacterial and antifungal activities against all the tested organisms in comparison with when the flavonoids from each plant were used singly (Table 4).

3.5 Minimum inhibitory concentrations of the flavonoids of extracts on bacteria and fungi isolate

The Minimum inhibitory concentrations (MICs) of the flavonoids extracts on the bacteria and fungi isolates are shown in table 5 while the minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the flavonoids extracts on the bacteria and fungi isolates are shown in table 6.

Table 3: antimicrobial activities of total flavonoids from leaves of *Psidium guajava*

	Concentrations (mg/mL)			Ampicillin	
BACTERIA	**40**	**80**	**120**	**50 mg/ml**	**DMSO**
Klebsiella pneumonaie	9.00±0.56	14.00±0.89	14.00±0.10	25.00±0.45	-
Micrococcus roseus	14.00±0.35	15.00±0.05	16.00±0.55	15.00±0.25	-
Streptococcus faecalis	7.00±0.55	16.00±0.10	15.00±0.43	-	-
Pseudomonas aeruginosa	10.00±0.15	11.00±0.05	14.00±0.05	-	-
FUNGI	**40**	**80**	**120**	**Vastatin (67 mg/L)**	**DMSO**
Aspergillius niger	-			15.00±0.25	-
Trichophyton tonsurans	-			20.00±0.45	-
Candida tropicalis	13.00±0.05	15.00±0.05	15.00±0.05	-	-

Values are mean ± SEM of 3 determinations.

 https://doi.org/10.53858/arocfn01011521

Table 4: Antimicrobial activities of combined total flavonoids from leaves of *Psidium guajava* and *Vernonia amygdalina*

	P guajava : *V amygdalina*			Ampicillin	
BACTERIA	**1:1**	**1:2**	**2:1**	**50 mg/ml**	**DMSO**
Klebsiella pneumonaie	14.05±0.45	10.05±0.03	15.05±0.05	25.00±0.45	-
Micrococcus roseus	16.00±0.09	15.03±0.56	12.00±0.55	15.00±0.25	-
Streptococcus faecalis	14.05±0.55	8.05±0.56	18.05±0.35	-	-
Pseudomonas aeruginosa	14.05±0.05	12.00±0.55	17.55±0.05	-	-
FUNGI	**40**	**80**	**120**	**Vastatin (67 mg/L)**	**DMSO**
Aspergillius niger	15.04±0.02	13.06±0.64	15.05±0.05	15.00±0.25	-
Trichophyton tonsurans	17.30±0.25	15.09±0.06	12.55±0.55	20.00±0.45	-
Candida tropicalis	19.55±0.06	15.05±0.35	17.50±0.35	-	-

Values are mean ± SEM of 3 determinations.

4.5 Discussion

Flavonoids (derivatives of phenylchromone ring) are a large group of compounds naturally occurring in higher and lower plants. The inhibitory activities of flavonoids against bacteria and yeast have been investigated by a number of researchers [4,16-17). *V. amygdalina* serves as vegetables and is used as a nutritive seasoning in the preparation of food. Apart from its nutritive value, it has been found to be effective in inhibiting the growth of microorganisms. Researchers have also studied the phytochemical and antimicrobial properties of leaf and stem extract [8]. In the present study, the flavonoids extract from *Vernonia amygdalina* exhibited a dose-dependent inhibitory effect on *Klebsiella pneumonaie, Micrococcus roseus, and Pseudomonas aeruginosa* with maximum inhibition diameters of 25.00±0.45, 15.00±0.05 and 16.00±0.34mm respectively (Table 1). Antifungal activity of the flavonoids from *Vernonia amygdalina* was observed only against *Candida tropicalis* (15.00±0.15- 17.00±0.05mm). This finding is in agreement with earlier work by Udochukwu *et al*. [18] who reported the phytochemical and antibacterial activity of *V. amygdalina. Streptococcus faecalis* was observed to be completely resistant to all concentrations of flavonoids from *V. amygdalina*. This resistance may have arisen from drug/phytochemical inactivating enzymes present in *Streptococcus faecalis.* Also, variations observed in the susceptibility of Gram-positive and negative bacteria could have resulted from their relative composition of cell wall components.

Table 5: Minimum inhibitory concentration (MIC) of the flavonoids extracts on the bacteria and fungi isolate

		M. roseus	*S. faecalis*	*P.aeruginosa*	*T. tonsurans*	*C. tropicalis*
***P. guajava* (mg/mL)**	40	0.012±0.00	0.002±0.00			
	80		0.0014±0.00			
	120		0.0003072±0.00			0.003072±0.00
***A.indica* (mg/mL)**	40		0.00256			
	80				0.002048±0.00	0.002048±0.00
***V. amygdalina* (mg/mL)**	80					0.002048±0.00
	120			0.001536±0.00		0.001536±0.00

Values are mean ± SEM of 3 determinations

Table 6: Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) of the flavonoids extracts on bacteria and fungi isolate

		M. roseus	*S. faecalis*	*P. aeruginosa*	*T. tonsurans*	*C. tropicalis*
***P. guajava* (mg/mL)**	40	8.0±0.00				
	80		16.00±0.00			
	120		24.00±0.00			0.003072±0.00
***A.indica* (mg/mL)**	40		8.00±0.50			
	80				0.0256±0.00	0.002048±0.00
***V. amygdalina* (mg/mL)**	80					0.002048±0.00
	120			24.00±1.50		0.003072±0.00

Values are mean ± SEM of 3 determinations

 https://doi.org/10.53858/arocfn01011521

The total flavonoids of *Azadirachta indica* exhibited antifungal activities against *A. niger, T. tonsurans*, and *C. tropicalis* only at higher concentrations of 80 and 120 mg/ml. Antibacterial activity of the flavonoids from *A. indica* was demonstrated against *Micrococcus roseus* (15.00±0.15 mm) and *Streptococcus faecalis* (17.00±0.05 mm) only at a higher dose of 120 mg/mL (Table 2). This finding confirms the studies of Rao et al. [19], which reported that *A. indica* possesses a wide spectrum of antibacterial activities. Also, results obtained show that the extracts of the neem plant inhibited the growth of *C. tropicalis* as corroborated by the work of Khan and Wassilew [20]. The inhibition of various microbial isolates used in this study by the flavonoids extract from leaf of A. indica suggested it is effective in the treatment of infection caused by the organisms [21]. Candida spp has been implicated in thrush [22]

The total flavonoids of *P. guajava* exhibited a dose-dependent inhibitory effect on *K. pneumonaie, M. roseus, S. faecalis,* and *P. aeruginosa* with maximum inhibition diameters of 14.00±0.10, 16.00±0.55, 15.00±0.43, and 14.00±0.05 mm respectively (table 3). Antifungal activity of the flavonoids from *P. guajava* was observed only against *Candida tropicalis* (13.00±0.05-15.00±0.05mm). The observed dose-dependent inhibitory effects of the flavonoids extract against the bacteria and fungi correlate with the findings from the previous studies which reported that increasing concentration of antimicrobial substance led to corresponding increase growth inhibition of microorganisms [23-25]. Therefore, more of the antimicrobial agents were able to diffuse into the inoculated nutrient agar as the extract concentration increase.

The combination of total flavonoids from leaves of *Psidium guajava* and *Vernonia amygdalina* at different ratios achieved synergetic antibacterial and antifungal activities against all the tested organisms in comparison with when the flavonoids from each plant were used singly (Table 4).

The 40 mg/ml of the total flavonoids of *P. guajava* had a significantly higher MIC value (0.0128) on *Micrococcus roseus* while 120 mg/ml of the total flavonoids of *P. guajava* had a significantly lower MIC value (0.0003072) on *S. faecalis*. For fungi, 120 mg/ml of total flavonoids of *P. guajava* had a significantly higher MIC value (0.003072) on *C. tropicalis* while 120mg/ml of total flavonoids of *V. amygdalina* had a significantly lower MIC value (0.00153) on *Candida tropicalis* (table 5). Similarly, For bacteria, 40mg/ml of the total flavonoids of *P.guajava* had higher MBC (24mg/ml) on *S. faecalis* while 120mg/ml of the total flavonoids of *V. amygdalina* had lower MBC value (4mg/ml) on *P. aeruginosa*. For fungi, 120mg/ml of total flavonoids of *P. guajava* had higher MFC value (0.003072 mg/ml) on *C tropicalis* while 120mg/ml of total flavonoids of *V. amygdalina* had a lower MFC value (0.00153mg/ml) on *Candida tropicalis* (table 6).

The lower range of MIC and MBC values of the flavonoids extracts from *P. guajava*, and *V. amygdalina* in this study translates to high antibacterial and anti-fungi potency on the extracts tested while the inhibitory activity is an indication of broad-spectrum activity against gram positive and gram negative bacteria [26] which implies that the phytochemical possessed broad-spectrum antibiotic compounds. However, based on the MBC values, flavonoids extract of *V. amygdalina* can be considered as a stronger anti bactericidal and anti-fungi agent compared with *P. guajava*, and *A. indica*. The results of the MICs and MBC revealed that the gram-positive bacteria tested were more susceptible to inhibition by the flavonoids extracts of the plants. The high inhibition of bacterial strains (*M. roseus* & *S. faecalis*) suggests that the flavonoids possesses higher spectrum antibacterial properties on gram +ve bacteria**.** Flavonoids are known to inhibit hydrolytic enzymes (proteases), microbial adhesion, and cell envelope transport proteins [27]. In addition, flavonoids form complexes with soluble and extracellular proteins of bacterial cell walls leading to their death [28].

5.0 Conclusion

The observed activity of the flavonoids extract from *A. indica, P. guajava* and *V. amygdalina* against the tomatoes spoilage microorganism suggests the usefulness of the extracts in treating bacterial and fungal-based food infections. A combination of flavonoids from *Psidium guajava* and *Vernonia amygdalina* can serve as a potential source of novel antimicrobial agents for food industry.

Authors' contributions

This work was carried out in collaboration between all authors. Author OI designed the study and performed the experiment. Author AYK supervised the work and revised the thesis. Author AAJ Co-supervised the work. All authors read and approved the final thesis of this manuscript.

Funding

No source of funding has been provided

Competing Interests

Authors have declared that no competing interests exist.

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