OPEN ACCESS

Online ISSN:2353-0391

Algerian Journal of Natural Products
www.univ-bejaia.dz/ajnp

Type of the Paper (Article)

Antibacterial Activity Of Essential Oil Of *Syzygium cumini* Leaf Against Pathogenic And Spoilage Bacteria Isolated From Cheese

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Received: 2020-03-13 /Accepted: 2020-06-28

Abstract: local cheese has been reported to be a high risk food in Nigeria due to the possible transmission of numerous spoilage and pathogenic bacteria. This study therefore investigated not only the spoilage and pathogenic bacteria associated with local cheese sold within Ilorin metropolis but also microbiological food safety of the food through the use of Syzygium cumini oil as a preservative. Samples of wara were collected from selected seller across the metropolis and analysed for the presence of different bacterial species using spread plate method on appropriate selective growth media. All the bacterial isolates were identified using biochemical test. Invitro therapeutic efficacy of S.cumini oil aginst foodborne isolates was carried out using Kirby-Bauer method. The most six prevalent bacterial isolates were Bacillus subtilis, Proteus vulgaris, Escherichia coli, Listeria monocytogene, Salmonella typhimurium and Staphylococcus aureus were isolated from the wara. Hydrodistilled leaves of Syzygium cumini yielded 0.32% (v/w) of essential oil. Examination of the oil by GC and GC/MS uncovered that the greater part of the oil was comprised by sesquiterpenes. Other outstanding constituents were Cis-Beta-Farnesene (1.40%), Beta-Ocimene (2.11%), Beta-bisabolol (1.48%), Beta-Ocimene (2.11%), and D-Limonene (2.89%). Antibacterial action was measured base on the zone of inhibition and minimum inhibitory concentration. The oil repressed the development of Proteus vulgaris at 25% concentration, Escherichia coli at 12.5% concentration, Listeria monocytogene at 6.25% concentration, Salmonella typhimurium at 12.5% concentration and Staphylococcus aureusat 6.25% concentration while no action was recorded against B. subtilis. Highest activity, represented by diameter of zone of clearance around the loaded wells was recorded for Staphylococcus aureus. Accordingly, the oil can be an elective method for averting spoilage in stored wara in this manner expanding its time span of edibility.

Keywords: *Syzygium cumini,* wara, *Antibacterial Activity, Essential Oil, P*athogenic, *spoilage Bacteria*

I. Introduction

Syzygium cumini L. Skeels have a place with *Myrtaceae* family, they are usually known as Black Plum in English, Jaman inUrdu, Jamun in Hindi, Jambu in Sanskrit. *S. cumini* is a huge evergreen tree indigenous to India, however they are found all through the Asian subcontinent, South America, Eastern Africa, Madagascar, etc (Li *et al*., 2009). The fruit pulp contains K, Ca, Na, P, Fe, Mn, Zn, Vitamin A and C, nicotinic corrosive, riboflavin, folic corrosive, maleic corrosive, sugar, and amino corrosive (Ayyanar *et al*., 2012). Different pieces of the tree have been utilized customarily for the treatment of different human infirmities. According to Ayurveda, the barks of S.cumini have bitter, digestive, astringent and wound mending properties. They are utilized for the treatment of biliousness, diarrhea, sore throat, thirst, bronchitis, asthma and ulcers. Unani System of medication portrays it to be a tonic for liver, to enhance blood, fortify teeth and gums and as indicated by Siddha arrangement of medication, it is semen advancing, haematinic and hypothermic (Balinga *et al*., 2007).

Essential oils are natural, complex compounds, volatile liquid, which have a very tough odor, they are soluble in lipid and organic solvents, and they are not often colored. They could be

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synthesized by all plant organs and are stored in epidermic cells or glandular trichomes secretary cells, canals, cavities, (Bozin *et al*., 2006).The study have shown that *S.cumini* contains larger volume of essential oil than other components present in traceable quantities. Pharmacological and therapeutic values of the essential oil can be attributed to the two major component of the essential oil essentially against some pathogenic microbes (Pichersky *et al*., 2006).

Wara (Cheese) is a milk derivatives, it is the curd produced by the coagulation of milk of certain mammals by rennet or related enzymes in the company of lactic acid. It is produced by adventitiousmicroorganisms(microorganisms coming from another source and not inherent or innate) (Raheem *et al*., 2009). Wara is an unripened cheese eaten in several parts of West Africa; coagulating fresh cow's milk with a leaf extract of Sodom apple (*Calotropis procera*) makes the cheese. A method involved in cheese processing requires obsolete equipment, usually neither theuse of starter culture nor the consideration of processing conditions such as standardization and optimixation, although very recently an alternative"lemon juice"which serves as coagulant and antimicrobial agent was introduced in the production of soft cheese (Adetunji *et al*., 2007). 'Wara' is frequently eaten in various forms either as normal cheese, a flavored snack and can be use to substitute meat in sauces or as fried cake or sandwich filling.

A range of preservation methods are well acknowledged literature.In an effort to search for berrer preservative to increase shelf life of cheese several method have been reported ranging from chemical and biological origin, among the chemicals are the use of proponic acid and 0.8 % sodium benzoate for a period of 8 days at 0.8% concentration(Joseph and Akinyosoye 2007). Both the oil and extract of garlic and ginger have been reported to poses preservative values (Friedman *et al.*, 2002; Ekwenye and Elegalam, 2005). Chemical composition and bioactive compounds in the essential oils responsible for their flavour, antimicrobial and antioxidant properties especially sulphur-containing compounds allicin(Oladipo and Jadesimi, 2012).

Due to the increasing awareness about hazard associated with the use of sunthetic chemical for the preservation of foods and food products, several efforts have been directed towards the use of natural substances as food preservatives and antioxidants (Peschel*et al.,* 2006). Flavonoids, tannins, anthocyanins and other phenolic constituents present in food of plant origin are potential antioxidants (Peschel*et al.,* 2006).

Though fungi toxicological effect of *S. cumini*against two genera of plant pathogenic and food spoilage fungi have been evaluated bit there is paucity of information regarding the antibacterial and antifungal effect of essential oil from *S. cumini* (Madhu *et al*., 2015), limited information is available regarding the activities of *S. cumini* leaf against both the pathogenic and spoilage bacteria.

Owing to the environmental and toxicological concerns necessitated the need to search for the effective preservative compounds of plant origin which will provide a better and safe substitute for chemical preservatives with hazardous outcome. Therefore, poor handling of cheese during processing and their possible transmission of numerous pathogens necessitated the needs for the microbiological evaluation of cheese sold in ilorin and the use of *S.cumini* oil as a preservative towards enhancing the safety and shelflife.

II. Materials and methods

II.I Experimental Section

Study Area

This study was carried out within Ilorin metropolis, a capital of Kwara State. The State shares local boundaries with Oyo, Ondo, Ekiti, Kebbi, Niger and Kogi. It is located between latitude $8^{o}5$ - $10^{o}4$'N and longitude $4^{o}55$' - $6^{o}5$E.the average temperature ranges between 27^{o}- 37^{o}C with a mean annual rainfall of 1,000 -1,500mm

Collection of samples

Samples of 'Wara' were bought from Oja Oba in Ilorin, from the Fulani sellers across the state in presterilized plastic containers and immediately taken to the lab for the Microbiological studies.

Plant material

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Syzygium cumini leaves were obtained from Ilorin, in Kwara State and Identified at the Herbarium of Plant Biology University of Ilorin, deposited at voucher specimens (UILH/001/1231).

Culture media

The following culture media were used in this study nutrient agar, nutrient broth and Mueller Hinton agar. The media used were prepared according to the manufacturer's instructions.

Isolation of bacteria

One gram of the sample was weighed into 9 ml of sterile water and matched with mortar and pestle. Then, serial dilution was carried out up to 10^{-5}. Samples were inoculated into sterile plate using Nutrient Agar by pour plate technique, incubated at 37°C for 24-48 hours. Distinct colonies after 24 hours of incubation were streaked on the sterile plate of NA and incubated at 30°C for 24 hours. The stock culture were prepared and stored in a refrigerator at 4^{o}C.

Identification of bacteria

The bacteria isolates were identified based on morphological and biochemical tests such as Gram and endospore staining, Motility test, microscopy, catalase test, Oxidase test, coagulase test, indole test, starch hydrolysis test and Citrate test using method of Fawole and osho (2002).

Extraction of essential oils from leaves

Leaves of *S. cumini* were air-dried. 200g of the dried leaves were pulverized and subjected to hydro-distillation using a Clevenger apparatus for 3h according to the British Pharmacopoea Specification (Pharmacopoea Specification 1980) for the isolation of essential oils according to the method recommended by Guenther. The volume of the extracted essential oil was recorded. The extracted essential oil was stored at 0°C in air-tight glass vials until used for the analysis.

Analysis of essential oil compounds

The phyto compounds were characterized using Gas-Chromatography-Mass spectrometry. Helium gas was used as a carriergas at 1.2mL/min while the MS operating parameters were ionization voltage, ion source temperature of 70ev and 230°C respectively. The percentage composition of the phyto compounds in the oil were determined based on the retention time and GC peak areas. Mass extrapolated from Literature(Jennings and Shibamito, 1980; Adams, 1995; Joulain and Koenig 1998).

Antibacterial Susceptibility Test

Antibacterial effect of the oil was determined using agar diffusion method as described by (Mohammed *et al.,* 2016). Six colonies were selected from distinct colonies on nutrient agars and grown for 6-8 hours in nutrient broth. The bacterial cells were adjusted to 0.5 McFarland standard. Individual isolates were streaked on the Mueller Hinton agar using sterile cotton swab. A well was dug using corkborer (6mm) on the seeded Mueller-Hinton agar. Control plates contained only sterile culture media. All the plates were incubated at 37°C for 24h. The plates were checked for the clear zone around the well and measured using ruler. Minimum inhibitory concentration was determined as lowest concentration of oil that inhibited bacterial growth in each case. The essential oil was diluted with Tween 80 to obtain concentrations of 25 and 50% (v/v) and 0.1ml each was transferred to the wells. An aliquot (0.1ml) of Tween 80 was used as control. Plates were incubated at 37ºC for 24h. Antibacterial activity was determined by clearance around loaded wells. MIC was defined as lowest concentration of oil that inhibited growth of bacteria. The concentration used for the MIC ranged from 6.25to 50.0%.

Determination of Minimum Inhibitory Concentration (MIC) of the essential oil *S. cumini*:

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Four concentrations (6.25 %, 12.5 , 25 and 50 % (v/v)) of the extracted oil were used. One milliliter of each dilution was added to 9ml of sterile nutrient broth in a test tube. A control tube was also prepared. The contents were thoroughly mixed and the tubes were inoculated with 0.lml of Mcfarland standard of the test isolates. The tubes was incubated at 37°C and examined for growth after 24 h *(*Andrews, 2001*)*. The least concentration of the extracts that did not permit any visible growth of the inoculated test isolates in the broth medium was regarded as the MIC in each case. Test tubes inoculated with the test isolates without the oil will serve as controls.

Determination of Minimum Bactericidal Concentration (MBC'S) of the essential oil *S. cumini*:

The concentrations of the oil used for the MIC which do not permit any visible growth were inoculated on plates containing the respective growth medium and incubated for 24 h. The least concentration which showed no growth after incubation was taken as the minimum bactericidal concentration *(*Andrews*,* 2001*)*.

Statistical analysis

The data were statistically evaluated by analysis of variance ANOVA by applying the level of significance (P≤0.05) using SPSS Statistical package for social sciences (SPSS, Version 17.0).

III. Results and discussion

Isolation and Identification of test organisms

Different bacteria were isolated from local cheese sold within Ilorin in varying degrees from different hawkers and characterized based on their colours, shape, elevation, edges, surface and pigmentation. six most prevalent bacteria were identified as *Bacillus subtilis, Proteus vulgaris, Escherichia coli, Listeria monocytogene, Salmonella typhimuriumand Staphylococcus aureus.*

Chemical Composition of Essential Oil of *S. cumini*

The phytochemical compounds in the essential oil of the *S.cumini* leaves are represented in peaks shown in GC-MS chromatogram. Each peak represents a compound as shown in Fig. 1 While the Pulverized leaves of *S.cumini* on hydro-distillation afforded oil in the yield of 0.32 % (v/w). The retention time, relative percentages and identities of the oil constituents of *S.cumini* leaves are shown in Table 1. A total of 14 compounds representing 100% of the oil were identified from their retention indices and mass spectra data. The major compounds that were identified by GC–MS were Trans-Alpha-Bergamotene (42.28 %), Cis-Beta-Fernesene (9.36 %), Alpha-Pinene (3.73 %), Alpha-Santalene (7.42 %), Alpha-Bergamotene (6.94 %), Beta-Pinene (5.20 %), and Trans-beta-Ocimene (5.08 %), along with some other minor components presented in trace amounts.

Effect of Oil Extract of *S. cumini* leaves on Isolates.

The susceptibility pattern of bacteria isolated from spoilt cheese to essential oil of *S. cumini* leaf were found to be susceptible to 25 and 50 % concentration of the oil except *B.subtilis* which was resistant and the diameter of zone of inhibition are shown in Table 2and Figure 2, respectively. All the isolates except *B. subtilis* were sensitive to the oil. Highest activity, represented by diameter of zone of clearance around the loaded wells was recorded for *S.aureus*, at both concentrations that was used (20 and 50 % (v/v)).At 25 % concentration,0, 0.9, 1.6, 1.8, 1.2and 2 mm was recorded for *B. subtilis, P. vulgaris, L. plantarum, Escherichia coli, Listeria monocytogene, Salmonella typhimurium*and *Staphylococcus aureus*respectively. While at 50 % concentration, 0, 1.3, 2.0, 2.3, 1.8 and 2.6mm was recorded for*B. subtilis, P. vulgaris, L. plantarum, Escherichia coli, Listeria monocytogene, Salmonella typhimurium*and *Staphylococcus aureus*,respectively.

Minimum inhibitory concentration and minimum bactericidal activity of the oil.

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The lowest MIC values recorded against *S. aureus* and *L. monocytogens* with the same MIC values of 6.25. Similarly, the same MIC values was recorded in *E. coli* and *S. typhimurium*, while the highest MIC values was found against *Proteus vulgaris* with 25 as shown in Table 3. Similarly, Minimum Bacteriocidal concentration recorded against *S. aureus, L*. *monocytogens* and *P. vulgaris* were 6.25, 12.5 and 50 respectively shown in Table 4.

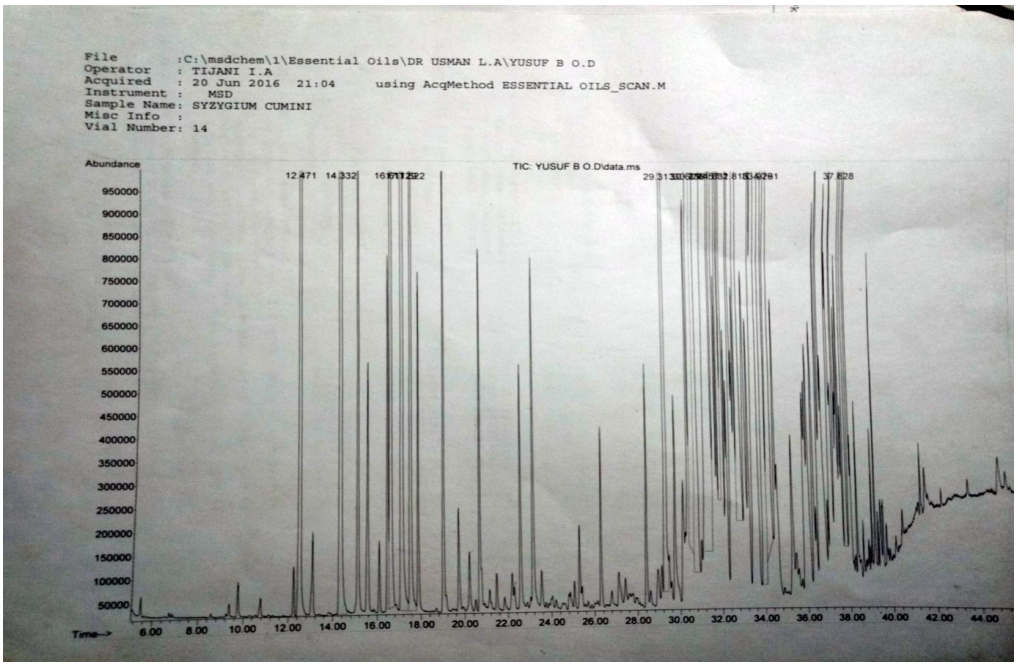


Figure 1: GC-MS Chromatogram Of the essential oil of Syzygium cumini Leaves

Table 1: Chemical composition (%) ofessential oil S. cumini leaves

S/N	Retention Time	Compounds	Area%
1	12.471	Alpha-Caryophylene	3.73
2	14.332	Beta – Caryophylene	5.20
3	16.611	D-Limonene	2.89
4	17.129	Trans-beta-Ocimene	5.08
5	17.522	Beta-Ocimene	2.11
6	29.313	Alpha-Copaene	3.73
7	30.601	Beta-Pinene	6.94
8	30.758	Alpha-Santalene	7.42
9	31.457	Alpha-Pinene	42.28
10	31.881	Cis-Beta-Farnesene	1.40
11	32.816	(E)-Beta-Famesene	9.36
12	33.979	Calamenene	5.02
13	34.261	Cubenene	3.37
14	37.828	Beta-bisabolol	1.48

Table 2: Antimicrobial Susceptibility Pattern of Oil on Isolates

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Concentration of essential oil of S. cumini % (v/v)			
Organisms	**Control**	25	50
Bacillus subtilis	-	-	-
Proteus vulgaris	-	+	+
Escherichia coli	-	+	+
Listeria monocytogene	-	+	+
Salmonella typhimurium	-	+	+
Staphylococcus aureus	-	+	+

KEY +: Sensitive to the oil -: Resistant to the oil

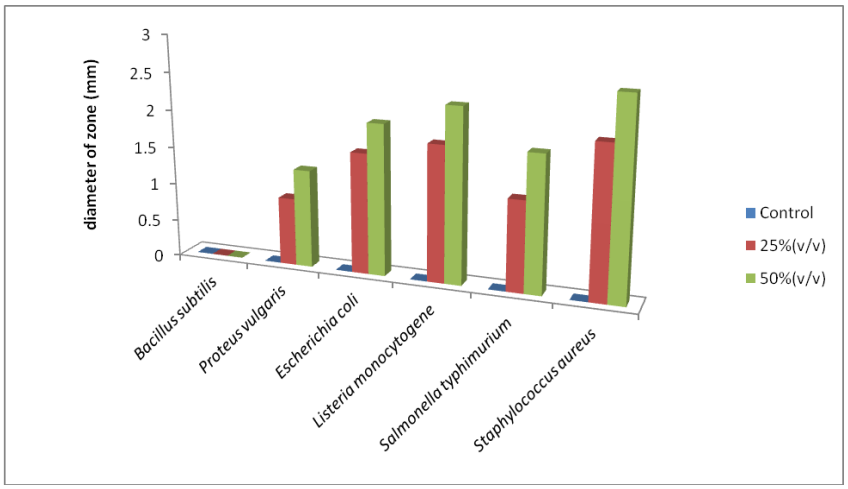


Figure 2: Sensitivities of isolates to Different concentrations of essential oil of S. cumini

Table 3: Minimum Inhibitory Concentration (MIC) of the Oil

S/N	Bacteria	Concentration (%v/v)
1.	*Bacillus subtilis*	NIL
2.	*Proteus vulgaris*	25.0
3.	*Escherichia coli*	12.5
4.	*Listeria monocytogene*	6.25
5.	*Salmonella typhimurium*	12.5
6.	*Staphylococcus aureus*	6.25

KEY NIL: Not Sensitive

Table 4: Minimum Bactericidal Activities of the Oil

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S/N	Bacteria	Concentration(%v/v)
1	*Bacillus subtilis*	NIL
2	*Proteus vulgaris*	50.0
3	*Escherichia coli*	25.0
4	*Listeria monocytogene*	12.5
5	*Salmonella typhimurium*	50.0
6	*Staphylococcus aureus*	6.25

KEY NIL: Not Sensitive

This study revealed the presence of pathogenic and spoilagebacteria and some of these bacteria isolated from the cheese might have contaminated the cheese during the milk collection and processing. Microbial spoilage of cheese is one of the major factors responsible for the food poisoning, nutritional and economic losses to the processors due to the organoleptic changes of the products.

Fourteen compounds, representing about 100% of the essential oils extracted from *S. Cumini* were categorized.*S. Cumini* oil contained a complex mixture of monoterpene hydrocarbons and oxygen containing mono- and sesquiterpenes, which are hydrocarbons with the general formula $(C_5H_8)n$. The major compounds that were identified by GC–MS were Trans-Alpha-Bergamotene (42.28 %), Cis-Beta-Fernesene (9.36 %), Alpha-Pinene (3.73 %), Alpha-Santalene (7.42 %), Alpha-Bergamotene (6.94 %), Beta-Pinene (5.20 %), and Trans-beta-Ocimene (5.08 %) and they are all sesquiterpenes which is one of the classes of terpenes. Sesquiterpenes are found naturally in plants and insectse.g. defensive agents or pheromones (*Martin et al.,* 2003).

Antibacterial properties exhibited in the current study can be attributed to the ability of the essential oil contained in the plants to scavenge free radicals which plays significant role in the prevention of some diseases such as brain dysfunction cancer heart disease and immune system decline (Kamatou and Viljoen, 2010). Incidentally, the presence of α-pinene in *S. cumini* essential oil contribute to its therapeutic and antimicrobial properties such as antibacterial, antifungal, anti-inflammatory, insecticidal and antioxidant properties and it is used traditionally as flavouring agent and antimicrobial material in food (Hajji *et al.,* 1993; Tantaoui-Elaraki *et al.,* 1993). The present results agree with the work of Mohammed *et al*. (2016), who also reported that the essential oils isolated from the air dried *S. cumini* leaves contain α-pinene, β-pinene, 1,3,6- octatriene and α-limonene which accounted for the antibacterial properties of the plant.

The result of the minimum inhibitory and bactericidal concentration of the oil has shown that the oil extracted from leaf of *S. cumini* exerted effects on all the bacterial isolated from wara except for *Bacillus subtilis.*

It was found that sensitivity of most isolate to this oil increases with increase in concentration of the oil extract.

Proteus vulgaris arod-shaped, nitrate-reducing, indole positive and catalase-positive, Gram-negative bacteriumthat inhabits the intestinal tracts of humans and animals which can be found in soil, water, and fecal matter and an opportunistic pathogen of humans was only slightly sensitive to the oil at 25% concentration, and the oil only excerted bactericicdal effect at a higher concentration of 50%.

Essential oil of *S. Cumini* has bactericidal effect on all the isolated organisms at varying concentration except *Bacillus subtilis* which is not sensitive to the at all.

Bacillus subtilis found in gut and gastrointestinal tract of ruminants and humanswas not sensitive to the oil may be because of formation of tough, protective endospore which allow it to tolerate extreme environmental condition (Bakkali *et al., 2008*)and probably produces certain enzymes that neutralize the effect of the extract.

Some of the organisms are only sensitive to a certain concentration gradient and an increase in concentration above or below the particular one will exert little or no effect on the isolate. This may be due to the production of certain enzymes by microbial cell that neutralize the oil effect is affected by the concentration of the essential oil i.e produced when the effect of the oil is already in to progress. The sensitive ones are those ones that cannot neutralize the effect of the oil. They cannot

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grow in the presence of the oil or they may contain the buffer species but are unable to synthesize them in the presence of the oil.

Conclusion

The essential oil extracted from the leaf of *S. cumini* was found to be active at low concentration against most of the isolates isolated from wara sold in Ilorin. Therefore, the oil can be an alternative means of enhancing the microbiological safety of wara.

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