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Polyphenol's characterization and antioxidant activity of extracts from unripen and fruit fly-infested guava waste biomass

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Abstract

A significant quantity of guava (*Psidium guajava* L.) fruit is annually wasted in Pakistan and other tropical regions due to the excessive cultivation of immature fruit and the infestation of pests such as the fruit fly (*Bactrocera dorsalis*). This wasted biomass is an underutilized source of bioactive chemicals, particularly polyphenols. This study aims to convert fruit waste into a valuable resource through an eco-friendly extraction, characterization and antioxidant activities of polyphenols. Using water-ethanol mixtures as a eco-friendly green solvents, polyphenols extracted from unripe wasted guava (UG) and fruit fly-infested guava (IG). The extracts were analyzed for total phenolic content (TPC), total flavonoid content (TFC), and the profiles of individual polyphenol compounds using HPLC-DAD. We employed the DPPH, ABTS, and FRAP, assays to evaluate the efficacy of the antioxidants. The IG extract exhibited a significantly elevated TPC (91.28 ± 2.91 mg GAE/g dw) and TFC (48.52 ± 2.31 mg QE/g dw) compared to the UG extract (TPC: 76.37 ± 3.15 mg GAE/g dw; TFC: 32.23 ± 2.57 mg QE/g dw). HPLC studies indicated that IG extracts included higher concentrations of gallic acid, catechin, and quercetin compared to other extracts. Consequently, IG extracts shown superior antioxidant activity across all assessments as DPPH was 24.98 ± 1.36 mg AAE/g for IG compared to 19.03 ± 0.91 mg AAE/g for UG. Guava waste infested with fruit flies is frequently seen as a complete loss; nonetheless, it serves as a superior source of polyphenols with enhanced antioxidant capabilities compared to unripe guava. The efficient application of biomolecules extracts a sustainable and scalable

method for converting guava waste into high-value nutraceutical components, thus promoting a circular economy in the agri-food sector.

Keywords; Unripe guava, infested guava, waste utilization, fruit waste biomass

1. Introduction

Guava (*Psidium guajava* L.), locally known as amrood, is cultivated in numerous rural regions of Pakistan. The fruit produced exhibit variations in size, shape, and flavor contingent upon the cultivar. The superior cultivars are pleasant, whereas others may be astringent. The fruit typically comprises 74–87% moisture, 13–26% dry matter, 0.5–1% ash, 0.4–0.7% fat, and 0.8–1.5% protein (Parvez et al. 2018). It contains a high concentration of ascorbic acid (vitamin C), surpassing that of most imported and domestic fruits. The fruit, namely the pink flesh kind, has a significant quantity of vitamin A (beta-carotene). Certain B vitamins, including thiamin (B1), riboflavin (B2), niacin, and pantothenic acid, are also present in the fruit. Moreover, it comprises a significant quantity of phosphorus, calcium, iron, potassium, and sodium (Bashir and Abu-Goukh 2003). Moreover, it is extensively consumed, processed for juice production, and utilized in medical formulations (Dange et al. 2020).

A significant amount of the guava harvest is wasted prior to reaching the consumers, despite its a considerable worth. In Pakistan, high number of guava grower, the postharvest losses are projected to the surpass 20-40 % (Kanwal et al. 2016). The main reason of the guava waste is, waste due to unripen fruit and more significantly, the infestation by the guava fruit fly, which renders the fruit unusable due to compositional and structural damage and possible the nutritional changes (Brown and Wills 1983). Fruit fly is extensively found over the Southeast Asia, Pakistan, the Pacific Islands, and southern region of China. This pest is known as the primary damage to the guava fruits and is also a significant to pest managed in other fruits, including papaya, mango, and citrus (Malo et al. 2005). The fruit fly is listed on the quarantine list of various countries, as per International Standards for Phyto-sanitary Measures (ISPM) No. 26 of the International Plant Protection Convention. Consequently, the treatment of guava fruits potentially contaminated with this fruit fly is important for exporting guava to nations that are free of the pest. Guava fruits must be managed in compliance with pest control protocols (Lin et al. 2020).

The infested guava (IG) is generally disposed of in orchards or landfills, exacerbating environmental problems and signifying a considerable waste of biological resources.

Although research has examined the poly phenolic composition of ripened guava fruits, investigations of its underused waste forms remain limited. No research has explicitly examined the bioactive potential of unripen and insect-infested guava waste or suggested a sustainable valorization strategy for this particular biomass. This research aims to examine the phenolic extracts and antioxidant activity of unripen and infested guava extracts from the local Punjab region.

2. Material and Methods

2.1. Chemicals Collection and preparation of fruit sample.

Unripen fruits and infested white fleshed guava varieties were collected from the orchards of Faisalabad origin located in Punjab. Fruits were chosen for consistency in size, color, and presence of infestations (infested with fruit flies exhibiting visible signs of infestation: sign of injection, larvae, and softened fruit). The fruits were thoroughly cleaned with tap water and then with distilled water. The visibly damaged and larval-infested portions of the samples were carefully excised using a sterile knife, and the adjacent pulp and peel tissue were gathered.

2.2. Chemicals

All utilized chemicals were of analytical or HPLC grade. The Folin-Ciocalteu reagent, gallic acid, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), fluorescein sodium salt, and other chemicals were acquired from Sigma-Aldrich, USA.

2.3. Extraction of extracts

The extracts were obtained using 80% methanol in distilled water and 0.1% formic acid, and then we employed ultra-sonication for 2 minutes. The samples were placed in an orbital shaker for 16 hours at 20 °C and 150 rpm following the addition of 30 mL of solvent to a 2 g sample in triplicate. This was conducted to determine the feasibility of extracting phenolic compounds. Subsequently, all samples were centrifuged at 8000 rpm for 15 minutes. The supernatant was collected and stored at −20 °C for subsequent use as an antioxidant. We employed a 0.45 µL syringe filter to purify the extracts for subsequent HPLC analysis using vials.

2.4. Determining the phenolic compounds and assessing their antioxidant activity

2.4.1. Total Phenolic Content

The methods used by (Ahmad et al. 2023) was employed to ascertain the total phenolic content (TPC) of the extracted fruit samples, with minor modifications. Initially, 25 µL of 25% Folin–Ciocalteu reagent (v/v) and 200 µL of Milli-Q water were combined with 25 µL of sample extracts. The samples was subsequently maintained at 25 °C for 5 minutes. Subsequently, 25 µL of 10% v/v sodium carbonate was incorporated into the reaction mixture, which was subsequently maintained in darkness for 60 minutes at 25 °C. The

absorbance was measured at 765 nm. The data are calculated as mg equivalents of Gallic acid (GAE) per gram of dry weight of the samples.

2.4.2. Quantification of Flavonoid Concentrations

We use the AlCl3 colorimetric method as described (Ivanišová et al. 2019) to quantify the total flavonoid concentration in the phenolic extract from Juniper Berries and Barberries. on this experiment, 80 µL of the sample extract was combined with 80 µL of a 2% aluminum chloride solution and 120 µL of a 50 g/L sodium acetate aqueous solution on 96-well plates. The reaction mixture was kept in the dark at 25 °C for 2.5 hours, and the absorbance was measured at 440 nm. The results are shown as milligram quercetin equivalents (QE) per gram of dry weight of the samples ($r^2 = 0.999$).

2.4.3. Determining the Total Tannins Content (TTC)

We utilized the modified methods of (Khan et al. 2021) to ascertain the TTC. We amalgamated 25 µL of the extract with 150 µL of a 4% vanillin solution (diluted in methanol) and 25 µL of a 32% sulfuric acid solution in a 96-well plate. Thereafter, we positioned the plate in an incubator at 25 °C for 15 minutes and measured the absorbance at 500 nm. We quantify the TTC in mg CE/g dw of the samples.

2.4.4. HPLC Analysis

The qualitative and quantitative analysis of phenolic compounds was performed using an HPLC system (Shimadzu Prominence-i, Japan) equipped with a DAD detector and a C18 column (Phenomenex Luna®, 250 mm × 4.6 mm, 5 µm). The methodology originated from Flores et al. (2015). The mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The gradient elution procedure was as follows: 0-5 minutes at 5% B; 5-15 minutes increasing from 5% to 20% B; 15-30 minutes increasing from 20% to 25% B; 30-40 minutes increasing from 25% to 50% B; 40-45 minutes increasing from 50% to 95% B; 45-50 minutes at 95% B; followed by re-equilibration. The flow rate was 1.0 mL/min, the injection volume was 20 µL, and the column temperature was maintained at 30°C. Detection was performed at 280 nm and 360 nm. Compounds were identified by comparing their retention times and UV spectra to those of reference standards. Quantification was achieved by external calibration curves for each standard and expressed as µg per gram of dry weight (µg/g dw).

2.4.5. DPPH Assay

We utilized modified approaches from (Ali et al. 2022) to evaluate the effectiveness of unripe and infected guava extracts in neutralizing DPPH free radicals. A 25 µL sample extract was combined with 275 µL of a 0.1 M DPPH solution in methanol. The reaction mixture was kept at room temperature in darkness for 30 minutes, and the absorbance was measured at 517 nm.

The findings were expressed as milligrams of ascorbic acid equivalents per gram of dry weight of the samples (mg AAE/g).

2.4.6. FRAP Analysis of Samples

We applied a modified version of the methodology performed by (Anwar et al. 2023) to evaluate the effectiveness of extracted guava in diminishing ferric ions. Formulate the FRAP reagent by amalgamating 300 mM sodium acetate buffer, 10 mM TPTZ, and 20 mM ferric chloride in a 10:1:1 (v/v/v) ratio. Furthermore, 20 µL of the sample extract was combined with 280 µL of the FRAP reagent in a 96-well plate. The reaction mixture was held at 37 °C for 10 minutes, and the absorbance was measured at 593 nm. The values were expressed as mg AAE/g.

2.4.7. ABTS Assay

We utilized a modified version of the approach developed by (Ahmad et al. 2023) to assess the effectiveness of the extracted guava in scavenging the ABTS radical. A 7 mM ABTS solution was combined with a 140 mM potassium persulfate solution. The reaction mixture was incubated in the absence of light for 16 hours to yield an ABTS+ solution. Ethanol was utilized to dilute the ABTS+ solution to an absorbance of 0.70 ± 0.02 at 734 nm. Subsequently, 10 µL of the sample extract was combined with 290 µL of the ABTS+ solution on a 96-well plate. The mixture was thereafter allowed to rest at 25 °C for 6 minutes, after which the absorbance was measured at 734 nm. The quantification was accomplished by establishing a standard curve for ascorbic acid values from 0 to 150 µg/mL in water. The data were presented as mg AAE per gram.

2.4.8. OH-RSA

The hydroxyl radical scavenging activity of unripe and infected guava extract was evaluated using the modified Fenton-type reaction method as described by (Gulcin 2020). A 50 µL extract was combined with 50 µL of 6 mM $FeSO_4 \cdot 7H_2O$ and 50 µL of 6 mM H_2O_2 (30%), then incubated at 25 °C for 10 minutes. Subsequent to incubation, 50 µL of 6 mM 3-hydroxybenzoic acid was introduced, and absorbance was quantified at a wavelength of 510 nm. **Discussion**

Polyphenol contents and antioxidant activities of UG and IG biomass

The comparative investigation of polyphenol content and antioxidant activity in guava biomass (Figure. 1) specifically between infected and unripe guava extracts reveals substantial insights into the biochemical effects of biotic stress. Tables 1 and 2 distinctly illustrate the variations in polyphenol concentration and antioxidant potential between the two extracts.

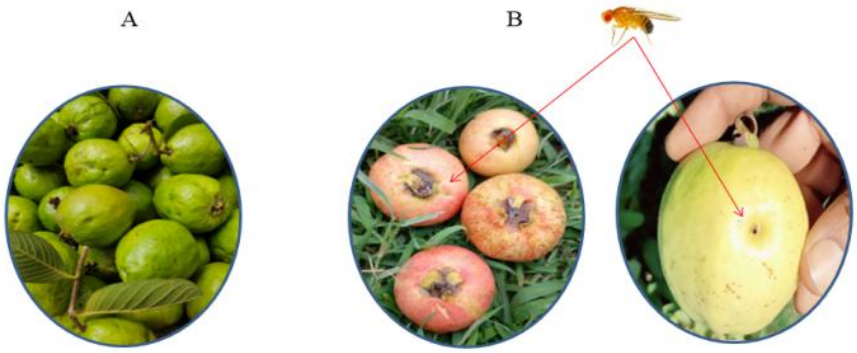

Figure 1. Unripen (A) and fruit fly infested guava (B) fruits

The infected guava extract exhibited a markedly elevated total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC) compared to the unripe guava extract (Table 1).

Table 1. Phenolic contents extracted from the unripe (UG) and infested guava (IG) biomass

Parameters	IG extract	UG Extract
TPC (mg GAE/g)	91.28 ± 2.91	76.37 ± 3.15
TFC (mg QE/g)	48.52 ± 2.31	32.23 ± 2.57
TTC (mg CE/g)	17.82 ± 1.98	14.47 ± 1.38

The infestation seems to have facilitated the formation of phenolic metabolites, as the total phenolic content (TPC) of infested guava (91.28 mg GAE/g) exceeded that of unripe guava (76.37 mg GAE/g). This process is elucidated by the activation of the phenyl propanoid pathway, a crucial pathway in plant secondary metabolism that is elevated as a defensive response to pest and pathogen assaults (Adamo 2022). Infested guava exhibited a considerably greater total flavonoid content (TFC) of 48.52 mg QE/g compared to unripe guava, which had a TFC of 32.23 mg QE/g. Stress frequently elevates the manufacture of flavonoids, which are crucial defensive chemicals with antibacterial and anti-oxidative properties(Schweikert et al. 2012). The increased flavonoid levels reflect an activated systemic response to mitigate oxidative damage caused by pest infestation. A similar trend was observed in tannin levels (TTC), with infected guava (17.82 mg CE/g) surpassing unripe guava (14.47 mg CE/g). Tannins, as defensive secondary metabolites, accumulate in damaged plant tissues and are recognized for their capacity to inhibit herbivory and microbial invasion (Hafsi and Delatte 2023).

Table 2. Anti-oxidant activity of extracts from the unripe (UG) and infested guava (IG) biomass

Parameters	Infested guava extract	Unripe Guava Extract
DPPH (mg AAE/g)	24.98 ± 1.36	19.03 ± 0.91
ABTS (mg AAE/g)	482 ± 7.32	412 ± 6.98
FRAP (mg AAE/g)	368 ± 5.24	297 ± 4.89

Antioxidant studies further confirmed the enhanced bioactivity of infected guava extracts. The DPPH radical scavenging activity was superior in infected guava (24.98 mg AAE/g) relative to unripe guava (19.03 mg AAE/g), signifying an increased hydrogen-donating capacity and efficacy in free radical neutralization. The ABTS assay, which quantifies both hydrophilic and lipophilic antioxidant activity, indicated that infected guava exhibited significantly higher values (482 mg AAE/g) compared to unripe guava (412 mg AAE/g). This signifies that infestation not only increases the concentration of antioxidant chemicals but also expands their spectrum of chemical actions. The FRAP assay, which quantifies the efficacy of antioxidants in reduction processes, indicated elevated results in infected guava (368 mg AAE/g) compared to unripe guava (297 mg AAE/g). A higher FRAP indicates that the chemical can donate more electrons. This is typically associated with elevated concentrations of phenolic and flavonoids (Ajagun-Ogunleye and Ebuehi 2020). The infected guava extract shows the significantly number of higher total phenolic content (TPC), total flavonoid content (TFC), and total tannin content (TTC) as compared to the unripe guava extract. The damage appears to have led to an accumulation of the phenolic metabolites, as the total phenolic content (TPC) of IG (91.28 mg GAE/g) increased that of unripe guava (76.37 mg GAE/g). This phenomenon is carried by the activation of the phenyl propanoid pathway, a vital process in the plants secondary metabolism that is increased as a defensive effect against any pest attack and disease (Zaynab et al. 2018).

Table 3. Quantification of individual phenolic compounds in unripe guava and infested guava biomass.

Sr. No.	Compounds	Unripe Guava Extract	Infested Guava Extract
		Hydroxybenzoic Acids	
1	Gallic acid	9869.15 ± 240.42	12537.91 ± 316.55
2	Protocatechuic acid	342.37 ± 14.2	505.04 ± 20.77
3	p-Hydroxybenzoic acid	153.48 ± 5.17	214.15 ± 7.44

4	Syringic acid	86.15 ± 0.97	127.26 ± 4.0
5	Vanillic acid	197.93 ± 6.53	280.59 ± 10.77
	Hydroxycinnamic Acids		
6	Chlorogenic acid	853.15 ± 40.97	1238.59 ± 51.88
7	Caffeic acid	241.82 ± 7.64	349.48 ± 14
8	p-Coumaric acid	309.04 ± 12.08	460.52 ± 17.44
9	Ferulic acid	120.15 ± 4.2	180.26 ± 6.33
10	Sinapic acid	75.6 ± 2.14	116.15 ± 2.91
	Flavanols		
11	Catechin	4518.48 ± 152.08	6208.37 ± 193.55
12	Epicatechin	1564.59 ± 84.75	1980.15 ± 97.44
13	Epigallocatechin gallate	564.52 ± 29.42	881.71 ± 40.77
	Flavonols		
14	Quercetin	786.26 ± 40.97	1782.15 ± 62.92
15	Quercetin-3-glucoside	453.48 ± 18.75	749.59 ± 29.66
16	Quercetin-3-galactoside	231.26 ± 10.97	238.37 ± 11.88
17	Kaempferol	95.48 ± 3.11	138.37 ± 4.97
18	Kaempferol-3-glucoside	164.59 ± 5.42	238.48 ± 9.66
19	Myricetin	342.37 ± 16.53	493.92 ± 24
20	Myricetin-3-rhamnoside	185.71 ± 7.64	271.6 ± 14
21	Rutin	564.52 ± 29.42	605.15 ± 34
22	Isorhamnetin	42.37 ± 1.25	71.6 ± 0.77

		Flavones	
23	Apigenin	53.48 ± -0.58	80.35 ± 1.44
24	Luteolin	64.59 ± 0.31	105.04 ± 4
		Flavanones	
25	Naringenin	120.15 ± 4.2	193.93 ± 7.44
26	Hesperidin	42.37 ± 1.25	81.71 ± 1.88
27	Ellagitannins	ND	7.3 ± 1.9
28	Punicalagin	1231.26 ± 63.12	1869.24 ± 84.11
29	Pedunculagin	453.48 ± 29.86	671.6 ± 40.77
		Other Phenolics	
30	Ellagic acid	675.6 ± 40.97	949.48 ± 51.88
31	trans-Resveratrol	9.04 ± 1.2	38.37 ± 2.4
32	Phloridzin	ND	16.15 ± 1.3

The comparative data of the concentrations of many phenolic compounds determined in the unripe and infected guava biomass are shown in Table 3. This shows that how biological stress significantly change the content of phytochemicals. Infested guava throut all the compounds shows increment amounts compared to the unripe guava across all categories, which signifying the presence of the phenyl propanoid pathway and stress-induced secondary metabolism in samples (Sharma et al. 2019).

Gallic acid was the highest in hydroxybenzoic acid catagory, with concentrations rising from 9869.15 to 12537.91 μg/g in the IG sample. Comparable changes were observed in the amount of protocatechuic, p-hydroxybenzoic, syringic, and the vanillic acids. These acids act as a important antioxidants and antibacterial agents (Kaurinovic and Vastag 2019).

The hydroxycinnamic acids were shown simultaneous enhancement. Chlorogenic acid increased from 853.15 to 1238.59 μg/g, whereas caffeic, p-coumaric, ferulic, and sinapic acids also

exhibited significant increases. These chemicals are crucial for radical scavenging and lignin production, hence enhancing the plant's structural integrity against pests (Shu et al. 2021). Catechin and epicatechin were the predominant flavanols, with catechin increasing from 4518.48 to 6208.37 µg/g. Epigallocatechin gallate also exhibited a considerable increase, corroborating the elevated antioxidant capacity demonstrated in Table 2.

Flavonols demonstrated considerable stress-induced accumulation, notably quercetin (786.26 to 1782.15 µg/g) and its glycosides, along with myricetin and rutin. Quercetin derivatives are acknowledged for their significant antioxidant and anti-inflammatory effects (Ali et al. 2023). Flavones (apigenin, luteolin) and flavanones (naringenin, hesperidin) increased, indicating an upregulation of flavonoid metabolism overall.

Ellagitannins, absent in unripe guava, were detected in infested guava. The concentrations of punicalagin and pedunculagin were elevated. These intricate tannins are associated with enhanced plant defense and improved human health (Li et al. 2016).

Ellagic acid, trans-resveratrol, and phloridzin are among the phenolics that had significant increases, with resveratrol rising over fourfold. This demonstrates the robust response of phytoalexins to stress. Infestation not only elevated existing phenolic levels but also induced the formation of novel chemicals. This illustrates the alterations in guava's biochemistry as a reaction to stress. This enhancement renders infested biomass a potential source of high-value nutraceuticals.

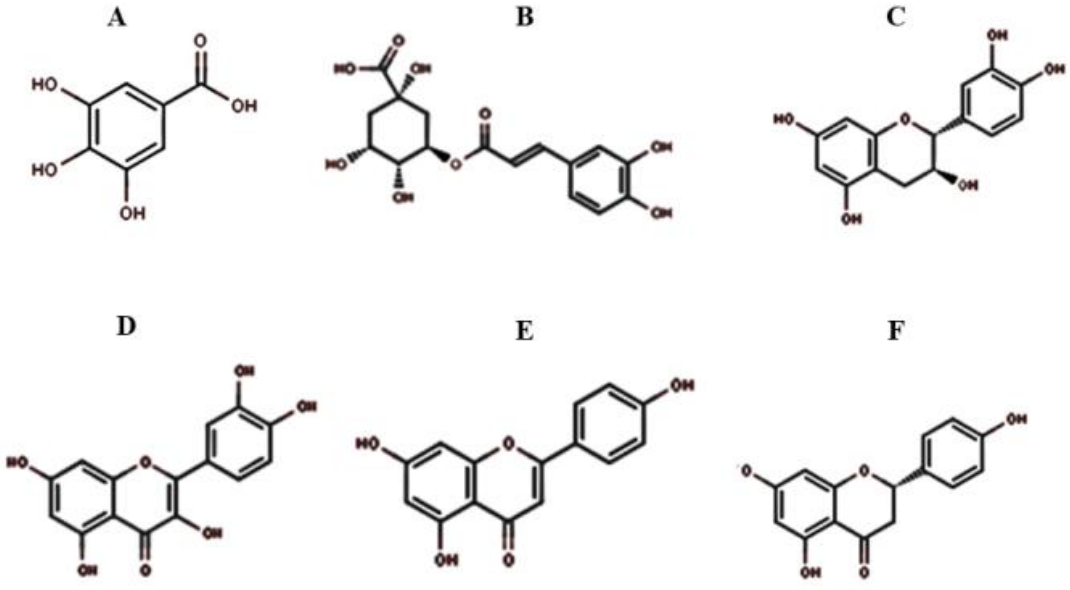

Figure 2. Chemical structure of some phenolic acids and flavonoids from guava biomass: Gallic acid (A), Chlorogenic acid (B), Catechin (C), Quercetin (D), Resveratrol (E) and Apigenin (F).

Figure 2 illustrates the chemical structures of phenolic acids and flavonoids often present in guava.These structures illustrate the structural diversity of many plant polyphenols, elucidating their numerous biological functions and health advantages.

This study effectively demonstrates the viability of converting two major guava waste streams, unripe fruit and fruit fly-infested fruits, into plentiful amounts of bioactive polyphenols via an environmentally sustainable extraction technique. The results describes that the infected guava possesses a greater phytochemical content and antioxidant capability than unripe guava. From an ecological perspective, the elevated polyphenols in IG illustrate a plant's triggered defense system. The incursion of fruit fly is regarded as a biotic stressor, triggering the jasmonic acid signaling pathway, which in turn amplifies the expression of genes encoding critical enzymes, including phenylalanine ammonia-lyase (PAL), the primary enzyme in the phenyl propanoid pathway (Valifard et al. 2015). This prompts the body to produce phenolic chemicals more rapidly to deter pests, prevent microbial invasion of the part, and facilitate the tissue healing. The IG extract's significantly enhanced antioxidant profile indicates it may serve as a superior natural substitute for synthetic antioxidants such as BHA and BHT, which are increasingly facing scrutiny from regulators and consumers due to potential health hazards(Husøy et al. 2019). Potential applications include the serving as a functional food component, a natural preservative in food products to enhance shelf life, or a nutraceutical supplement in capsule format. This research offers a practical answer to a pressing agricultural waste problem in Pakistan and other guava-producing countries. Agricultural producers and agro-industries could enhance their profitability by selling this "waste" biomass to extraction factories specializing in high-value botanical extracts. This would save their expenses on waste disposal and prevent environmental impacts.

Conclusion

This study definitively demonstrates that guava infested with fruit flies, now regarded as agro-food waste, is a markedly greater source of polyphenol antioxidants with increased bioactivity relative to unripe guava. The stress induced by the infestation prompts the fruit to produce additional beneficial compounds, including gallic acid, catechin, and quercetin. We can transform this underutilized biomass into a valuable resource in an efficient and environmentally sustainable manner by employing an eco-friendly, optimized extraction technique. This technology eliminates agro-food waste and introduces innovative approaches to produce cost-effective, natural antioxidants for the food and nutraceuticals sector. This will also enhance the sustainability and circular bio-economy.

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