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Original Article

IN VITRO ANTIBACTERIAL ACTIVITY OF SELECTED UNDERUTILIZED PLANTS AND CYTOTOXIC PROPERTY OF *TERMINALIA CATAPPA*

SOMARATHNA THIKSHANI[1,3,6], PREMAKUMARA GAS[2], AKBARSHA A. MOHAMMAD[3], KADALMANI BALAMUTHU[4,5,6], WEERAKKODY SEVWANDIKA NIMSHA[6*]

[1]Department of Food Science and Technology, Faculty of Graduate Studies, University of Sri Jayewardenepura, Nugegoda, Sri Lanka, [2]Industrial Technology Institutes, 396, Bauddhaloka Mw, Colombo, Sri Lanka, [3]Mahatma Gandhi-Doerenkamp Center, Bharathidasan University, Tiruchirappalli 620024, India, [4]Department of Animal Science, School of Life Sciences, Bharathidasan University, Tiruchirappalli, India, [5]UGC National Centre for Alternatives to Animal Experiments, Bharathidasan University, Tiruchirappalli, India, [6*]Department of Agricultural and Plantation Engineering, Faculty of Engineering Technology, The Open University of Sri Lanka, Nawala, Sri Lanka Email: nweer@ou.ac.lk

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ABSTRACT

Objective: This study aimed at determining the antibacterial activity of the underutilized plants of Sri Lanka, "Kottamba" (*Terminalia catappa),* "Purpurata" *(Alpinia purpurata)* and "Harankaha" *(Curcuma zedoaria),* against food-borne pathogens. Chemical composition and *in vitro* cytotxicity of the most active antibacterial plant extract(s) were examined.

Methods: Crude rhizome extracts were obtained for all plants whereas in respect of *T. catappa,* the red pericarp of the fruit was used. The antibacterial activity was determined using the agar disc diffusion and broth dilution assays. Total phenol content, Gas Chromatograph-Mass Spectrometry analysis and cytotoxicity assay were conducted only with the plant which showed the most effective antibacterial activity.

Results: *T. catappa* extract showed significantly ($p<0.05$) high DIZ (19.6±0.47 mm) against *S. aureus* 113. *A. purpurata* showed DIZ (16.3±0.94, 15.0±1.00, 14.3±0.57 mm) against *L. monocytogenes* V7 (1/2a), *S. aureus* 25925 and *S. aureus* MSSASS 25D. The MIC of *T. catappa* ethanol extract was 10 mg/ml, while MBC was 80 mg/ml for *S. aureus* 113. The phenolic content of *T. catappa* ethanol extract was 81.54±1.28 mg GAE/g dry sample and the major compound (31.86 %) was 2, 5-Furandione, 3 methyl. The No-Observed Adverse Effect Concentration (NOAEC) of this extract for COS7 cells was 100 µg/ml whereas for 3T3 it was 300 µg/ml. This indicates that the extract is cytotoxic only at a very high concentration, suggesting that at lower concentrations the extract could be used as a food preservative.

Conclusion: The results indicate that *T. catappa* has potential antibacterial activity as a safe bio-preservative.

Keywords: *Terminalia catappa*, Antibacterial activity, GC-MS, NOAEC, Cytotoxicity

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INTRODUCTION

Food-borne diseases are a global issue with significant impact on human health. The widespread use of antibiotics promotes antibacterial resistance which in turn increases the burden of health care. In the present scenario, the emergence of multiple drug resistance to human pathogenic organisms has necessitated a search for new antimicrobial substances, synthetic as well as natural. However, consumers are hesitant to use synthetic drugs in view of the side effects [1]. It is evident that over time consumers look for natural antimicrobials to prevent food-borne infectious diseases. Particularly, the antimicrobial activity of plant oils and extracts has been the basis for most of the preservatives, pharmaceuticals, alternative medicines and natural therapies [2].

About 80 % of the world population relies on herbal medicines as primary source of health care [3]. But the potential of higher plants as source of new drugs is still largely unexplored. Among the estimated 250,000-500,000 plant species, only a small percentage has been investigated from phytochemical perspectives, and the fraction submitted to biological or pharmacological screening is even smaller [4]. Therefore, several more plants should be investigated to better understand their medicinal properties, safety and efficiency.

Among the underutilized plants of Sri Lanka, *Terminalia catappa*, *Alpinia purpurata* and *Curcuma zedoaria* are used in the preparation of traditional medicines since ancient times [5]. Antibacterial activity has been reported in the fruit [6], leaf [7], bark [8] and root [9-10] of *T. catappa*, mostly the green variety. Leaf [11], fruit [12], and root [13] of *A. purpurata* have been investigated for antibacterial activity against *S. aureus*, *E. coli*, and *Salmonella* sp. However, to the best of our knowledge, there has been no report of antimicrobial activity of the *A. purpurata* varieties grown in Sri Lanka. *Curcuma zedoaria* Rosc has been reported for antimicrobial [14], antifungal [15] and other activities. Nevertheless, no antibacterial testing of *C. zedoaria* has been carried out against opportunistic pathogens such as *L. monocytogenes*.

Safety aspects of any natural chemical compound need to be investigated since otherwise it may lead to deleterious effects in man and animals. On the other hand, repeated administration of doses can produce unwanted side effects in tissues and organs. Therefore, toxicity testing is required for the most potential antibacterial active plant extracts. However, there are no pharmacological safety studies on *T. catappa*. Therefore, the objective of this study has been to find the antibacterial property of *T. catappa*, *A. purpurata* and *C. zedoaria* against selected food-borne pathogens. Further, total phenol content and cytotoxic property of the most active antibacterial extract were also determined. The novelty of the study lies in finding plants with antimicrobial property, with cytotoxicity being induced only at concentrations that are not toxic, so as to ensure efficacy as food preservative but safe for human/animal consumption, and to decipher the potentially active phyto-compound (s).

MATERIALS AND METHODS

Plant materials

T. catappa and *A. purpurata* were collected from the premises of the Open University of Sri Lanka Nawala. *Curcuma zedoaria* was

collected from the medicinal garden of the Nature Secret Pvt Ltd, Millewa, Horana, Colombo, Sri Lanka. Voucher specimens were placed in the herbarium of the Department of Agricultural Plantation Engineering, The Open University of Sri Lanka.

Chemicals and reagents

n-Hexane, Trypton Soya Broth (TSB), Trypton Soya Agar (TSA), Mueller-Hinton Agar (MHA), Violet Red Bile Glucose Agar (VRBG), Xylose Lysine Desoxycholate Agar (XLD), Dulbecco's modified Eagle's Medium (DMEM), Tween 20, Dimethyl sulphoxide (DMSO), Folin Ciocalteu Reagent, Sodium Carbonate and Gallic Acid were obtained from Sigma-Aldrich Chemical Co., (St. Louis, MO, USA). Phosphate-Buffered Saline (PBS) and 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) were purchased from HiMedia Laboratories (Mumbai, India). Fetal Bovine Serum (FBS), Trypsine-EDTA, Penicillin and Streptomycin were obtained from Invitrogen (USA). All other chemicals and reagents were of analytical grade.

Preparation of extracts

The rhizomes of *A. purpurata* and *C. zedoaria,* and red pericarp of *T. catappa* were used for extraction. Fresh rhizomes and fruits, respectively, were cleaned by washing in running tap water. The rhizomes were peeled off and cleaned. The rhizomes and fruit pericarp were sliced and oven-dried at 40 °C for 24 h. The slices were then powdered to fine particles using a grinder (National Super Blender, Taiwan, Model MX-TIIOPN) for 5 min at adequate intervals. The powders were stored at- 20 °C until use.

N-Hexane and ethanol were used as solvents for extraction. The extract was prepared by adding 10 g of each powder to 100 ml of n-hexane or ethanol and agitated for 24 h at 30 °C in a rotary shaker (Stuart® Orbital Shaker SSL1, UK). The mixture was filtered using Whatman No 1 filter paper under vacuum and the filtrate was evaporated under vacuum at 40 °C using a rotary evaporator (KIA RV 5, Switzerland). The concentrated extract was filter-sterilized through a 0.45 µm filter units (Millex® HA, Germany). The filtrate was subjected to N_2 flux under heat (40 °C) for 3 h until a hexane-free extract was obtained. Finally, the concentrated extract was re-dissolved in DMSO to make a 0.5 g/ml stock solution and stored at 4 °C until use.

Test micro-organisms and cell culture

The antibacterial activity of the extract was determined by using food-borne bacteria. *S. aureus* 113, *S. aureus* MSSA SS 25D, *S. aureus* MSSA SS 21D, *Listeria monocytogenes* Scott A (4b), *L. monocytogenes* V7 (1/2a), and *L. monocytogenes* EDG were obtained from University of Queensland, Brisbane, Australia. *S. aureus* ATCC 29213, *S. aureus* ATCC 49476, *S. aureus* ATCC 25925, *L. monocytogenes* ATCC 7644, *Escherichia coli* (*E. coli*) ATCC 1858 and *Salmonella typhimurium* (*S. typhimurium)* ATCC 14028 were obtained from American Type Culture Collection (Manassas, USA). The bacterial strains were confirmed using Gram staining method. Baird Parker Agar with egg yolk tellurite emulsion was used for the identification of *S. aureus s*trains. *E. coli* and *S. typhimurium* were identified using VRBG agar medium and XLD agar medium, respectively. All bacterial strains were stored in 80 % glycerol at- 80°C as frozen stock cultures. Working cultures were grown and maintained in Trypton Soy Agar (TSA).

Monkey kidney (COS7) and mouse fibroblast (3T3) cells were obtained from National Center for Cell Science (NCCS), Pune, India. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, and with 20 ml each of penicillin and streptomycin as antibiotics, and 1.2 g Na_2HCO_3 was added to it, in a humidified atmosphere of 5% CO_2 and 95% air, in a CO_2 incubator (Thermo Scientific, USA).

Disk diffusion assay

Antibacterial activities of the extracts were evaluated using slightly modified disk diffusion method described previously by Barry [16]. A single colony of bacteria was grown in 2 ml Trypton Soy Broth (TSB) at 37 °C for 18 h. The content was centrifuged (Centurion Scientific Ltd, UK) at 10000 x g for 10 min to obtain the bacterial pellet. The supernatant was removed and the bacterial pellet was re-suspended in 1 ml sterile 0.85 % NaCl. The preparation was pipetted to 9 ml of sterile 0.85 % NaCl solution to obtain 5 x 10^5CFU/ml. Then, 100 µl of dilute bacterial suspension was spread on Mueller Hinton Agar (MHA) plates. An aliquot (10 µl) of the plant extract was pipetted on to a 5.5 mm diameter sterile paper disc (Whatman No1) on the agar surface. Streptomycin (100 µg/ml) served as the positive control while DMSO-containing disc served as the negative control. The plates were then inverted and incubated at 37 °C for 18 h. Microbial inhibition was determined, in triplicate, by measuring the Diameter of inhibition around each disc and recorded as DIZ in millimeter.

Broth dilution assay

The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration *(*MBC*)*, were determined against *S. aureus* 113 and *S. aureus* ATCC 29213 with some modifications of the method described by Hennekinne *et al.* [17] with the plant showing the strongest antibacterial activity for which quantitative two-fold serial dilutions of extracts were performed. Thus, two-fold serial dilution of *T. catappa* ethanol extract was made with Mueller Hinton Broth (MHB). After 160 µl of *T. catappa* extract was added to the first tube containing 2 ml of MHB, the tube was kept in a 50 °C water bath for 5 min for solublizing the content, and two-fold serial dilution was made up to the seventh tube. Since solubility of the extract was low, one drop of Tween 20 (4.6 mg) was added to solublize the extract. An aliquot of 10 µl (5 x 10^5CFU/ml) of the test organism was added to each test tube.

Streptomycin (100 µg/ml), MHB containing the microorganism and media only were used as positive-, negative-and media control. The tubes were incubated, without shaking, at 37 °C. The tubes were visually examined for the lowest concentration of extract which showed clear solution after 24 h and 48 h. The concentration in the lowest serial dilution of the extract at which growth did not occur on broth was recorded as MIC. All the tubes that did not show any turbidity were used to take 100 µl of each suspension and spread on the surface of TSA plates. Then the plates were incubated at 37 °C, for 24 h and 48 h. The lowest extract concentration at which no bacterial growth was observed on TSA plate was taken as MBC. All assays were performed independently three times each in triplicates. The same procedure was followed for *A. purpurata* ethanol extract in quantification of MIC and MBC for *L. monocytogenes* Scott A and *E. coli* as these bacteria responded with a strong antibacterial activity in the disk diffusion assay.

Total phenol content

The total phenol content of the extract of *T. catappa* was determined using Folin-Ciocalteu colorimetric method adopted from Shan *et al.* [18] with some modifications. An aliquot of 100 µl of an appropriate dilution of the extract was oxidized for 4 min with 0.5 ml of Folin-Ciocalteu reagent. The reaction was neutralized with 0.5 ml of sodium carbonate (75 g/l). After two hour incubation at 26 °C the absorbance of the color was measured at 760 nm in a spectrometer (6300, Jenway, UK). Gallic acid was used as the standard to plot a curve where concentrations of 0, 1, 5, 10, 25 75, 100, 150 mg/ml were prepared using deionized (DI) water. A standard curve was plotted using the absorbance obtained using the procedure described previously. Absorbance of the extract was determined using a standard curve of Gallic acid and the results are given in milligram of Gallic Acid Equivalent (GAE) per gram of dry sample. All tests were performed independently three times each in triplicates.

Chemical composition of the plant

T. catappa crude ethanol extract was analyzed by GC-MS using an Agilent make model 6890 gas chromatograph with a HP-5 MS column and an Agilent 5973 mass selective detector. The temperature was first held at 50 °C for 2 min and then raised gradually to 250 °C at 10 °C/min interval and held at 250 °C for 8 min. The carrier gas was helium at a flow rate of 0.9 ml/min and injection volume was 1 µl. The identities of the main component peaks were confirmed by comparison of their retention time with those of reference compounds using Wiley W9N08 data base library. The percentage of each compound was calculated as the ratio of the peak area to the total chromatographic area.

MTT assay for assessment of cell viability

Cell viability, which would reveal the cytotoxic property of the plant extract, was evaluated using the MTT colorimetric assay with some modifications [19]. Cells were seeded in 96-well plates at 5 x 10^3cells/well and incubated for 24 h at 37 °C. The cells were treated with the extract at increasing concentrations within 0-1000 µg/ml for 24 h, at 37 °C. Dilution of stock solutions was made in the culture medium yielding final extract concentrations in the DMSO concentration of 0.1 %. This concentration of DMSO did not affect the cell viability [20]. DMSO was used as the solvent control. Control cells were incubated in culture medium only. Experiments with each extract concentration were conducted in triplicates on the same batch of cells. After 24 h incubation, 20 ml of MTT solution (5 mg/ml in PBS) was added to each well, and incubated for 3 h at 37 °C. The medium was then removed and 100 ml of DMSO was added to each well to dissolve the purple formazan product. The absorbance was measured at 570 nm using a 96-well plate reader (Bio-Rad, Hercules, CA, USA). Data were collected for triplicates independently three times and used to calculate the respective means and standard deviations. The percentage inhibition was calculated from this data using the following formula, and the IC_{50} was calculated. The IC_{50} is defined as concentration of the test substance at which cell viability is decreased to 50 %.

$$\text{Percentage of cell Inhibition} = \frac{\text{Mean OD (control)-Mean OD(treatment)}}{\text{Mean OD (control)}} \times 100$$

Morphological assessment of cell death using AO/EB fluorescent assay

The morphological characteristics of cells indicating apoptosis and necrosis were assessed by AO/EB staining [21]. The cells were cultured in 6-well plates and treated with IC_{50} and no toxicity concentrations (130 µg/ml, 300 µg/ml, respectively) of *T. catappa* extracts for 24 h against COS7 and 3T3 cells, respectively. The treated and untreated cells were centrifuged (3000 rpm for 4 min) and incubated with AO and EB solutions (1 part of 100 mg/ml each of AO and EB in PBS) and observed in a fluorescent microscope (Carl Zeiss, Jena, Germany) using a UV filter (450-490 nm).

Three hundred cells per sample were counted, in duplicate, and scored as viable or dead, and if dead whether by apoptosis or necrosis as judged from nuclear morphology and cytoplasmic organization. Then, percentages of apoptotic and necrotic cells were calculated. The appropriate morphological features were photo-graphed.

Assessment of nuclear features using hoechst 33528 staining

The nuclear morphological features of the cells were assessed using Hoechst 33528 staining [22]. The cells were cultured in 6-well plates and treated with *T. catappa* extracts alone, at their respective IC_{50} concentrations and no toxicity concentration, for 24 h. After incubation, the treated and control cells were harvested and stained with Hoechst 33258 (1 mg/ml in PBS) for 5 min at room temperature. A drop of cell suspension was placed on a glass slide, and covered with a cover slip. At random 300 cells, in triplicate, were observed at x400 in the fluorescent microscope fitted with a 377-355 nm filter. Then, the percentage of cells reflecting pathological changes was calculated.

Statistical analysis

The triplicate data were subjected to analysis of variance of the general linear model using SPSS statistical software, at 5% significance level. Means were compared using Turkey's simultaneous test set at $P<0.05$.

RESULTS

Antibacterial activities of the extracts

The mean DIZ of *A. purpurata*, *T. catappa* and *C. zedoaria,* measured in disk diffusion assay, are shown in the table 1. A significant ($p<0.05$) variation was found in antibacterial activity between n-hexane and ethanol extracts for each of the plants tested. The ethanol extract of *T. catappa* showed significantly ($p<0.05$) higher overall DIZ, 19.6±0.47 and 19.3±0.47 mm, against *S. aureus* 113 and *S. aureus* ATCC 29213, respectively, but not hexane extract. Further, *T. catappa* ethanol extract showed significantly ($p<0.05$) larger DIZ against all *S. aureus* strains tested except *S. aureus MSSA SS 21D*. *A. purpurata* extract produced a significantly ($p<0.05$) larger DIZ with *L. monocytogenes V7 (1/2a)* irrespective of the solvent but not so when tested against *S. aureus* 113. However, no significant result in DIZ was obtained with hexane extract of *A. purpurata* against *S. aureus* 25925 and *S. aureus* ATCC 49476. *A. purpurata* ethanol extract produced significant ($p<0.05$) inhibition (9.3±0.57 mm) against *E. coli*. Similarly, *C. zedoaria* produced significant ($p<0.05$) inhibition (8.6±0.57 mm) of *S. typhimrium* ATCC 14028. The disk diffusion assay for *C. zedoaria* hexane extract showed higher inhibition of 11.6±0.57 and 12.0±0.00 mm against *S. aureus* 113 and *S aureus* MSSA SS 21D, respectively. No significant ($p>0.05$) difference in DIZ between *A. purpurata* ethanol, and *T. catappa* and *C. zedoaria* hexane extracts was observed against *E. coli and S. typhimurium.* These values were always smaller than or equal to the DIZ obtained with the Gram–positive bacteria.

Table 1: Diameter of inhibition zone (mm) of the plant extracts

Micro-organism	*A. purpurata*		*T. catappa*		*C. zedoaria*	
	Hexane	Ethanol	Hexane	Ethanol	Hexane	Ethanol
SA 25925	15.0±1.00^{cdef}	11.3±0.57^{ijk}	6.0±0.00^{w}	17.6±0.47^{bc}	10.0±0.00^{mn}	9.0±0.00^{pq}
SA MSSA SS 25D	14.3±0.57^{defg}	9.3±0.57 ^{op}	6.0±0.00^{w}	19.0±0.81^{ab}	9.0±0.00^{pq}	7.0±0.00^{uv}
SA ATCC29213	14.3±0.57^{defg}	10.6±0.57^{klm}	6.0±0.00^{w}	19.3±0.47^{a}	12.0±0.00^{ghi}	7.0±0.00^{uv}
SA MSSA SS 21D	14.0±0.00^{efgh}	10.3±0.57^{lm}	9.6±0.00$^{n\ op}$	11.6±0.57^{hij}	11.3±0.57^{ijk}	9.0±0.00^{pq}
SA ATCC 49476	15.0±1.00^{cdef}	11.3±0.57^{ijk}	6.0±0.00^{w}	18.6±0.94^{ab}	9.3±0.57 ^{op}	10.0±0.00^{mn}
*SA*113	13.0±1.73^{fgh}	12.3±0.57^{ghi}	6.0±0.00^{w}	19.6±0.47^{a}	11.6±0.57^{hij}	10.0±0.00^{mn}
LM Scott A	14.0±0.64^{efgh}	13.6±0.57^{fgh}	6.0±0.00^{w}	12.6±0.57^{ghi}	7.3±0.57^{tu}	8.6±0.57^{qr}
LM V7 (1/2a)	16.3±0.94^{bcd}	15.6±0.57^{cde}	6.0±0.00^{w}	13.3±0.57^{fgh}	7.0±0.00^{uv}	6.0±0.00^{w}
LM EGD	14.3±0.57^{defg}	11.3±0.57^{ijk}	6.6±0.57^{vw}	11.0±0.00^{jkl}	10.3±0.57^{lm}	8.0±0.00^{rs}
LM ATCC7644	13.0±1.73^{fgh}	11.0±0.00^{jkl}	6.0±0.00^{w}	12.6±0.57^{ghi}	10.0±0.00^{mn}	9.0±0.00^{pq}
E. coli	7.6±2.88st	9.3±0.57 ^{op}	6.6±0.57^{vw}	6.0±0.00^{w}	6.0±0.00^{w}	6.0±0.00^{w}
S. typhimrium	7.6±2.88st	6.6±0.00^{vw}	6.0±0.00^{w}	6.0±0.00^{w}	6.0±0.00^{w}	8.6±0.57^{qr}
Streptomycin	17.0	18.0	18.0	5.5	18.0	18.0
DMSO	6.0	6.0	6.0	6.0	6.0	6.0

SA = *S. aureus*, LM= *L. monocytogenes.**Means inhibition (mm)±S. D of three replicates with different lowercase letters is significantly (P<0.05) different.

Broth dilution assay

The MIC and MBC values obtained at 24 and 48 h using broth dilution assay for the ethanol extract of *T. catappa* and *A. purpurata* are presented in table 2. The MIC value of *T. catappa* extract against S. aureus 113 strain was 10 mg/ml at 24 h whereas MBC was found to be 80 mg/ml irrespective of the strain, and the value did not change at 48 h. The MIC for *A. purpurata* hexane extract was 5 mg/ml and 10 mg/ml against *L. monocytogenes V7* and *E. coli*, respectively.

Table 2: The MIC and MBC values of the plant extracts against food-borne pathogens

Plant	Name of the organism	Concentration (mg/ml) of extract			
		MIC		MBC	
		24 h	48 h	24 h	48 h
T. catappa	*S. aureus*113	10	10	80	80
(ethanol)	*S. aureus* ATCC29213	10	10	80	80
A. purpurata	*L. monocytogenes* V7 (1/2a)	5	5	20	20
(n-hexane)	*E. coli*	10	10	>20	>20

MIC of *T. catappa* indicates activity within the range of the concentrations tested (0.625-80 mg/ml), and *A. purpurata* within the range of the concentration tested (1.25–20 mg/ml).

Total phenol content (TPC)

The antibacterial activity of plant extracts might be attributed to the presence of bioactive plant compounds. TPC of *T. catappa* was found to be 81.54±1.28 mg/GAE/g in ethanol extract.

GC-MS analysis

The GC-MS data are presented in fig. 1. The compounds identified in the spectrum are presented in table 3. with only chemical compounds greater than 1 % are shown. The *T. catappa* ethanol extract contained 13 compounds at >1 %, accounting for 97.9 % of the total composition of the extract. The major chemical compound, the one obtained at the highest concentration in ethanol extract, was 2, 5-furandione, 3-methyl as identified from Wiley W9N08 data base library (31.86 %). The second most abundant compound was 2-fural carboxaldehyde (29.33 %). The other compounds such as furane (15.66 %) and cyclophentanol (4.51 %) were also identified in the ethanol extract.

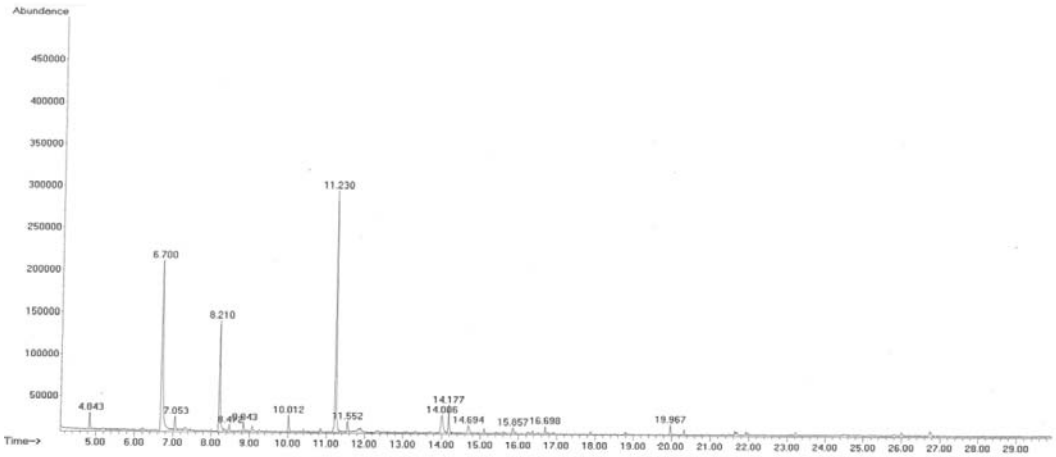

Fig. 1: GC-MS chromatogram of *T. catappa* ethanol extract

Table 3: GC-MS data of *T. catappa* ethanol extract

Peak*	Chemical compound	Percentage
4.84	2-Furancarboxaldehyde	2.18
6.70	2,5-Furandione	31.86
7.05	2-Furalcarboxyadehyde,, 3-methyl	1.96
8.21	Furan	15.60
8.843	4,5-Diamino-2-hydroxypyrimidine	1.23
10.01	4H-pyran-4-one	2.24
11.23	2-Furalcarboxyadlehyde	29.33
11.55	3-hydroxythiophenol 4-mercaptophenol	1.46
14.00	Cyclophentanol	4.51
14.17	L-Glutamic acid 5-ethyle ester	3.49
14.69	D-allose	1.79
15.85	Methyl. beta-d-ribofuranoside	1.02
19.96	Hexadeconoic acid	1.27
	Total	97.90

*peak time in minutes

Cyto-toxic activity of crude extracts of *T. catappa*

Cytotoxic activity of *T. catappa* ethanol extract was carried out against COS7 and 3T3 cells at different concentrations to determine the respective IC_{50} by MTT assay. The results of different concentrations of *T. catappa* extract treatment at 0 to 1000 µg/ml are graphically represented in fig. 2 and 3. IC_{50} values of *T. catappa* ethanol extract for COS7 and 3T3 were found to be 375 µg/ml and 750 µg/ml, respectively. It was found that the ethanolic extract showed 0 % inhibition of cell at the concentration 130 µg/ml and 300 µg/ml, for COS7 and 3T3, respectively. Therefore, 130 µg/ml and 300µg/ml concentration against COS7 and 3T3, respectively, are considered as the non-toxic concentrations.

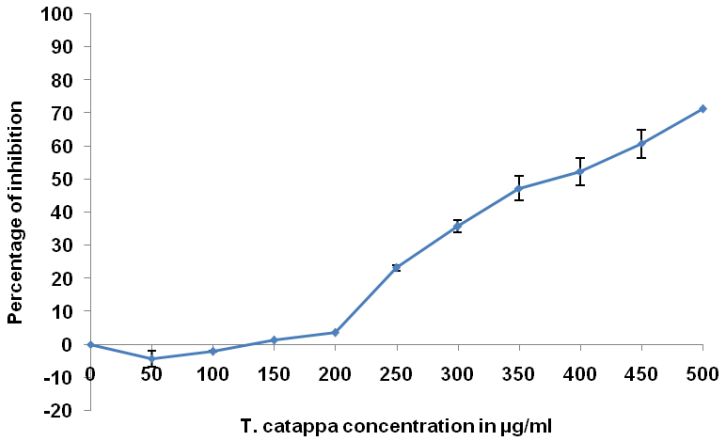

Fig. 2: Cytotoxicity of *T. catappa* crude extract against COS7 cell line (µg/ml). Values represent the mean±SD of the three determinants

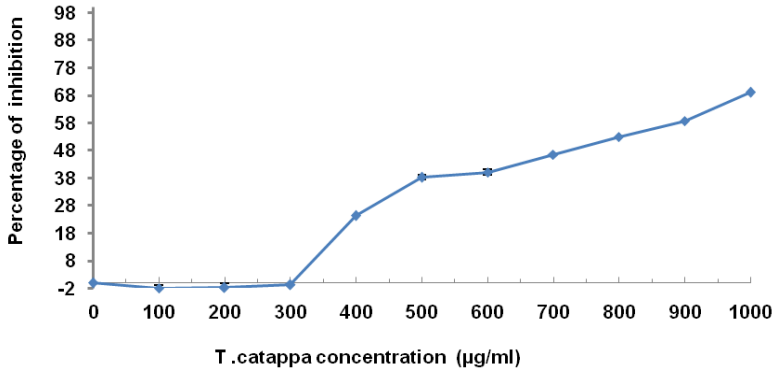

Fig. 3: Cytotoxicity of *T. catappa* crude extract against 3T3 cell line (µg/ml). Values represent the mean±SD of the three determinants

Microscopic features of apoptosis and/or necrosis were observed adopting AO/EB staining. In this test dead cells are permeable to EB and fluoresce orange-red, whereas live cells are permeable to AO only and, therefore, fluoresce green. The viability and membrane integrity of the cells were determined based on the fluorescence pattern. The morphological changes observed in the treated cells were classified based on the fluorescence emission as follows: i) viable cells having highly organized nuclei fluoresced green; ii) early apoptotic cells which showed nuclear condensation fluoresced orange green; iii) late apoptotic cells with the chromatin highly condensed or chromatin fragmented fluoresced orange to red; and iv) necrotic cells fluoresced orange to red without chromatin fragmentation. Data on cells indicating apoptotic and necrotic morphologies (fig. 4), induced on treatment with the IC_{50} concentration of *T. catappa* for 24 h, and collected from manual counting, are presented in fig. 5, which reveal that the *T catappa* IC_{50} is highly efficient in bringing about early apoptosis but little necrosis was produced. However, 130 and 300 µg/ml *T. catappa* (no toxicity concentration) did not induce apoptosis in both cell lines.

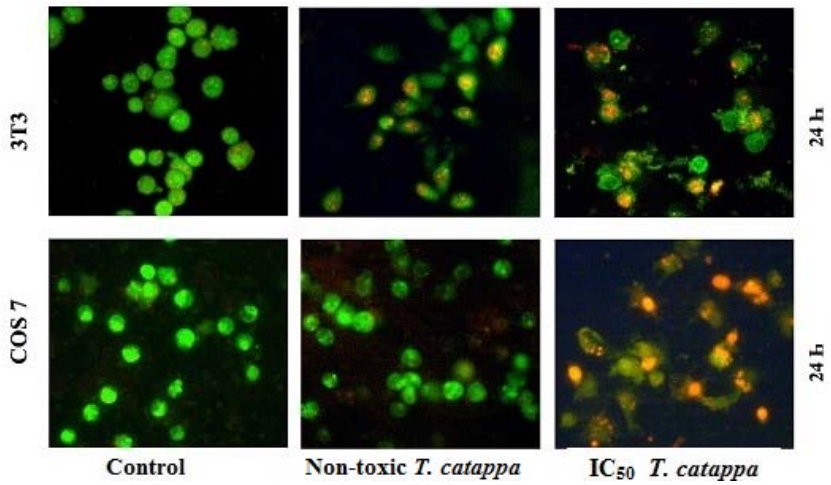

Fig. 4: Morphological changes observed in control and *T. catappa*-treated (24 h) 3T3 and COS7 cells stained with acridine orange and ethidium bromide

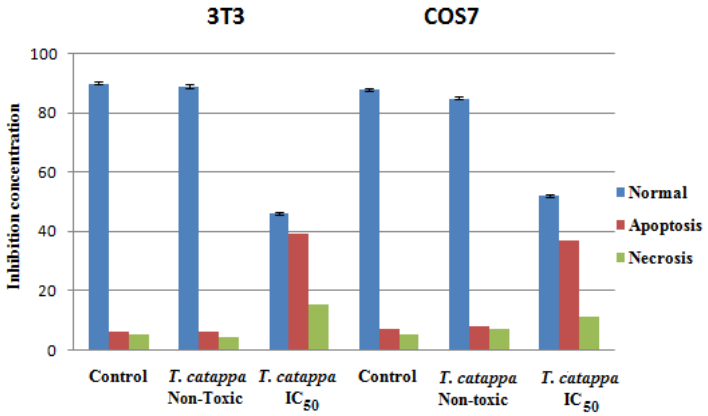

Fig. 5: Percentage of normal, apoptotic and necrotic cells. Data are expressed as mean±SD of three independent experiments. P<0.05 compared to control

Hoechst 33528 staining was adopted to find the morphological changes in the nucleus as caused by treatment of IC_{50} concentration and non-toxicity concentration of *T. catappa* (fig. 6). In control cells the nuclear chromatin was in full while after treatment with the IC_{50} of *T. catappa* extract for 24 h, changes such as chromatin marginalization, condensation and fragmentation were noticed. These observations revealed that exposure of *T catappa* IC_{50} concentration, led to chromatin fragmentation which is a characteristic feature of apoptosis. However, treatment with no toxicity concentration indicated a fairly good percentage of normal cells. Data collected from manual counting of cells with normal and abnormal nuclear features are shown in fig. 7.

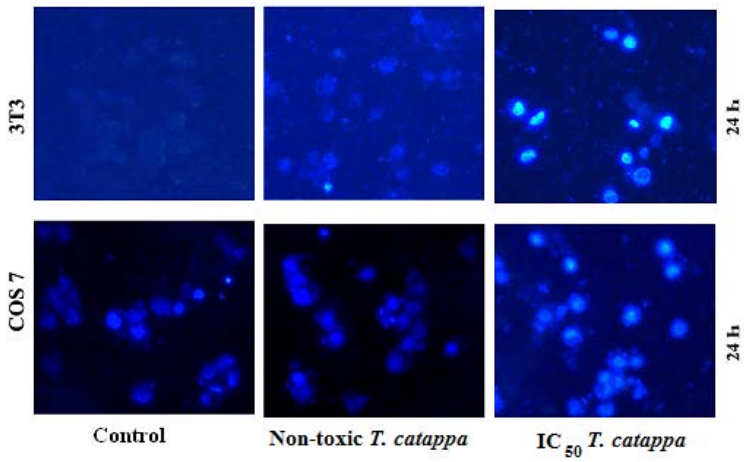

Fig. 6: Morphological features of nuclei observed for control and extract-treated cells stained with Hoechst 33258

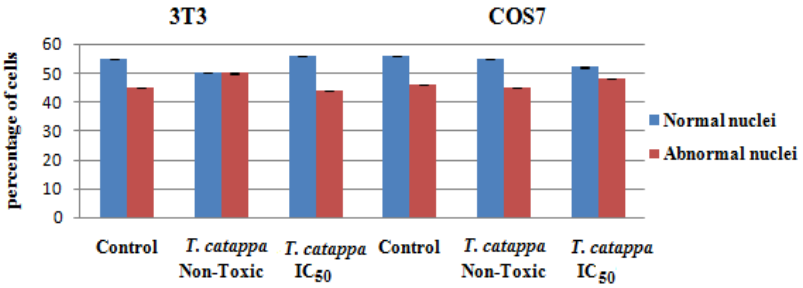

Fig. 7: Percentage of cells with normal and abnormal nuclei. Data expressed as mean±SD of three independent experiments. P<0.05 compared to control

DISCUSSION

The ethanol extract of *T. catappa* showed significantly higher DIZ against *S. aureus* 113 and, *S. aureus* ATCC 29213, respectively, than the other plants/extracts. According to Krishnaveni *et al.* [23] DIZ of *T. catappa* undiluted crude ethanol leaf extract was 7.0 mm against *S. aureus* which is a low DIZ value compared to our data. Similarly, 9 mm of DIZ was reported for *E. coli* whereas in our study it was 6.0 mm. According to Neelavathi *et al.* [24] *S. aureus* and *E. coli* DIZ were 17.00 and 13.00 mm, respectively, for 300 µg extract which is comparable to our results.

Further, there has been no study for antimicrobial activity of *T. catappa* against *L. monocytogenes* and *S. typhimurium* [25].

T. catappa root methanol extract exhibited MIC of 0.065 mg/ml against *E. coli* and the chloroform extract exhibited MIC of 0.4 mg/ml against *S. aureus* [26]. Rajarajan *et al.* [27] reported that fruit pulp ethanol extract showed MIC of 0.5 mg/ml against *Actinobacilus*. Further, Yazdi [6] reported that *T. catappa* leaf methanol crude extract showed MIC of 20 mg/ml against *E. coli* whereas in our study the MIC values were 10 mg/ml and 5 mg/ml against *S. aureus* 113 and *L. monocytogenes* for *T. catappa* pericarp hexane extract. Obawemi *et al.* [28] reported the MIC for *S. aureus* as 1.25 mg/ml which does not agree with our data. These differences may be due to the differences in concentrations of the crude extracts, plant parts, strains and solvent type used for the extraction. Further, for *A. purpurata* ethanol extract the MIC was<10 µg/ml against *S. aureus* and 1 mg/ml as MIC for *E. coli* [29]. It was reported that *A. purpurata* leaf oil extract was active against *S. aureus* strains at MIC 10 g/ml. For Gram-negatives it was more than 1000 g/ml [30]. However, in our study the MIC was 5 mg/ml and 1 mg/ml for *L. monocytogenes* and *E. coli*, respectively.

The medicinal plant extracts used tended to precipitate once added to water-based broth media, whereas the emulsifying agent Tween 20 did not reduce the opaqueness. As a result, visual observation of the end point reading lacked precision [31-32]. Therefore, we practiced determination of MBC for each plant extract against the bacteria tested even though it was tedious. MBC values for all tested strains of *S. aureus* were found to be 80 mg/ml for *T. catappa* whereas for *L. monocytogenes* it was 20 mg/ml for *A. purpurata* ethanol extract. For *T. catappa* young leaves, the MBC was effective at 100-145 mg/ml [7].

We observed varying degrees of sensitivity of bacteria to the plant extracts tested. Generally, the trend in sensitivity was *S. aureus* 113>*L. monocytogenes*>*E. coli*. This trend in sensitivity is consistent with other research findings [11] since the *S. aureus* cell wall is permeable to antimicrobial agent [33]. Further, as observed, the MIC values for these extracts were lower than the MBC values for the tested bacteria which were in agreement with other studies [7]. However, we found that MBC values were not always two times the MIC [11].

Abdulkadir *et al.* [34] showed that TPC content of *T. catappa* leaf methanol extract was 285.70 mg GAE/g whereas for fruit extract it was 117.01 mg GAE/g. Marques *et al.* [35] reported the TPC of fruit as 142.84±2.09 GAE/g. The possible explanation for these differences may be due to the differences in plant material used, since plant leaf contain more phenolic compounds than fruit pericarp, and the differences in the solvent used in extraction. Since phenol compounds are efficiently extracted in polar organic solvents than non-polar solvents, the former showed higher antibacterial activity than the hexane extract of *T. catappa*. Therefore, we can speculate that the strong antimicrobial activity of *T. catappa* is due to the phenol compounds.

GC-MS analysis of the study demonstrated that the major chemical compound of *T. catappa* ethanol extract was 31.86 % of 2, 5-Furandione, 3 methyl. The second compound identified was 2-Furalcarboxaldehyde. According to Krishnaveni *et al.* [36, 37], Propane 1, 1-diethoxy content was 48.58 % in nut and 48.69 % in stem. In contrast, Marques *et al.* [35] reported Z-pytol as the major compound at 42.1 % in leaves and ethane 1,1-diethoxy 24.1% in bark [38]. However, no researcher has yet identified the chemical composition of fruit of *T. catappa*. These observations confirm that the plant part and extraction method have a great implication on the chemical compounds that would be obtained.

To find the cytotoxic effect of *T. catappa* fruit extract two mammalian cell lines were used. Since it is known that different cell lines might exhibit different sensitivities towards a cytotoxic compound, the use of more than one cell line is considered necessary in the detection of cytotoxic compounds [39]. Bearing this in mind, two different human cell lines were used in the present study. *T. catappa* showed high IC_{50} value against COS7 and 3T3, 375 and 750 µg/ml, respectively. However, so far no toxicity data has been reported in literature for *T. catappa* fruits. Selectivity Index (SI) calculated as SI = ratio of toxicity to activity = IC_{50}/MIC. For *T. catappa* SI= 300/10 = 30 for COS7, and SI = 130/10 = 13 for 3T3. Higher SI value indicates more selectivity for bacteria and less toxicity for cells. Therefore, the ethanolic extract of *T. catappa* showed less or no toxicity to human cells.

The morphological assessments were carried out to visualize the toxicity effect of the plant extracts on particular cell lines. Less than 10 % cell apoptosis was observed at 130 µg/ml and 300 µg/ml of *T. catappa* whereas treatment of the higher dose of *T. catappa* resulted in increase in the number of cells in apoptosis. However, the effects of these concentrations on AO/AB were not significantly different with the control values. Therefore, the results suggest that extracts of this particular plant may not induce apoptosis at these concentrations. Further, significant difference was not observed with the normal to abnormal nuclei in control treatment with no toxicity concentration in Hoechst staining. On the other hand, NOAEC (no-observed adverse effect concentration) is defined as the concentration at which there is no cytotoxicity [40]. Therefore, NOAEC for COS7 cells was 130 µg/ml whereas for 3T3 it was 300 µg/ml. However, these findings need to be confirmed using different human cell lines. Also, bioavailability of the extract needs to be evaluated in order to identify minimum/tolerant concentration of *T. catappa* to avoid health risks in the human/animals.

CONCLUSION

The present study aimed at identifying the antibacterial activity of the selected underutilized Sri Lankan plants against food-borne bacteria. MIC values for ethanol extracts of *T. catappa* and A. purpurata were ≤ 10 mg/ml for the tested bacteria. MBC of *T. catappa* was 80 mg/ml for *S. aureus* 113 while 20 mg/ml for *L. monocytogenes* V7. The major chemical compound of the ethanol extract of *T. catappa* was 2, 5-Furandione, 3 methyl (31.86 %). The IC50 of *T. catappa* against COS7 and 3T3 at were 375 and 750 µg/ml, respectively, which are indeed very high values, much above those used in food preservation. However, the respective non-toxic concentrations of *T. catappa* revealed that they were not mediating apoptotic cell death. In conclusion, *T. catappa* pericarp is a potential source of bio-preservative but additional research and clinical trials are needed for the product development to strengthen the use of *T. catappa*.

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AUTHOR CONTRIBUTION

Experimental part of the work and writing of the manuscript were done by the first author, TS, as part of her PhD study. Dr. G. A. S. Premakumara conducted the GC-MS analysis and interpreted the data. Cytotoxicity analysis was designed and interpreted by Dr. Mohammad A. Akbarsha; he also corrected the MS critically. Dr. Balamuthu Kadalmani executed the cytotoxicity assay and trained TS in animal cell culture. Nimsha S. Weerakkody, as the Ph. D. supervisor played crucial role in designing the study, methodology development and preliminary correction of the manuscript.

CONFLICT OF INTERESTS

Authors declare no conflict of interest

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