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A comparative evaluation of antibacterial and antioxidant potential of phenolic-rich hydro ethanol extracts of leaf, seed and fruit pulp of *Syzygium cumini* (L.) Skeels alone and in combination

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Abstract
Food products require protection both from microbial spoilage and contamination as well as oxidative deterioration. Synthetic antimicrobials and antioxidants are generally used as preservatives to combat the problems, but they have accumulated evidence that they could be toxic and carcinogenic. Therefore, innovation should continue to seek more potent safe and effective natural alternatives to synthetic preservatives especially from plant origin. The aim of this study was to evaluate and compare antibacterial and antioxidant potential of phenolic-rich hydro ethanol extracts of different plant parts (leaf, seed and fruit pulp) of *Syzygium cumini* (L.) Skeels alone and in combination along with chemical analysis and evaluation of cytotoxic potential, if any, using standard methods. At their individual effect, leaf extract was found to be most effective antibacterial agent against the studied foodborne bacterial pathogens *Listeria monocytogenes* and *Salmonella typhimurium* whereas fruit pulp extract was found to be most effective antioxidant agent. In combination, synergistic antibacterial and antioxidant interactions between leaf extract and fruit pulp extract was observed. No linear relationship between total phenolic and flavonoid content and their antibacterial as well as antioxidant efficacy was observed. The leaf extract and fruit pulp extract combination did not show any cytotoxic potential at recommended dosage level. The results provide evidence that phenolic-rich hydro ethanol extracts of leaf and fruit pulp of *Syzygium cumini* may serve as a potential source of more potent safe and effective antibacterial and antioxidant compounds which may serve as promising natural alternatives to synthetic preservatives in food and pharmaceutical industries.

Keywords: *Syzygium cumini*, polyphenols, antimicrobials, antioxidants, synergistic interactions natural preservatives

Introduction
Foods are spoiled and contaminated by various factors. The most common causes include microbial growth and contamination as well as oxidative deterioration [1]. Although, several preservation techniques have been developed to assure food safety and quality, in recent years, consumers are becoming more aware of health risks associated with synthetic preservatives and are looking for safer, healthier food options. This has led to growing demand for natural food preservatives from other sources especially from plant origin [2, 3]. Relevant literature reveals that plant polyphenols play an important role as a new strategy to combat microbial growth and contamination as well as oxidative deterioration because polyphenols regulate cellular redox-potential [4-7]. It has also been reported by several researchers that a combination of various extracts of berries may exhibit additive, synergistic, or even antagonistic interactions due to presence of structurally diverse major polyphenolic compounds [8, 9]. *Syzygium cumini* (L.) Skeels (Fam: *Myrtaceae*) commonly known as Jamun or Indian blackberry is a medicinal plant rich in polyphenols [10, 11]. All parts of the plant have been reported to have health benefit effects [12, 13]. Antimicrobial and antioxidant activities of extracts of different plant parts of *Syzygium cumini* at their individual effects have been reported by several workers [14, 15]. But, knowledge about their

antimicrobial and antioxidant efficacy resulting from interactions between the extracts of different plant parts of *S. cumini* is lacking. This knowledge is particularly important to develop more potent, safe and effective natural antimicrobial and antioxidant blend from *S. cumini* which may serve as safe and effective promising natural alternative to synthetic preservatives. The present investigation has therefore been designed to evaluate and compare antibacterial and antioxidant potential of phenolic-rich hydro ethanol extracts of different plant parts (leaf, seed and fruit pulp) of *S. cumini* alone and in combination along with chemical analysis and evaluation of cytotoxic potential with a view to shed some light on these important aspects.

Methodology

Collection and processing of plant materials

Fresh matured leaves and fully ripe fruits of *Syzygium cumini* were procured from the local market (Baranagar Bazar Market, Kolkata, India). To remove any impurities adhered over the leaves and fruits surfaces, they were washed thoroughly under running tap water. *Syzygium cumini* fruits were manually peeled to separate pulps and seeds. The leaves, seeds and fruit pulps of *S. cumini* were then dried in a hot air oven at 40°C until a constant weight is reached and the dried materials were powdered separately using a grinder.

Preparation of phenolic-rich hydro ethanol extracts of different plant parts of *S. cumini*

The phenolic-rich hydro ethanol extracts of different plant parts of *S. cumini* were prepared following the method described by Bae *et al.* [16] with slight modifications**.** Briefly, 100 g powder of each plant parts (leaf, seed and fruit pulp) of *S. cumini* were placed in three 500 ml conical flasks containing 300 ml of 70% hydro ethanol in each flask and mixed thoroughly. They were kept for 24h at room temperature with occasional shaking and filtered using Whatman No. 1 filter paper. The obtained residue was re-extracted twice following the same procedure and filtered. The filtrates of each plant parts were then pooled separately. Each of the pooled filtrates was then evaporated to dryness using a rotary evaporator (bath temp. 40°C).The dried mass of each of the hydro ethanol extracts of different plant parts of *S. cumini* was then dissolved separately in 150 ml distilled water and partitioned (3×) successively with an equal volume of n-hexane, chloroform, ethyl acetate and n-butanol. The ethyl acetate fraction of each plant parts was taken, pooled separately, filtered and concentrated to get dried mass of phenolic-rich hydro ethanol extracts of different plant parts of *S. cumini*. The dried mass of phenolic-rich hydro ethanol extracts of leaf, seed and fruit pulp of *S. cumini* were then kept at -20°C for experimental purposes.

Estimation of total phenolics content

The total phenolic content (TPC) of ethyl acetate fraction of hydro ethanol extract of leaf, seed and fruit pulp of *S. cumini* was estimated quantitatively using Folin-Ciocalteu reagent, with gallic acid as the standard following the method described by Mcdonald *et al.* [17] with slight modifications. Briefly, a volume of 0.5 ml of each test extract (100 µg/ml) was mixed with 1 ml of Folin-Ciocalteu reagent (diluted 1:10 with deionized water), vigorously shaken and kept for 5 min at room temperature. Thereafter, 3 ml of 2% Na_2CO_3 solution was added and the mixture was allowed to stand for 2h with intermittent shaking for color development. The absorbance of the resulting blue color was measured at 760 nm. The total phenolic content was estimated from the standard curve prepared with different concentrations (3.125-100 µg/ml) of gallic acid and expressed as mg Gallic acid equivalent /g dry extract (mg GAE /g DE). Each experiment was repeated thrice.

Estimation of total flavonoids content

The total flavonoid content (TFC) of each test extract was estimated using aluminum chloride colorimetric method as described by Zhishen *et al.* [18] with slight modifications. In brief, 0.5 ml of each of the test extracts was taken in 10 ml test tubes containing 2 ml distilled water. To each of the test tubes, 0.15 ml of 5% $NaNO_2$solution was added and kept for 10 min at room temperature. Then 0.15 ml of 10% $AlCl_3$solution was added and kept for 2 min. Now, 1 ml of 1M NaOH solution was added and the volume was adjusted to 5 ml with distilled water. The mixture was kept for 10 min at room temperature and then absorbance of the resulting solution was measured at 510 nm. The total flavonoid content was estimated from the standard curve prepared with different concentrations (3.125-100 µg/ml) of quercetin and expressed as mg Quercetin equivalent /g dry extract (mg QE /g DE). Each experiment was repeated thrice.

Microorganisms used

The foodborne bacterial pathogens used in the present investigation were selected based on their relevance in the food industry. The bacterial pathogens used were pure reference standard foodborne bacteria *Listeria monocytogenes* (MTCC 657*)* and *Salmonella typhimurium* (MTCC 3224) as indicator strains of Gram-positive and Gram-negative foodborne bacteria respectively. These two strains were procured from the Institute of Microbial Technology, Chandigarh, India and were maintained following standard CLSI guidelines [19].

Standardization of inoculum size

For standardization of bacterial inoculum size, test bacterial strains were incubated for 3-6 h at 30°C for *L. monocytogenes* and37°C for *S. typhimurium* until the culture attained a turbidity of 0.5 McFarland Unit. The final inoculum size was adjusted to $5x10^5$ CFU/ml following CLSI guidelines [19].

Determination of individual antibacterial effect

Determination of Inhibition Zone Diameter (IZD) by agar well diffusion method

Individual antibacterial efficacy of each test extract of *S. cumini* against the studied bacteria was determined by modified agar well diffusion method [20] for the determination of inhibition zone diameter (IZD). Briefly, on sterilized nutrient agar plates, one ml of inoculum ($5×10^5$ CFU/ml) of each bacterial strain was spread evenly with a glass rod spreader and six mm diameter wells were bored on the surface of agar plates. Then, 100 µl ((1000 µg/ml) of each of the test extracts reconstituted with 0.2% dimethylsulfoxide (DMSO) were given into the wells of agar plates and kept for 2h at room temperature to allow diffusion of test extracts. The plates were then incubated for 24h at 30ºC for *L. monocytogenes* and 37ºC for *S. typhimurium*. After incubation, inhibition zone diameter (IZD) was measured in milimeter. Ciprofloxacin (10 µg/ml) was used as reference standard antibiotic (positive control) and 0.2% DMSO as negative control. Each experiment was repeated thrice.

Determination of Minimum Inhibitory Concentration (MIC) by microbroth dilution method

Individual antibacterial activity of test extracts of *S. cumini* against the studied bacterial pathogens was also evaluated by microbroth dilution method following CLSI guidelines [19] for

the determination of minimum inhibitory concentration (MIC). Briefly, 100 µl of each test extract in varying concentrations ranging from 7.81 µg/ml to 1000 µg/ml was given in each well of 96-well microtiter plates containing 90 µl of nutrient broth. Then, 10 µl of working inoculum suspension (5×10^5 CFU/ml) of test bacteria was given to each well. The plates were then incubated for 24 h at respective temperature (30°C for *L. monocytogenes* and 37°C for *S. typhimurium*). After incubation, 40 µl of INT solution (0.4 mg/ml) was added to each well of microtiter plates and incubated for 6 h. The microtiter plates were then examined to observe change in color. A change in color from faint yellow to red-purple occurs when viable microorganisms in wells interact with INT solution. The lowest dilution of the extract with no visible color change was considered as the MIC for the test extract. Each experiment was repeated thrice.

Determination of rate and extent of bacterial killing by Time Kill-kinetics assay

The rate and extent of bacterial killing by the test extracts at their individual effect against the studied bacteria were determined by time kill-kinetics assay following the method described by Levinson [21] for the determination of bactericidal or bacteriostatic activity of test extracts. Briefly, in each well of microtiter plate, 90 µl of nutrient broth, 10 µl of bacterial suspension (5×10^5 CFU/ml) and 100 µl of each of the test extract at different concentrations (0.5×MIC, 1×MIC, 2×MIC and 4×MIC) were added and mixed thoroughly. The plates were then incubated for 24h at 30ºC for *L. monocytogenes* and 37ºC for *S. typhimurium*. Then 10 µl sample was removed from wells at different time intervals (0-, 3-, 6-, 12- and 24-h of incubation), and diluted serially with nutrient broth. 100 µl of diluted aliquots were then spread evenly on nutrient agar plates and incubated for 24h at respective temperature (30ºC for *L. monocytogenes* and 37 ºC for *S. typhimurium*). After incubation, agar plates with 30 to 300 colonies were used for viable counting. Log_{10}CFU/ml was plotted against time for construction of time kill-kinetics curves. Each experiment was repeated thrice. The antibacterial effect of test extracts was considered 'bactericidal' when the reduction in colony count by the test extract against the studied bacteria at 24h of incubation was found to be ≥ 3 log_{10} CFU/ml and 'bacteriostatic' when this reduction was < 3 log_{10}CFU/ml compared to respective controls [22].

Determination of combined antibacterial effect

Determination of minimum inhibitory concentration (MIC) in combination by microbroth dilution method

The MIC values of test extracts in combination against the studied bacterial pathogens were determined following CLSI guidelines [19]. Briefly, in each well of microtiter plate containing 90 µl of nutrient broth, 10µl of bacterial inoculums (5×10^5CFU/ml) and 100µl of test extracts in combination (1:1) at their 1/32×MIC to 4×MIC concentrations were added and incubated for 24 h at 30 °C for *L. monocytogenes* (MTCC 657) and at 37 °C for *S. typhimurium* (MTCC 3224). Now, for the determination of MIC of test extracts in combination, same procedure was followed as done before for individual MIC determination. Each experiment was repeated thrice.

Determination of fractional inhibitory concentration (FICI) by checkerboard assay method

Based on the MIC values of test extracts alone and in combination, their fractional inhibitory concentration indices (FICI) values against both the studied bacterial pathogens were determined by checkerboard assay method using the following formula to know the type of antibacterial interactions (additive, synergistic or antagonistic) of test extracts in combination against the studied bacteria [23].

FICI (FIC_1 +FIC_2)=MIC of test extracts A (TE_A) and test extract B (TE_B) in combination /MIC of test extract A (TE_A) alone + MIC of test extracts A (TE_A) and test extract B (TE_B) in combination/ MIC of test extract B (TE_B) alone.

The type of antibacterial interactions between two test extracts (TE_A and TE_B) were interpreted as follows: Synergy (FICI ≤ 0.5); Additive (0.5< FICI≤4); Antagonistic (FICI>4) [23].

Determination of individual and combined antioxidant efficacy: Evaluation of antioxidant efficacy alone and in combination by DPPH (2-2-diphenyl-1-picryl hydrazyl) radical scavenging assay method

DPPH free radical scavenging activity of test extracts of leaves, seeds and fruit pulps of *S. cumini* alone and in combination (1:1 v/v) was evaluated quantitatively using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay method [24]. Briefly, in test tubes containing 3.9 ml of 0.1mM solution of DPPH in methanol, 100 µl of test extract alone and in combination (1:1) at varying concentrations (7.8 µg/ml -1000µg/ml) were added, shaken vigorously and the tubes were allowed to stand in dark for 30 min. The control tube received no test extract and methanol was used for zero adjustment. Absorbance of the samples was measured at 517 nm. DPPH free radical scavenging activity by the test extracts alone and in combination was calculated according to the following formula.

$$(\%)\ \text{Free radical scavenging} = \frac{A(blank) - A\,(sample)}{A\,(blank)} \times 100$$

Where $A_{(sample)}$ is the absorbance of DPPH solution after reacting with a given concentration of test extract and $A_{(blank)}$ is the absorbance of DPPH solution with methanol blank instead of test extract. Each experiment was repeated thrice. IC_{50} values of test extracts alone and in combination were determined.

Determination of antioxidant combination Index (CI) by classical isobologram analysis

To investigate the type of antioxidant interactions (additive, synergistic or antagonistic) of test extracts in combination, an isobologram analysis based on IC_{50} values alone and in combination was performed. The classical isobologram combination index equation (CI) was used for analyzing the data [25].

$$CI = (D)_1/(Dx)_1 + (D)_2/(Dx)_2$$

Where, $(D)_1$ and $(D)_2$ are theIC_{50} value of two different test extracts in combination; $(Dx)_1$ and $(Dx)_2$ are the individual IC_{50} values of two different test extracts that were in combination. On the basis of CI values, the type of antioxidant interactions between the test extracts were interpreted as follows. CI < 1: synergistic; CI = 1: additive; CI > 1: antagonistic [26].

Chemical Analysis

LC-MS/MS analysis

Chemical analysis of fruit pulp extract of *S. cumini* has already been done in our previous study [27]. In the present investigation,LC-MS/MS analysis of only leaf extract of *S.cumini*that showed synergistic antibacterial and antioxidant interactions with fruit pulp extract in combination was

performed at Central Instrumentation Facility (CRF), Indian Institute of Technology Kharagpur, India.Briefly, after dilution of the test leaf extract by a 100-fold factor, the sample was analyzed using an LC-MS/MS system (Waters, Milford, MA, USA). The LC-MS/MS system was equipped with a photodiode array detector operating at a wavelength of 250 nm. The analytical C18 column (Waters) was used as the stationary phase. The device consists of a combined integrated and automated fraction collector. The Quattro microTM Application Programming Interface (API) in conjunction with the updated mass-Lynx 4.7 software was used to control the instrument and collect the data. A mobile phase consisting of an aqueous solution containing 0.1% formic acid (A) and methanol acidified with 0.1% formic acid (B) was used for gradient elution at a flow rate of 0.5 mL/min. Mass spectra were recorded in the mass-to-charge ratio (m/z) range of 50-2000 at 400 °C and a nebulizing gas flow rate of 10 L/min. Both negative and positive ionization modes were used and a chromatogram was recorded at a wavelength of 350 nm. Tentative identification of peaks in the LC-MS/MS chromatogram were established based on LC-MS/MS spectral data and the measured reference spectra of polyphenols identified in the literature [28-36].

Cytotoxicity study

Brine shrimp lethality assay

The cytotoxic potential of test extracts that showed synergistic antibacterial and antioxidant interactions in combination was evaluated using brine shrimp lethality assay following the method described by Meyer *et al.* [37]. Briefly, for hatching brine shrimp eggs, artificial sea water was prepared by dissolving 38 g of sea salt in 1 L of distilled water. Brine shrimp eggs (*Artemia salina*) were incubated in artificial sea water at 26°C for 48h. This time period was allowed for the shrimp eggs to hatch and mature as nauplii (larva). Then 10 test tubes containing 4.5 ml of artificial sea water were taken and in each of the test tubes 10 nauplii were given. Then, 100 µl of test extracts in combination (1:1) at varying concentrations (7.81 µg/ml-2000 µg/ml) were given in nine test tubes. The control tube received no test extract. All the tubes were then incubated for 24h at room temperature (25º±1ºC). After incubation, number of nauplli alive was counted with the help of a magnifying glass and per cent mortality against various concentrations of test extracts in combination was calculated. A curve was plotted taking percent mortality versus concentration of test extracts in combination to determine 24h LC_{50} (50% lethal concentration) of test extracts in combination.

Statistical Analysis

Data were expressed as mean±S.D. of triplicate experiments. Data were statistically analyzed by one-way ANOVA and Tukey's post hoc test using SPSS software (Version 18.0) with the level of significance set at $p < 0.05$.

Results

Table 1 shows the results of inhibition zone diameter (IZD) of leaf, seed and fruit pulp extracts of *S. cumini* against the studied bacteria *L.monocytogenes* (MTCC 657) and *S. typhimurium* (MTCC 3224) at their individual effect. All the tested extracts showed varying degrees of strain specific growth inhibition activity against the studied bacteria with IZD ranged from 12.60±0.80 mm to 27.00±0.63 mm. Based on IZD values, leaf extract was found to be most effective antibacterial agent against both the studied bacteria (IZD: 27.00±0.63 mm against *L. monocytogenes* and 23.00±0.63 mm against *S. typhimurium*) followed by fruit pulp extract (IZD: 20.60±0.80 mm against *L. monocytogenes* and 17.20±0.74 mm against *S. typhimurium*) and seed extract (IZD: 15.50±0.49 mm against *L. monocytogenes* and 12.60±0.80 against *S. typhimurium*). IZD values of ciprofloxacin was found to be 28.20±0.66 mm against *L. monocytogenes* and 22.20±0.66 mm against *S. typhimurium.*

Table 1: Inhibition zone diameter (IZD) of phenolic-rich hydro ethanol extracts of different plant parts of *Syzygium cumini* against foodborne bacteria *Listeria monocytogenes* (MTCC 657) and *Salmonella typhimurium* (MTCC 3224)

Treatment	Inhibition Zone Diameter (IZD) (mm)	
	Microorganisms	
	Listeria *monocytogenes* (MTCC 657)	Salmonella *typhimurium* (MTCC 3224)
LE#$*	27.00±0.63	23.00±0.63
SE	15.50±0.49	12.60±0.80
FPE	20.60±0.80	17.20±0.74
Cip (positive control)	28.20±0.66	22.20±0.66
DMSO (negative control)	-	-

Results are mean ±S.D. of triplicate experiments. LE: leaf Extract; SE: Seed Extract; FPE: Fruit Pulp Extract; CIP: Ciprofloxacin; DMSO: Dimethlsulphoxide.One-way analysis of variance (ANOVA) revealed significant difference ($p < 0.05$) among the extract treated groups against both the studied bacteria. #Tukey's Posthoc test revealed significant difference ($p<0.05$) between the test extracts treated groups against both *L. monocytogenes* and *S. typhimurium*. $Leaf extract was found to be almost equipotent to ciprofloxacin against both the studied bacteria on activity basis.

The MIC values of test extracts of *S. cumini* alone and in combination against the studied bacteria are shown in Table 2. On the basis of MIC values, at their individual effect, leaf extract showed highest antibacterial efficacy against both the studied Gram-positive and Gram-negative bacteria [MIC: 41.00±1.09 µg/ml against *L. monocytogenes* and 51.20±0.97 µg/ml against *S. typhimurium*] followed by fruit pulp extract [MIC: 62.60±0.52 µg/ml against *L. monocytogenes* and 89.20±1.47 µg/ml against *S. typhimurium*] and seed extract [MIC: 69.40±0.79 µg/ml against *L. monocytogenes* and 93.60±1.01 µg/ml against *S. typhimurium*]. In combination, leaf extract/fruit pulp extract blend was found to be most effective antibacterial agent against both *L. monocytogenes* (MIC: 10.80±0.74 µg/ml) and *S. typhimurium* (MIC: 16.40±0.49 µg/ml) compared to other tested possible combinations: leaf extract/seed extract [MIC: 35.40±0.48 µg/ml (*L. monocytogenes*) and 70.60±0.80 µg/ml (*S. typhimurium*)] and seed extract/fruit pulp extract combination [MIC: 50.80±0.74 µg/ml *(L. monocytogenes*) and 83.60±0.80 µg/ml µg/ml (*S. typhimurium*)].

Table 2: Minimum inhibitory concentration (MIC) of phenolic-rich hydro ethanol extracts of different plant parts of *Syzygium cumini* against *Listeria monocytogenes* (MTCC 657) and *Salmonella typhimurium* (MTCC 3224)

Test extracts alone and in combination	Minimal inhibitory concentration (µg/ml)	
	Microorganisms	
	Listeria monocytogenes (MTCC 657)	*Salmonella typhimurium* (MTCC 3224)
LE	41.00±1.09	51.20±0.97
SE	69.40±0.79	93.60±1.01
FPE	62.60±0.52	89.20±1.47
LE+SE	35.40±0.48	70.60±0.80
LE+FPE[$]	10.80±0.74	16.40±0.49
SE+FPE	50.80±0.74	83.60±0.80

Results are mean ±S.D. of triplicate experiments. LE: Leaf Extract; SE: Seed Extract; FPE: Fruit Pulp Extract. At their individual effect, leaf extract exhibited significantly higher ($p<0.05$) antibacterial activity against both the studied bacteria compared to other tested extracts. [$]Leaf extract/fruit pulp extract combination showed significantly higher antibacterial potential against both the studied bacteria over other tested extracts in combination.

Table 3 shows the results of fractional inhibitory concentration indices (FICI) values of test extracts in combination against the studied bacteria. On the basis of FICI values, leaf extract/fruit pulp extract combination exhibited synergistic antibacterial interactions against both *L. monocytogenes* (FICI: 0.43) and *S. typhimurium* (FICI: 0.50). Other tested possible combinations showed additive antibacterial effects against both the studied bacteria with FICI ranged from 1.37 to 2.47.

Table 3: Fractional inhibitory concentration indices (FICI) values of phenolic-rich hydro ethanol extracts of different plant parts of *Syzygium cumini* against foodborne bacteria *Listeria monocytogenes* (MTCC 657) and *Salmonella typhimurium* (MTCC 3224)

Microrgnisms	Test extracts in combination (1:1)								
	LE+SE			LE+FPE			SE+FPE		
	FIC	FICI	Remarks	FIC	FICI	Remarks	FIC	FICI	Remarks
Listeria monocytogenes (MTCC 657)	0.86	1.37	ADD	0.26	0.43	S	0.73	1.54	ADD
	0.51			0.17			0.81		
Salmonella typhimurium (MTCC 3224)	1.59	2.47	ADD	0.32	0.50	S	0.89	1.82	ADD
	0.88			0.18			0.93		

LE: Leaf Extract; SE: Seed Extract; FPE: Fruit Pulp Extract. S: Synergistic (FICI ≤ 0.5); ADD: Additive (FICI: 0.5 > ADD ≤ 4); Leaf extract/fruit pulp extract combination showed synergistic antibacterial activity against both *L. monocytogenes* (FICI: 0.43) and *S. typhimurium* (FICI: 0.50). Other tested possible combinations showed additive antibacterial effect against both the studied bacteria (FICI ranged from 1.37 to 2.47).

The rate and extent of bacterial killing by the test extracts of *S. cumini* at their individual effect against *L. monocytogenes* and *S. typhimurium* are shown in Fig 1 and Fig 2 respectively. From these two Figures (Fig 1 and Fig 2), it was observed that test extracts exhibited time-dependent strain-specific antibacterial activity against both the studied bacteria. The rate and extent of bacterial killing by the test extracts at their individual effect were found to be in the following decreasing order: leaf extract > fruit pulp extract > seed extract. Moreover, leaf extract was found to have bactericidal activity (reduction in colony count by the leaf extract was found to be ≥ $3\log_{10}$ CFU/ml at 24h compared to control sample) against both *L. monocytogenes* (Fig 1) and *S. typhimurium* (Fig 2) whereas fruit pulp extract and seed extract showed bacteriostatic activity (reduction in colony count by fruit pulp extract and seed extract was found to be < $3\log_{10}$ CFU/ml at 24h compared to their respective controls) against both *L. monocytogenes* (Fig 1) and *S. typhimurium* (Fig 2).

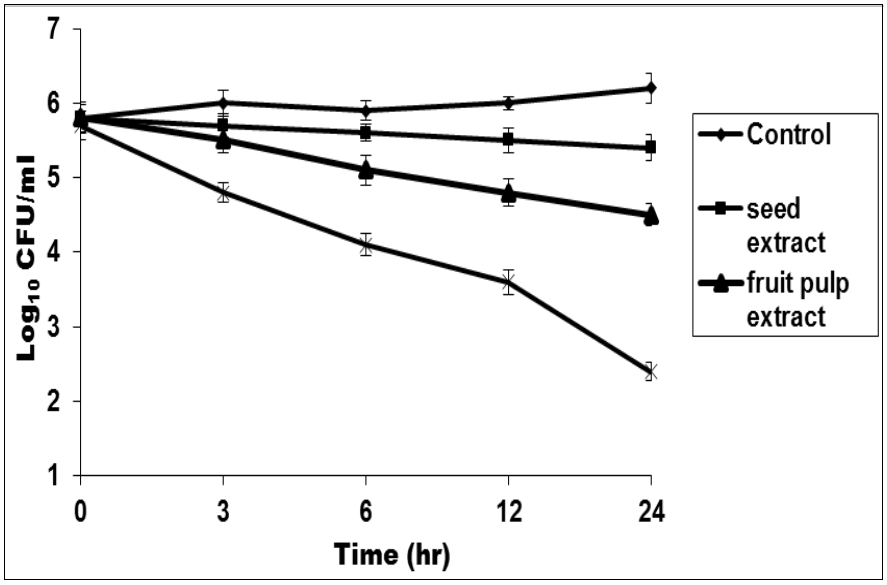

Fig 1: Time-kill-kinetics curves of phenolic-rich hydro ethanol extracts of leaf, seed and fruit pulp of *Syzygium cumini* against foodborne bacteria *Listeria monocytogenes* (MTCC 657)

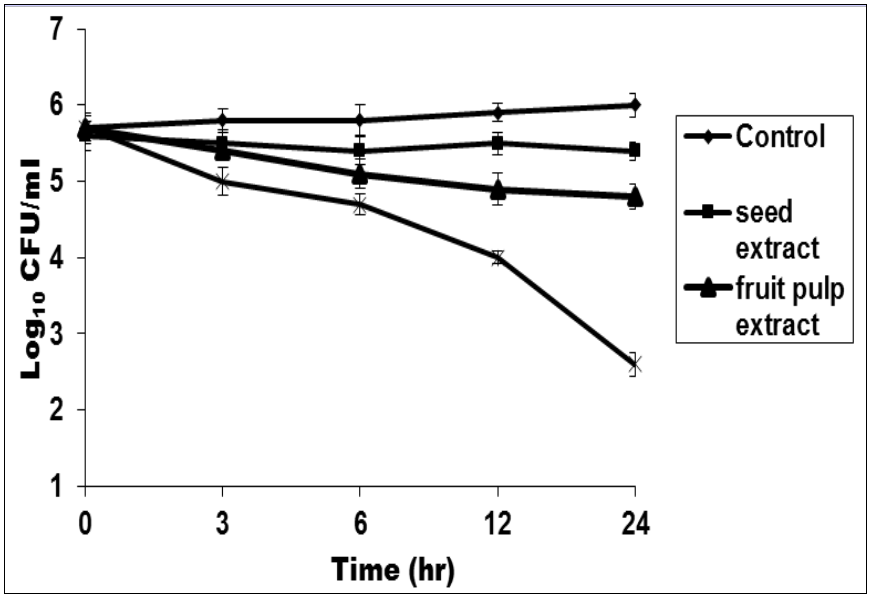

Fig 2: Time-kill-kinetics curves of phenolic-rich hydro ethanol extracts of leaf, seed and fruit pulp of *Syzygium cumini*against foodborne bacteria *Salmonella typhimurium* (MTCC 3224)

Table 4 shows the results of antioxidant efficacy (IC_{50}) of test extracts at their individual effect and type of antioxidant interactions in combination (CI) in DPPH radical scavenging method. On the basis of IC_{50}values, fruit pulp extract was found to be most effective antioxidant agent (IC_{50}: 25.33±0.39 µg/ml) followed by leaf extract (IC_{50}: 30.56±0.50 µg/ml) and seed extract (IC_{50}: 34.43±0.78 µg/ml). On the basis of combination indices (CI) values, only leaf extract/fruit pulp extract combination showed synergistic antioxidant interactions [CI: 0.72 (<1)] whereas other tested possible combinations showed additive antioxidant effect: CI: 1 for leaf extract / seed extract combination and CI: 0.99 (≈1) for seed extract / fruit pulp extract combination.

Table 4: Antioxidant efficacy of phenolic-rich hydro ethanol extracts of different plant parts of *Syzygium cumini* at their individual effect and their type of antioxidant interactions in combination

Test extracts	IC_{50} (µg/ml)	$CI_1 = (D)_1 / (Dx)_1$	$CI_2 = (D)_2 / (Dx)_2$	$CI = CI_1 + CI_2$	Remarks
LE	30.56±0.50	-	-	-	-
SE	34.43±0.78	-	-	-	-
FPE	25.33±0.39	-	-	-	-
LE + SE	16.26±0.34	0.53	0.47	1.0	ADD
LE+FPE	10.13±0.17	0.33	0.39	0.72	S
SE+FPE	14.60±0.57	0.42	0.57	0.99 (≈1)	ADD

Results are mean ±S.D. of triplicate experiments. LE: Leaf Extract; SE: Seed Extract; FPE: Fruit Pulp Extract; S: Synergistic; ADD: Additive

Table 5 shows the results of total phenolic and flavonoid content of test extracts. Leaf extract was found to possess higher concentration of both total phenolics [(TPC: 21.56±0.97 mg GAE/g D) and total flavonoids (TFC: 9.50±0.53 mg QE/g DE)] followed by seed extract [TPC:16.53±0.93 mg GAE/g DE); (TFC: 5.43±0.20 mg QE/g DE)] and fruit pulp extract [(TPC: 12.83±0.45 mg GAE/g DE); (TFC:4.66±0.25 mg QE/g DE)].

Table 5: Total phenolics and total flavonoids content of phenolic-rich hydro ethanol extracts of different plant parts of *Syzygium cumini*

Test extracts	Total phenolics (mg GAE/g dry extract)	Total flavonoids (mg QE/g dry extract)
LE	21.56±0.97	9.50±0.53
SE	16.53±0.93	5.43±0.20
FPE	12.83±0.45	4.86±0.25

Results are mean ±S.D. of triplicate experiments. LE: Leaf Extract; SE: Seed Extract; FPE: Fruit Pulp Extract

Fig 3 shows the chromatogram of six major components of *S. cumini* leaf extract obtained by LC-MS/MS analysis and Table 6 shows the spectral data for tentative identification of these six major components. The tentatively identified six major compounds of *S. cumini* leaf extract based on LC-MS/MS analysis data and the measured reference spectra of polyphenols identified in the literature were (1) Quinic acid derivative;(2) Quercetin-3-O-(6″-malonyl-glucoside)-7-O-glucoside; (3)1,2,2′- triferuloylgentibiose;(4) Rosmarinic acid 3′-glucoside; (5) Dehydrodiferulic acid; and(6) Procyanidin dimer digallate (A-type)].

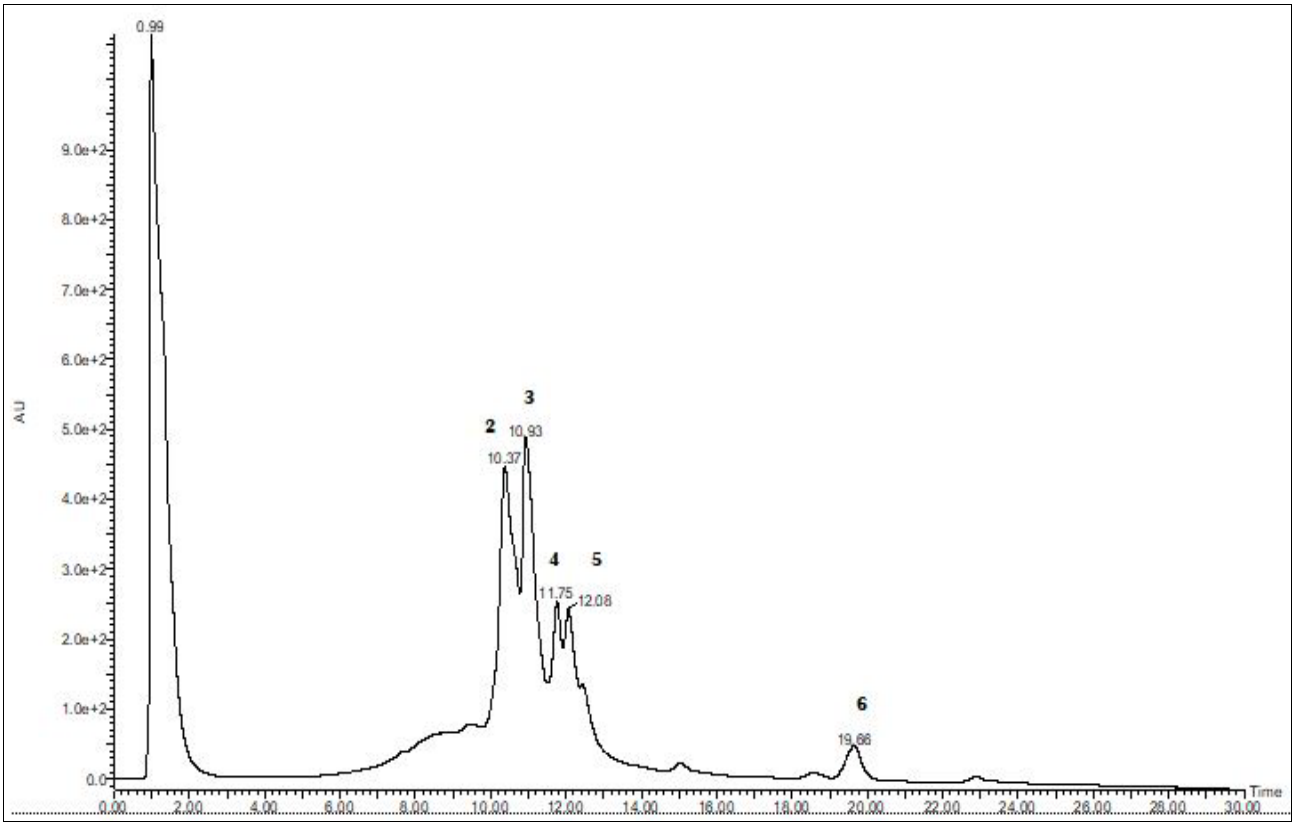

Fig 3: LC-MS/MS chromatogram of phenolic-rich hydro ethanol extract of *Syzygium cumini* leaves.

Note: (1) Quinic acid derivative, (2) Quercetin-3-O-(6″-malonyl-glucoside) -7-O-glucoside, (3) 1,2,2′- triferuloylgentibiose, (4) Rosmarinic acid 3′-glucoside, (5) Dehydrodiferulic acid, (6) Procyanidin dimer digallate (A-type)

Table 6: Tentative identification of major components of phenolic-rich hydro ethanol extract of *Syzygium cumini* leaves

Peak Number	R_T (Min)	$UV_{\lambda max}$ (nm)	MS$[M+H]^+$ (m/z)	MS/MS Fragments $[M+H]^+$ (m/z)	Tentative Identification	References
1	0.99	255-280	380.8163	367.2822, 191.6654 (100),182.7710, 168.7024, 133.8560	Quinic acid derivative	[28, 29]
2	10.37	266, 289	710.9446	464.0740, 318.2782 (100), 218.4076, 193.7479, 190.4562	Quercetin-3-O-(6″-malonyl-glucoside)-7-O-glucoside	[30]
3	10.93	235, 322	872.8481	522.3003, 464.1414, 318.2782 (100)	1,2,2-triferuloylgentiobiose	[31]
4	11.75	290, 330	522.0307	505.8549, 318.4801 (100), 193.4792	Rosmarinic acid 3′-glucoside	[32, 33]
5	12.08	203, 224, 289	384.7220	344.3253, 234.6061, 193.5464 (100), 152.9176	Dehydrodiferulic acid	[34]
6	19.66	210-280	878.4446	709.8658 (100), 573.0591	Procyanidin dimer digallate (A-type)	[35, 36]

Results of cytotoxic potential of *S. cumini* leaf extract / fruit pulp extract combination are shown in Table 7. The leaf extract and fruit pulp extract in combination did not show any noticeable cytotoxic effect at recommended dosage level and LC_{50} of leaf extract/fruit pulp extract combination was found to be > 2000 µg/ml (Table 7).

Table 7: Cytotoxic potential of phenolic-rich hydro ethanol extracts of leaf and fruit pulp of *Syzygium cumini* in combination in brine shrimp lethality assay

Concentration (µg/ml) of leaf extract/fruit pulp extract in combination (1:1 v/v)	No. of nuplii taken	No. of nuplii alive after 24h	% mortality (24h)	24h LC_{50} (µg/ml)
Control	10	10	0	> 2000
7.81	10	10	0	
15.62	10	10	0	
31.25	10	10	0	
62.50	10	10	0	
125	10	10	0	
250	10	10	0	
500	10	09	10	
1000	10	09	10	
2000	10	08	20	

Discussion

Food preservation is important to ensure food availability, safety, and quality of food [38]. Despite the worldwide development of new ranges of food preservation techniques, the microbiological spoilage and contamination as well as oxidative deterioration of food components pose major challenges for food safety and quality [39, 40]. This not only leads to economic losses but also poses health risks to consumers [41]. Different approaches have been employed to address the problem but use of antimicrobials and antioxidants as preservatives are the most effective, convenient and economical means [1, 42]. Moreover, antimicrobials and antioxidants are also used in health-related areas due to their ability to protect the body against infections as well as free-radical directed oxidative damage which contributes to the etiology of many chronic health problems such as cardiovascular and inflammatory disease, cataract, and cancer [43, 44]. Synthetic antimicrobials and antioxidants are generally used as preservatives to food industry, but they have accumulated evidence that they could be toxic and carcinogenic [45]. Owing to the safety and toxicological concerns regarding synthetic preservatives, in recent years the search for more potent, practically nontoxic antimicrobial and antioxidant natural compounds especially from plant origin have been intensified [46]. Relevant literature reveals that berries are rich in polyphenols and have strong antioxidant as well as antimicrobial properties due to presence of structurally diverse major bioactive compounds [47-49]. Moreover, these structurally diverse major phenolic compounds of berries may undergo additive, synergistic or antagonistic interactions in combination. This is because the aromatic ring confer to the polyphenols their resonance structure due to declocalization of the electrons in the molecules. When structurally diverse polyphenols are mixed, their electrons can be delocalized from one compound to another and so can easily react with target molecules or free radicals leading to additive, synergistic or antagonistic effects [50, 51]. Keeping these in mind, in the present work, we have evaluated and compared antibacterial efficacy of phenolic-rich hydro ethanol extracts of different plant parts of *Syzygium cumini* alone and in combination against Gram-positive and Gram-negative foodborne bacterial pathogens as well as their antioxidant efficacy taking the advantages of their possible synergistic interactions. Because synergistic interactions have proven to be an essential feature in antimicrobial and antioxidant treatment due a number of important considerations *viz*. (a) they increase the activity of plant extracts/active components; (b) they thwart drug resistance; (c) they lower amount of required doses, which facilitates the reduction of both cost and adverse/toxic side effects, and (d) they increase the spectrum of activity. It is therefore a field with immense opportunity to protect food both from microbial spoilage and contamination as well as oxidative deterioration at sufficiently low concentration with higher antimicrobial and antioxidant efficacy [52, 53].

To achieve our goal, we first evaluated individual antibacterial potential of test extracts against the type strains of foodborne bacterial pathogens *L. monocytogenes* (MTCC 657) and *S. typhimurium* (MTCC 3224). The individual antimicrobial activity against the studied bacteria was evaluated by the determination of inhibition zone diameter (IZD) and minimum inhibitory concentration (MIC). Based on threshold limits of antimicrobial susceptibility testing [**(**IZD≥11mm and MIC<100µg/ml)] [54, 55], all the extracts tested were found to be active against the studied bacteria and exhibited varying degrees of strain specific antibacterial activity. On the basis of IZD and MIC values, leaf extract was found to be most effective antibacterial agent against both the studied bacteria followed by fruit pulp extract and seed extract (Table 1 and Table 2).

Next, the rate and extent of bacterial killing by the test extracts at their individual effect against the studied bacteria were determined by Time Kill-kinetics assay to know their bacteriostatic or bactericidal efficacy, if any. From the time kill-kinetics curve, the leaf extract was found to have bactericidal activity (reduction in colony count was ≥3 $\log_{10}$CFU/ml compared to control group at 24h) against both *L. monocytogenes* (Figure 1) and *S. typhimurium* (Figure 2) whereas other tested extracts (seed extract and fruit pulp extract) showed bacteriostatic activity (reduction in colony count was < 3 $\log_{10}$CFU/ml compared to respective controls at 24h) at their individual effect (Figure 1 and Figure 2). Possible reason behind higher antibacterial potential of leaf extract compared to other tested extracts is not clear right now. Relevant literature reveals that *Syzygium cumini* leaves are known to be very rich in gallic acid, ellagic acid and tannins [56]. Acylated flavonol glycosides, kaempferol, myricetin, and other polyphenols were also isolated from *Syzygium cumini* leaves [56, 57]. It is therefore presumed that higher antibacterial activity of leaf extract against the studied bacteria may be due to presence of high concentration of antibacterial phenolic compounds in the extract. This is because plant phenolic acids, flavonoids and tannins have been reported to have strong antimicrobial properties and since the different molecules vary in their structure and chemical composition, they can display various antimicrobial effects, such as permeabilization and destabilization of the plasma membrane or inhibition of extracellular enzymes [58, 59].

Now, combined antibacterial efficacy of test extracts against the studied bacteria was evaluated by checkerboard assay method and fractional inhibitory concentration indices (FICI) values were determined to assess the type of antibacterial interactions (additive, synergistic or antagonistic) of test extracts in combination against the studied bacteria [23]. Based on FICI values, among the possible combinations tested, only leaf extract/fruit pulp extract combination showed synergistic antibacterial interactions (FICI ≤ 0.50) against both the studied bacteria *L. monocytogenes* (FICI: 0.43) and *S. typhimurium* (FICI: 0.50) whereas other tested possible combinations showed additive antibacterial interactions against the studied bacteria (FICI ranged from 1.37 to 2.47). No antagonistic antibacterial effect (FICI > 4) of test extracts in combination was observed (Table 3).

Next, antioxidant efficacy of test extracts alone and in combination was evaluated by DPPH radical scavenging method followed by isobologram analysis to know their individual antioxidant efficacy (IC_{50}) as well as their type of antioxidant interactions (additive, synergistic, antagonistic) in combination (CI) [25]. Based on IC_{50} values, fruit pulp extract was found to be most effective antioxidant agent followed by leaf and seed extracts at their individual effects. In combination, leaf extract and fruit pulp extract showed synergistic antioxidant interactions (CI: 0.72) whereas other tested possible combinations showed additive antioxidant effects (CI:1) (Table 4). Possible reason behind higher antioxidant efficacy of fruit pulp extract at its individual effect over other tested extracts is not clear right now. It has been reported by several workers that among the plant polyphenols, flavonoids have strong antioxidant property due

to presence of a number of aromatic hydroxyl groups especially in the B ring of their molecular structure. The hydroxyls in the B ring are the first active stations in traversing the oxidation chain. The three structural groups of flavonoid compounds are responsible for determining the scavenging activity of free radicals and antioxidant activity of flavonoids. Therefore, it is noteworthy that flavonoids can exhibit different antioxidant activities according to their structure [60, 61]. Relevant literature also reveals that *S. cumini* fruit pulp is rich in anthocyanins [62, 63] and anthocyanins are a sub-group of flavonoids containing flavylium cation (HA^+) in their molecular structure in addition to a number of aromatic hydroxyl groups (-OH) [64, 65]. This peculiar structure of anthocyanins make them more strong antioxidant than other flavonoids [66]. Therefore, it is presumed that higher antioxidant activity of fruit pulp extract of *S. cumini* over other tested extracts may be due to presence of anthocyanins as major components of the extract.

It is well documented that major components of plant extracts such as phenolics and flavonoids exhibited a number of pharmacological properties including antioxidant and antimicrobial activities [67, 68]. Moreover, plant phenolics and flavonoids are potential substitutes for bioactive agents in pharmaceutical and medicinal sections to promote human health and prevent and cure different diseases [69, 70]. We, therefore, in the present investigation determined the total phenolic and flavonoid content of test extracts. The leaf extract was found to possess high concentration of both total phenolics and flavonoids followed by seed and fruit pulp extracts (Table 5). From the foregoing findings it was observed that the leaf extract exhibited highest antibacterial potential whereas fruit pulp extract showed highest antioxidant potential among the extracts tested. These findings indicated that there is no linear relationship between total phenolics and flavonoids content of tested extracts with their antibacterial and antioxidant activities. This may be due to the fact that higher polyphenolic levels do not always mean a better at microbial or antioxidant activity, but this is mostly due to the qualitative composition of the extract and the relative concentration as well as different mechanism of action of active compounds present in the extracts [5, 50, 51, 71].

The synergistic antibacterial and antioxidant interactions between leaf extract and fruit pulp extract in combination observed in the present investigation is not clear right now. In the present investigation, LC-MS/MS analysis revealed that leaf extract possesses phenolic acids and flavonoid derivatives as major components of the extract. On the other hand, in our previous study, anthocyanins were found to be the major components of fruit pulp extract [27] and these findings are in corroboration with the findings of other workers [10, 56, 57, 62, 72]. Relevant literature reveals that structurally diverse polyphenols of berries undergo interaction among themselves leading to additive, synergistic or antagonistic effects. This is because when structurally diverse polyphenolic compounds are mixed, their electrons can be delocalized from one compound to another and so can easily react with target molecules or free radicals leading to additive, synergistic or antagonistic effects [50, 73]. It is therefore presumed, that the synergistic antibacterial and antioxidant interactions of leaf extract/fruit pulp extract of *S. cumini* in combination observed in the present investigation may be due to interactions between structurally diverse major polyphenolic compounds present in the extracts.

Now, the test leaf extract and fruit pulp extract that showed synergistic antibacterial and antioxidant interactions among themselves were subjected to cytotoxicity study in combination using brine shrimp lethality assay. Brine shrimp lethality assay was used because this assay is considered to be a useful tool for preliminary assessment of toxicity. This assay has advantages of being rapid (24h), inexpensive and simple. It easily utilizes a large number of organisms for statistical validation. *Artemia* nauplii have been suggested for use as a model for several preliminary evaluation of pharmacological and ecotoxicological activities of compounds or crude plant extracts of greater complexity. The lethality of the test sample in a simple zoological organism like the brine shrimp (*Artemia salina*) has been utilized by many researchers and has proven to be a useful tool in screening various chemical compounds found in various bioactivities [74, 75]. According to Meyer *et al.* [37] crude plant extract is considered to be non-toxic, if the LC_{50} is found to be $\geq$ 1000 µg/ml. In the present investigation, test leaf extract/fruit pulp extract combination did not show any cytotoxic potential at recommended dosage level and LC_{50} of leaf extract/fruit pulp extract combination was found to be > 2000 µg/ml (Table 6) and can therefore generally be considered as a non-toxic antibacterial and antioxidant blend.

Conclusions

Thus, phenolic-rich hydro ethanol extracts of leaf, seed and fruit pulp of *Syzygium cumini* exhibited varying degrees of antibacterial and antioxidant activities of which leaf extract was found to be most effective antibacterial agent against foodborne Gram-positive and Gram-negative bacterial pathogens whereas fruit pulp extract was found to be most effective antioxidant agent at their individual effects. In combination, leaf extract/fruit pulp extract blend showed synergistic antibacterial and antioxidant interactions without any cytotoxicity at recommended dosage level whereas other tested possible combinations showed additive antibacterial and antioxidant effects. No linear relationship between total phenolics and flavonoids content of tested extracts and their antibacterial as well as antioxidant activity was observed. It is presumed that these differences in activities of the tested extracts at their individual as well as combined effects may be due to presence of structurally diverse major phenolic compounds in the extracts. The results provide evidence that phenolic-rich hydro ethanol extracts of leaf and fruit pulp of *Syzygium cumini* may serve as potential source of more potent safe and effective natural antibacterial and antioxidant blend in food and pharmaceutical industries. The foregoing findings may help the researchers in the field of study to develop more potent safe and effective antimicrobials and antioxidants as promising natural alternatives to synthetic preservatives by the interactions between different plant extracts having antimicrobial and antioxidant efficacy taking the advantages of their possible synergistic interactions. This report may serve as a footstep on these important aspects. To the best of our knowledge, this paper is the first to study comparative antibacterial and antioxidant efficacy of extracts of different plant parts of *Syzygium cumini* at their individual effect as well as resulting from interactions between the tested extracts.

Ethical considerations

This study does not contain any studies with human participants or animals performed of any of the authors.

Author Contributions

We all contributed actively to achieve success in this work.

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Competing Interest
The authors declare no conflict of interest.

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