IN-VITRO ANTI-TUBERCULAR ACTIVITY OF SYZYGIUM AROMATICUM EXTRACT AGAINST MYCOBACTERIA SPECIES

BABAYI, H.[1,2*]; OLADOSU, P. O.[3]; OLATUNJI, K. T.[3]; ABOYEJI, D. O.[1]; AMADI, E. D.[4] & LAWAL, F.[1]

[1]Department of Microbiology, School of Life Sciences,
Federal University of Technology, P.M.B. 65, Minna, Niger State, Nigeria
[2]Centre for Genetic Engineering and Biotechnology,
Federal University of Technology, P.M.B. 65, Minna, Niger State, Nigeria
[3]National Institute of Pharmaceutical Research and Development,
Microbiology and Biotechnology Department Abuja, Nigeria
[4]Department of Pharmaceutical Microbiology,
University of Ibadan, Ibadan, Oyo State, Nigeria
E-mail: acadbabayi@yahoo.com **Phone No:** +234-803-370-0406

Abstract
Tuberculosis (TB) is one of the leading infectious diseases in the world and its control is being complicated by the increasing emergence multidrug resistant and extensively drug-resistant strains. Therefore, the urgent need to develop new anti- TB drugs from medicinal plants cannot be overemphasized. In this study, the in-vitro anti-tubercular activity of Syzygium aromaticum (clove) extract was investigated against Mycobacterium bovis (BCG), M. smegmatis and M. tuberculosis $H_{37}R_V$ using micro-broth dilution method. The activity was compared with rifampicin (0.04 µg/ml). The phytochemical composition of the extract was determined using the method of Association of Official Analytical Chemists (AOAC). The ethylacetate extract contained alkaloids (47.79 mg/100g), saponins (103.70 mg/100g), flavonoids (6.84 mg/100g), tannins (98.35 mg/100g) and phenols (252.55 mg/100g) respectively. The extract at 50% v/v concentration inhibited the growth of Mycobacterium bovis, M. smegmatis and M. tuberculosis with minimum inhibitory concentration of 0.2% v/v, 0.1% v/v and 0.2% v/v, respectively. The results of the study showed that extract of S. aromaticum may have therapeutic value in the treatment of TB and support the local use of the plant in the treatment of tuberculosis.

Keywords: Minimum inhibitory concentration (MIC), *Mycobacterium* species, Phytocomponents, *Syzygium aromaticum* Extract, Tuberculosis

Introduction
Tuberculosis is an infectious disease caused by *Mycobacterium tuberculosis*. Tuberculosis typically attacks the lungs, but can also affect other parts of the body (Ridandari & Panjaitan, 2019). Although, the disease has become rare in high income countries, it is still a major public health problem in low- and middle-income countries. Tuberculosis remains a major world health problem, causing 1.75 million deaths annually and one-third of the world population is estimated to be latently infected with tuberculosis (WHO, 2018). Approximately 10% of those latently infected will develop active disease, but this risk is significantly increased by factors weakening the immune response, such as HIV infection (Esmail *et al*., 2018).

Tuberculosis (TB) is believed to be a contagious disease transmitted mainly through sharing contaminated food and eating utensils, and droplet infection (Tabuti *et al.,* 2010). This disease is identified by the African traditional system by signs and symptoms that include cough, whooping cough, labored breathing and weight loss (Rewari *et al*., 2021). Since the detection of tubercle *bacilli* as the causal agent by Robert Koch in 1882, and after the introduction of first anti-TB drugs by Schatz and Waksman in 1940, great developments

have transpired in the fields of biology, microbiology, pathogenesis, immunology, drug therapy and, recently, molecular genetics of this disease and its etiological agent. However, TB is still a worldwide problem (Rewari *et al*., 2021). Currently, about one-third of the world's population is assumed to be infected with TB. It is responsible for 1.4 million deaths per year (Elsayed *et al.,* 2021).

The therapy of tuberculosis has limitation on patients and a grave burden for the healthcare system. Drug-susceptible tuberculosis requires at least six months of therapy (Chong *et al.,* 2021). A remedy for multi-drug resistant tuberculosis requires two years of management with poorly tolerated and not as much of helpful drugs (Samuels *et al.,* 2018). The Bacillus Calmette-Guerin (BCG) vaccine discovered nearly a century ago, confers merely part protection (Olson, 2021). Therefore, the search for new effective drugs against TB is now more important. Natural products derived from medicinal plants and their analogs may play a critical role in anti-TB drug discovery. These plants have limited toxicity and potentials to mitigate compliance issues during protracted administration.

Syzygium aromaticum is an evergreen tree with sanguine flowers belonging to the family Myrtaceae that grows in tropical climates and has been widely used in Ayurveda and Chinese traditional medicines for over 2000 years (Vicidomini *et al.,* 2021). Arabic traders brought it to the Western world in the fourth century A.D., and in medieval Europe, it became very popular as a medicinal spice. Indigenous to the Moluccas, this tree is cultivated in several countries of Asia and Africa, including India, Indonesia, Madagascar, Malaysia, Sri Lanka, and Zanzibar (Vicidomini *et al.,* 2021).

The dried flower bud of this plant is indicated by the English name "clove", derived from the Latin word "clavus" (nail), as the shape resembles that of a small-sized nail. Cloves are currently used in three different forms, as whole dried buds, ground spice, and essential oil. Though all forms share similar bio-medically relevant properties, they differ in the degree of potency, with the oil showing the highest potency and, thus, often being diluted with almond oil. Whole cloves, containing a good amount of oil in their interiors, are still endowed with a medium potency, whilst ground cloves are the least potent form, as, in this form, the spice generally loses most of the essential oil (Thielmann *et al*., 2019).

Cloves have long been used in both traditional medicine and for culinary purposes and serve to produce an essential oil known since ancient times in food flavorings, traditional medicine, and perfume production (Vicidomini *et al.,* 2021). Even though cloves are mostly used as a nutritional spice for food in the Western world, in the past, they have constituted a remedy for a variety of health concerns, with the clove anesthetic (due to eugenol), stimulating, antimicrobial, antifungal, antiviral, and antiseptic properties having been known for centuries (Thielmann *et al.,* 2019). The current study is aimed at investigating the antitubercular activity of *Syzygium aromaticum* extract against *Mycobacterium tuberculosis, M. bovis* and *M. smegmatis.*

Materials and Methods

Collection and Identification of *Syzygium aromaticum*: Dry form of cloves were collected from Kure market, Niger State, Nigeria in the month of April, 2021 and were transferred into sterile plastic bag and transported to the Laboratory of the Department of Biological Sciences, Federal University of Technology, Minna for identification by an Ethnobotanist. It was identified as *Syzygium aromaticum.* Voucher specimen was deposited in the Herbarium unit of the Department. Information gathered from the survey included vernacular names and the parts used in the preparation of herbal antibacterial remedies and the diseases they are used to treat.

Laboratory Preparation of *Syzygium aromaticum*: The collected flower bud was washed with distilled water to remove soil and dirt in the sample and air dried at room temperature (25±2 ℃) for 2 days. The dried clove was pulverized using an electric blender. The powdered form of the clove was stored in a dark sterile plastic container.

Extraction of *Syzygium aromaticum*: Four hundred gram (400 g) of the clove powder was refluxed in 2000 ml of ethyl acetate for 6 hours. It was shaken vigorously for proper mixture of the sample and solvent. The setup was allowed to cool off after some time and filtration was done using a muslin cloth. The filtrate was concentrated using a rotary evaporator at 40℃. The extract was dried in a dessicator over anhydrous $CuSO_4$. The extract was transferred into vials and stored pending analysis. Prior to analysis, the extract was dried at 40℃ in an oven to remove the residual solvent which may interfere with test results.

Quantitative Phytochemical Screening of *Syzygium aromaticum* Extract: The extract of *Syzygium aromaticum* was screened for phytochemical compounds using the method of Association of Official Analytical Chemists (AOAC) (2005), the plant was screened for alkaloids, flavonoids, saponins, phenols and tannins.

Source of Mycobacterial strains: The test Mycobacterial strains were: *Mycobacterium tuberculosis*, *M. bovis* and *M. smegmatis*. The organisms were obtained from Department of Microbiology and Biotechnology laboratory, National Institute for Pharmaceutical Research and Development, Abuja, Nigeria.

Identification of Mycobacterial strains: The method described by NIPRD (2018) was employed in the identification and confirmation of mycobacterial species*. Mycobacterium tuberculosis, M. smegmatis* and *M. bovis strains* were inoculated into different conical flask containing Middlebrook 7H9 broth supplemented with 0.05% (v/v) glycerol and 10% OADC (oleic acid – albumin – dextrose – complex) respectively. The set up were incubated at 30℃ for 7 days. The identities of isolates were confirmed by Ziehl-Neelsen staining technique.

Standardization of *Mycobacterium* strains: Mycobacterial strains were standardized to 10^6 cfu/ml using the NIPRD (2018) technique. Inoculated into 50 ml of sterile 7H9/tween/ADC broth was 50 µL of *Mycobacterium* species stock culture. The setup was incubated for 7 days at 30℃. After incubation, the optical density was measured to be 0.30 OD in UV spectrophotometer at 520 nm. The resultant optical density (OD) was further diluted in ratio of 1:1000 by diluting 50 µl culture in 450 µl broth. The turbidity of the culture was compared with 0.5 McFarland turbidity standards. The standardized culture was then used for anti-tubercular screening.

Reconstitution of Extract: Specifically 1 ml of the 100% concentration of the extract obtained in liquid form after extraction was dispensed into slant bottle, 0.5 ml of Dimethyl sulfoxide (DMSO) was added to dissolve the extract and 0.5 ml of sterile Middlebrook 7H9/ADC broth was also added to the dissolved sample and homogenized to obtain 50 % (v/v) concentration.

Screening of *Syzygium aromaticum* Extracts for Anti-tubacular Activity: Assay was performed according to Clinical and Laboratory Standard Institute (2019). Specifically 50 µL of 7H9/ADC broth was dispensed into sterile wells of the 96 microwell plate from row 2-12. Exactly 100 µl of stock concentration of crude extract was transferred into well 1 of the microwell plate in duplicate. Specifically 50 µL of the solution in well 1 was transferred to well 2, mixed thoroughly and repeated through to well 11 where 50 µL was discarded. Well

12 served as the organism viability control (OVC) containing only the broth and test organism. The wells (1-12) were inoculated with 50 µl of standardized microorganisms and incubated at 37℃ for 7 days. Control wells with varying concentration of rifampicin (25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, 0.20, 0.10, 0.05, 0.025 and 0.01 µg/ml) were also set up. After the 7 days incubation period, 25 µL of tetrazolium dye was added to the wells. The plates were further incubated for 2 hours and observed for absence or presence of microbial growth by colour change in the wells. Colourless wells were interpreted as no growth of test organisms (activity of extract), while a change in initial colourless form to pink indicated growth of test organisms (no activity of extract). Minimum inhibitiory concentration (MIC) was defined as the lowest drug/extract concentration that prevented the colour change (growth of the organism).

Determination of Minimum Bactericidal Concentration: Specifically, 50 µL of the last well before the MIC was diluted in phosphate buffer pH 7.0 to neutralize the antimicrobial agent. The diluted solution was inoculated in 10 ml 7H9 Middle brook broth and incubated for 3 weeks at 30℃. The set up was observed for turbidity after the incubation period. Absence of turbidity was taken as bactericidal activity of the concentration before the MIC well (NIPRD, 2018).

Results

Phytochemical Components of *Syzygium aromaticum* Extract:

The quantitative phytochemical components of *Syzygium aromaticum* extract is shown in Table 1. The *Syzygium aromaticum* (cloves) extract contained phenol (252.55 mg/100g), saponins (103.70 mg/100g), tannins (98.35 mg/100g), alkaloids (47.79 mg/100g) and flavonoids (6.84 mg/100g).

Anti-tubercular Activity of *Syzygium aromaticum* Extract Against Mycobacterium Species:

The results of anti-tubercular activity of clove extract against Mycobacterium species are presented in Table 2. The clove extract tested at 50% (v/v) to 0.20% (v/v) concentration inhibited the growth of *Mycobacterium bovis* BCG, *M. smegmatis* and *M. tuberculosis* $H_{37}R_V$. However, the extract was more potent on *M. smegmatis* inhibiting at 0.10% (v/v) concentration.

Minimum Inhibitory Concentration of Clove Extract:

The minimum inhibitory concentration (MIC) of the clove extract against *Mycobacterium bovis* BCG, *M. smegmatis* and M. *tuberculosis* $H_{37}R_V$ was 0.2% v/v, 0.1% v/v and 0.2% v/v, respectively (Table 2).

Minimum Bactericidal Concentration of Clove Extract:

The minimum bactericidal concentration (MBC) of the extract against *M. bovis* BCG, *M. smegmatis* and *M. tuberculosis* $H_{37}R_V$ was 0.39% v/v, 0.20% v/v and 0.39% v/v, respectively (Table 2).

Table 1: Phytochemical components of *Syzygium aromaticum* ethylacetate extract

components	Extract	Quantity(mg/100g)
Saponins	+	103.70
Phenols	+	252.55
Tannins	+	98.35
Alkaloids	+	47.79
Flavonoids	+	6.84

+: present, mg: milligram, g: gram

Table 2: Anti-tubercular Activity of *Syzygium aromaticum* Extract against *Mycobacterium species*

Concentration Extract (%v/v)	Antitubercular activity M1 M3	M2	Rifampicin (µg/mL)	Antitubercular activity (Rifampicin)	MIC M1 M3	M2	MBC M1 M3	M2
50	+	+	25	+	0.20		0.39	
	+				0.10		0.20	
					0.20		0.39	
25	+	+	12.5	+				
	+							
12.5	+	+	6.25	+				
	+							
6.25	+	+	3.13	+				
	+							
3.13	+	+	1.56	+				
	+							
1.56	+	+	0.78	+				
	+							
0.78	+	+	0.39	+				
	+							
0.39▫▫	+	+	0.20	+				
	+							
0.20**	+	+	0.10	+				
	+							
0.10	_	+	0.05*	+				
	_							
0.05	_	_	0.025	-				
	_							
0.025	_	_	0.01	-				
	_							

+: Activity
-: No Activity
*: Minimum inhibitory concentration
▫: Minimum bactericidal concentration

M1: *Mycobacterium bovis*
M2: *Mycobacterium smegmatis*
M3: *Mycobacterium tuberculosis*

Discussion

Plants are the important source of diverse range of bioactive principles. This is due to the fact that plants have an almost limitless ability to synthesize chemical compounds of therapeutic value (Gowrish *et al.,* 2015). The most important bioactive compounds of plants are alkaloids, flavonoids, steroids, terpenoids, tannins and phenolic compounds (Gowrish *et al.,* 2015). These compounds are synthesized by primary or secondary metabolism of the plants. The quantitative screening of the crude extracts of *Syzigium aromaticum* revealed the presence of some important bioactive compounds like saponins, phenols, tannins, alkaloids and flavonoids in varied concentration, with high amount of phenolic compounds. This may be the main reason for its significant anti-tubercular activity.

Phenols have been reported by several authors to inhibit efflux system, proteasome, mycolic acid biosynthesis in Mycobacteria (Gröblacher *et al*., 2012; Zheng *et al*., 2014; Mazlun *et al*., 2019). Nantogo *et al*. (2018) reported that saponins are active bio-surfactants that have several therapeutic functions including immunostimulatory, antimicrobial and antioxidant activities. They also reported that flavonoids have strong influence against *Mycobacterium tuberculosis* and inhibit *M. tuberculosis* growth within human. Alkaloids inhibit *Mycobacterium* species from growing by interfering with permeability and causing loss of cellular components (Obakiro *et al.,* 2020). According to Kaur and Kaur (2017), the identified phytochemicals such as phenols, alkaloids, tannins, flavonoids, terpenes, xanthones were frequently isolated from medicinal plants with antitubercular activity. Al-Darraji *et al.* (2013) reported the synergetic impact of these components.

Rhadika *et al.* (2020) observed that the activity of different plant extracts depend on the solvent used for the extraction. Abdulmumin *et al*. (2011) reported that ethylacetate is a mid-polar solvent with strong affinity for bioactive compounds with potent anti-tubercular activity. Therefore, it is not surprising that ethylacetate used for the extraction in the present study was able to extract both lipophilic and non-lipophilic compounds. The lipophilic nature of the components in the extract enhanced the penetration of the hydrophobic outer membrane of *Mycobacterium* species to exert inhibitory effects (Mohammed *et al.,* 2018).

The anti-tubercular activities of various extracts of *S. aromaticum* have been reported by many investigators (Barua *et al.,* 2014; Kaur and Kaur, 2015; Fadipe *et al.,* 2020; Mounika *et al.,* 2020). The anti-tubercular data generated in the present study indicated that the Mycobacterial species were susceptible to *S. aromaticum* ethyl acetate extract at different concentration. Thus, *S. aromaticum* may be a rich source of biomolecules that can be used for suppressing the activity of *Mycobacterium* species.

In the present investigation, the potent anti-tubercular activity of rifampicin (0.05 µg/mL) was expected in selecting rifampicin as control drug due to its specificity to *Mycobacterium* species. The use of this standard drug was not to compare its activity with that of the plant extract rather it was used as a negative growth control to validate the assay procedure together with positive control. Again, the MIC of the plant extract for *Mycobacterium bovis, M. smegmatis* and *M. tuberculosis* were 0.20% v/v, 0.10% v/v and 0.20% v/v respectively, while rifampicin MIC was at 0.05 µg/mL. It is possible that upon purification of the extract, the active components may become highly concentrated to exhibited higher activity.

Chloroform extracts of *Pterolobium stellatum*, *Persea americana* and *Otostegia integrifolia* have shown minimum inhibitory concentration (MIC) values of 0.312, 2.5 and 0.312 mg/mL respectively against *M. tuberculosis* strain $H_{37}R_V$ (Kahaliw *et al.,* 2017). Barua *et al.* (2014) reported that methanolic extract of *S. aromaticum* L, exhibited anti-tuberculosis activity at a range of 0.8 to 100 µg/mL against *M. tuberculosis* strain $H_{37}R_V$. Study undertaken by

Mounika *et al.* (2020) showed that *S. aromaticum*, and other plant extracts exhibited anti-tubercular activity, the proportion of inhibition of these plant extracts for *M. tuberculosis* $H_{37}R_V$, was found to be 0.8 µg/ml, 50 µg/ml, 12.5 µg/ml and 50 µg/ml respectively. In the current study, an MIC of 0.2% v/v, 0.1% v/v and 0.2% v/v of the extracts was recorded against the Mycobacterial strains. It was also observed that the MBC of the extracts was 0.39% v/v, 0.2% v/v and 0.39% v/v for *M. bovis*, *M. smegmatis* and *M. tuberculosis* $H_{37}R_V$ respectively. This result is similar to the MIC values against the Mycobacterial strains. This result also indicates that *Syzigium aromaticum* showed bactericidal action against *Mycobacterium bovis, M. smegmatis* and *M. tuberculosis* $H_{37}R_V$. The activity observed from the medicinal plant extract in the present study may be attributed to the presence of phytochemical compounds (alkaloids, flavonoids, phenols, saponins, tannins).

Conclusion

This study investigated the ethylacetate extract of *Syzigium aromaticum* exhibited significant activity on *Mycobacterium bovis, M. smegmatis* and *M. tuberculosis* due to the presence of saponins, alakaloids, phenols, tannins and flavonoids. The present study therefore suggests that *S. aromaticum* may be a very effective medicine in the treatment of tuberculosis.

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