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ANTIMICROBIAL AND CELLULAR METABOLIC INHIBITORY PROPERTIES OF THE ETHANOLIC EXTRACT FROM THE BARK OF 'LUNAS-BAGON' (LUNASIA SP.)

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ABSTRACT: This study was conducted to evaluate the antimicrobial and cellular metabolic inhibitory properties of the ethanolic extract from the bark of 'Lunas-bagon', Lunasia sp. Extracts were evaluated using the agar well diffusion method for antimicrobial assay. The standard phytochemical screening was done for the determination of compounds that can be considered toxic and MTT assay for the effects of the extract on cellular metabolism. Results showed that the extracts have ability to inhibit selected Gram-positive and Gram-negative bacterial isolates indicating that the extracts are effective antibacterials. Results from the MTT assay revealed at doses lower than 100.0 ug/ml, extracts will not inhibit cellular metabolism of the splenocytes. Higher dose, however shows reduction of metabolic activity which may indicate the onset of apoptosis in cells. Basic biochemical tests indicated the absence of cyanogenic glycosides and anthraquinones, thus the extracts may not have toxic effects. Identification of bioactive compounds in through GC-MS analysis showed the presence of compounds that are known as antimicrobials and also effective in the treatment of selected health-related problems and conditions. The information generated in this study clearly indicates that the folk medical uses of 'Lunas-bagon', Lunasia sp. have antimicrobial and biochemical bases.

INTRODUCTION: Conventional drugs may serve as effective medicines and therapeutics, but many rural and poor patients prefer natural remedies to treat selected health-related problems. For centuries, medicinal plants and herbs played a major role in medicine and therapeutics for worldwide primary healthcare [1 - 8].

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This is based on the belief that plant-derived natural products contain phytomedicinal compounds and secondary metabolites that can serve as drug prototypes, pharmacological probes and drug precursors [9].

It was therefore claimed that knowledge of traditional application of medicinal plants is useful for community health care practices and for future drug discovery [10 - 17]. Increasing interest in the application of traditional medicine has therefore gained renewed attention to the global use of traditional/complementary and alternative medicine [TCAM] in all regions of the developing and industrialized countries [18].

One of the most popular ethnomedicinal and herbal plant species of the genus Lunasia related to the vine variety used in this study is *Lunasia amara* Blanco found not only in the Philippines but also in other Southeast Asian countries [19]. In the Philippines, the identified two varieties, amara and babuyanica (Merr.) were the focus of many research endeavors on their pharmacological properties. Decoctions of bark and leaves have been used to effectively treat swollen limbs and other skin diseases, stomach problems, envenomation and poisoning while the sap from the bark has been used as eye drops for inflamed or irritated eyes [20].

The plant is also known to possess several bioactive compounds with essential biological, medicinal and therapeutic properties [21 - 23] such as essential oils from its foliage and eleven different quinoline types and alkaloids isolated from the bark [24]. The species used in this study known locally as 'Lunas-bagon' (Lunasia sp.) has the bark of its vine used by the residents, traditional healers and the indigenous peoples (Manobos) in the treatment of infections and other health related illnesses. This was believed to be effective and are used instead of the shrub *Lunasia amara* not only because of its accessibility but also its efficacy when compared to the tree/shrub *Lunasia amara*.

It was therefore the objective of this study to evaluate selected biological properties of the Lunasia sp. vine variety based on the folk medical uses of the plant. Since the local people use the bark of the vine soaked in local wine or alcoholic drinks to treat internal and skin infections, the information served as basis for the antimicrobial and cellular metabolic inhibitory properties evaluation in this study. Understanding these biological properties of Lunasia sp. may help in a clearer understanding of the scientific basis of the efficacy of the plant in the treatment of health-related problems of the local inhabitants of the area.

MATERIALS AND METHODS:

Informal Interview and Field Observation: To be able to verify the information on Lunasia sp., key informants comprised of the local people, traditional healers and the indigenous peoples of the province of Agusan del Sur were interviewed. The survey centered on the folk medical uses of Lunasia sp. varieties. A snowball sampling method was employed for the respondents composed of local residents, traditional healers and Manobos. The key informants were interviewed based on a series of semi-structured questions related to the origin of knowledge, varieties and parts used, traditional preparations, modes of application and medical uses. The survey was initiated with an informed prior consent of the key informants. Participation of the members of the non-government indigenous people's organization of Bayugan City was also sought to help in the conduct of the interview.

Collection of Plant Material: The barks of the Lunasia sp. vine were collected directly from the plants growing in a midland well-drained rainforest of Mt. Ararat of Bayugan City, Agusan del Sur in July 2016. The collections were made possible with the help and consent of local residents and Manobos who are not only familiar with the area of collection but also with the plant in question. The plant was observed to have a long and woody stem clinging to other species of trees for vertical support and to gain access to well-lit regions near well-drained or moist areas like the sides of the rivers and creeks. Photographs of the plant and its parts were captured for taxonomic keys and identification following the list of medicinal plants of Philippines [25]. Confirmation of the identification was done and verified by a botanist and systematist. Lunasia sp. vine variety voucher specimen was collected and preserved in the herbarium.

Preparation of Crude Ethanolic Extract: Plant extract was prepared based on the modified procedure of the key informants instead of the local alcoholic drink. One hundred fifty (150.0) grams of powdered bark was soaked in 500.0 ml of absolute ethanol for 3 weeks with regular stirring. The supernatant was filtered using Whatman No. 1 (Whatman, UK) filter paper. The filtrate was concentrated using a rotary evaporator to a temperature at 45 °C. The crude extract was collected and allowed to completely dry at room temperature. The obtained viscous crude extract was stored in storage vials for antimicrobial, cellular metabolic inhibitory properties, phytochemical screening and GC-MS analysis.

***In-vitro* Antimicrobial Assay:** Agar well diffusion method (**Fig. 1**) was used for antimicrobial assay against selected test microorganisms from the Microbiological Research and Services Laboratory in Natural Sciences Research Institute, University of the Philippines in Diliman. The microbial suspensions of Gram-negative bacteria - *E. coli* (UPCC 1195), *Klebsiella pneumoniae* (UPCC 1360), *Pseudomonas aeruginosa* (UPCC 1244), *Salmonella typhimurium* (UPCC 1368) and Gram-positive bacteria - *Bacillus subtilis* (UPCC 1295) and *Staphylococcus aureus* (UPCC 1143) was prepared in 0.1% peptone broth.

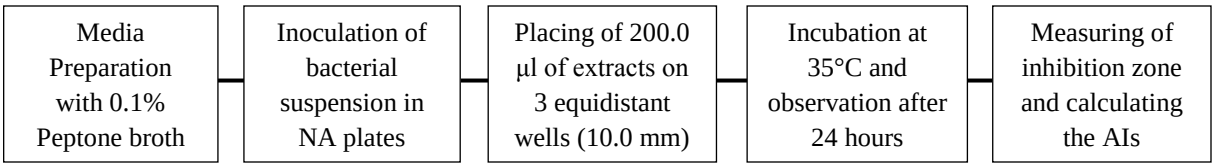


FIG. 1: DIAGRAM OF THE *IN-VITRO* ANTIMICROBIAL ASSAY USING THE AGAR WELL DIFFUSION METHOD

Absolute ethanol was used as the negative control and chloramphenicol (30.0 µg) was used as the positive control. Pre-poured Nutrient Agar (NA) plates about 3.0 mm thick, were inoculated with the respective microbial suspension by swabbing the agar surface. The swab was streaked over the entire agar surface. This procedure was repeated two more times, rotating the plate 60 °C each time to ensure even distribution of the inocula. Three (3) equidistant wells were made on the agar plate using a cork borer (10.0 mm) and 200.0 µl of the extract was placed in each of the wells. The plates were incubated at 35°C and observed after 24 hours.

The inhibition zone was measured and the mean diameter of the inhibition zones was calculated (**Fig. 2**). The antimicrobial index (AI) was computed by subtracting the diameter of the well from the diameter of the clearing zone divided by the diameter of the well.

***In-vitro* MTT Assay of Splenocyte Proliferation:** The MTT assay of Lunasia sp. vine bark ethanolic extract to assess its potenial as an antimicrobial agent against Gram-positive and Gram-negative bacteria was determined on BALB/c mice splenocytes. Modified MTT assay [26] was used to determine the cellular metabolic activity, hence, the cell proliferation. Two hundred fifty milligrams (250.0 mg) of dried extract was dissolved in 40.0 ml of 5% DMSO in PBS were tested for the stimulation or suppression of splenocyte proliferation.

To evaluate the splenic lymphocytes, the spleen cell suspension containing (1×10^6 cells/ml) were seeded in a 96-well culture plate (200.0 µl/well) in triplicates. Splenocytes were treated with (10-200 µg/ml) of the extract (in triplicates).

The positive controls, Concanavalin A (ConA) (10.0 µg /ml) and Lipopolysaccharide (LPS) (10.0 µg/ml) were added to each well separately for priming T cells and B cells, respectively. The plates were incubated at 37 ºC in a 5% humidified CO_2 incubator for 48 h. To each well, 100.0 µl of a DMSO working solution was added and let it stand for 10 min. The ELISA plate reader was set to shake the plate for 5 minutes and the absorbance was evaluated to read the optical density at 570 nm absorbance. The percent (%) cell proliferation was calculated.

$$\% \text{ cell proliferation} = [[OD_{sample} - OD_{control}] / OD_{control}] \times 100\%$$

The cytotoxicity data was analyzed using one-way analysis of variance (ANOVA) with Levene´s test for the homogeneity of variance from the means based on $p<0.05$ level of significance. All statistical analyses were done using the Paleontological Statistics software (PAST) version 3.14 [27].

Phytochemical Screening: The phytochemical screening of the bark ethanolic extract was carried out using the standard phytochemical methods [28] and modified according to the Laboratory Manual for the UNESCO Sponsored Workshop on the Phytochemical, Microbiological, and Pharma-cological Screening of Medicinal Plants at the Department of Chemistry, U.P. Diliman. A 3-point scale (+ turbid, ++ moderate and +++ heavy) in scoring was based on the Handbook of Philippine Medicinal Plants [29].

Gas Chromatography - Mass Spectrometry [GC-MS] Analysis: GC-MS analysis was performed following the protocol of Chipiti *et al.,* [30] with modifications to identify the compounds present in the ethanolic extract. The extract was diluted with chloroform and subjected to Agilent Technologies 7890AGC system coupled with (an Agilent) 5975C Mass Selective detector. A HP-5MS capillary column (30 m x 0.25 mm internal diameter, 0.25 µm film thickness) was applied. The carrier gas was helium with the injector temperature at 320 °C. The initial oven temperature was at 70 °C which was programmed to increase to 280 °C at the rate of 10 °C/min with a hold time of 4 min at each increment. Injections of 1 µL were made in split mode with a split ratio of 100:1.

The mass spectrometer was operated in the electron ionization mode at 70 eV and electron multiplier voltage at 1859 V. Other MS operating parameters were as follows: ion source temperature 230 °C, quadrupole temperature 150 °C, solvent delay 3 min and scan range 33 - 550 amu. The compounds were identified by direct comparison of the mass spectrum of the analyte at a particular retention time to that of a reference standard found in the National Institute of Standards and Technology (NIST) library. The total GC-MS running time lasts 45 minutes. At least 80% similarity index was considered significant [31].

RESULTS AND DISCUSSION: The ethno-medicinal uses of Lunasia sp. in Agusan del Sur based on the accounts of the key informants revealed that traditional ethnomedicinal uses include as an anti-inflammatory (for wounds, bites, skin diseases, fever, ulcer, nausea, heartburn and gastroenteritis), anti-motility (diarrhea), anti-histamines (for skin allergies and itchiness), antiparasitic (for malaria), antibacterial (for skin diseases and stomach troubles), anti-toxin (for food poison, poisoning, tetanus, snake and insect venom) and antiviral (for rabies, chikungunya and dengue) (**Table 1**).

TABLE 1: ETHNOMEDICINAL USES OF THE LUNASIA SP. IN AGUSAN DEL SUR, PHILIPPINES

S. no.	Preparation	Modes of application	Traditional medical use
1	Infusion	External (Rubbing)	For burn, cuts, bruises and wound healing; and for treatment of bites (insects, snakes and dogs), skin diseases and allergies.
2	Tincture	Internal (Oral)	For the treatment of stomach troubles like diarrhoea, gastroenteritis, vomiting, ulcers and heartburn; and cure for rabies, malarial, chikungunya and dengue infections

Treatments were either by the use of an infusion with coconut oil or a tincture with local wine into the bark of the vine. The modes of application include external application by rubbing the surface of affected or infected parts such as wounds, allergies, skin infection and bites of dogs, snakes and insects. Oral application by drinking the alcohol-tinctured preparation is for treatment of stomach troubles, poisoning, ulcer, nausea, diarrhea, gastroenteritis or as an antitoxin, antibacterial and antiviral treatment. The information generated from the key informants shows similarities of other reported traditional use of *Lunasia amara* Blanco in the treatment of various health issues ranging from treatment of a variety of health issues and conditions ranging from snake bites, stomach troubles and diarrhea [20], gastralgia and adynamic conditions of digestive system [32], infected eyes, swollen limbs and skin diseases [33], tuberculosis [15], tropical ulcers [21], bacterial infections and diseases [24]& infertility [34, 35].

Since the tincture and infusion of the plant are generally good source for treatments of food poisoning, toxinoses, diarrhea, stomach troubles, wounds, skin diseases and infections, then antimicrobial properties of the extract were therefore evaluated.

The results of antibacterial test revealed the extract has positive activity against tested organisms (**Table 2** and **Fig. 2**). The negative control does not show any inhibition. The anti-bacterial activities of

Lunasia sp. vine bark ethanolic extracts is in concurrent with early studies showing the anti-bacterial activities of the alcoholic extracts from leaves of *L. amara* against Gram-positive bacteria *i.e. B. subtilis* ATCC 6633 and *S. aureus* ATCC 25923 and multidrug resistant strains of tuberculosis *M. smegmatis* ATCC 607, *M. tuberculosis* H_{37} Rv and *M. avium* [36].

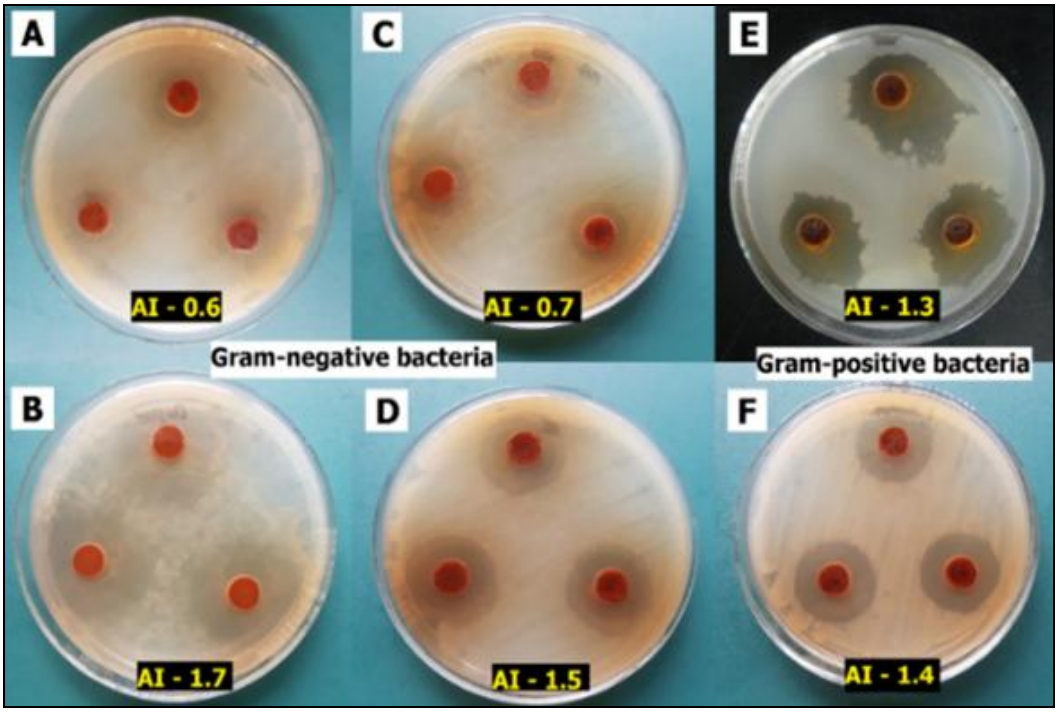


FIG. 2: THE ANTIMICROBIAL INDICES (AIS) OF GRAM-NEGATIVE BACTERIA: *E. COLI* (A), *K. PNEUMONIAE* (B), *P. AERUGINOSA* (C) AND *S. TYPHIMURIUM* (D); AND GRAM-POSITIVE BACTERIA: *B. SUBTILIS* (E) AND *S. AUREUS* (F) USING AGAR WELL METHOD IN TRIPLICATES OF THE LUNASIA SP. VINE BARK ETHANOLIC EXTRACT

TABLE 2: ANTIMICROBIAL ACTIVITY OF BARK ETHANOLIC EXTRACT (6.25mg/ml) AND POSITIVE CONTROL (30.0 µG CHLORAMPHENICOL)

UPCC Test Organism	Test Sample	Inhibition zone (mm)				Antimicrobial Index (AI)
		R1	R2	R3	Mean	
Gram-negative bacteria						
E. coli UPCC 1195	'Lunas-bagon' extract	16	16	17	16.33	0.6
	Chloramphenicol		27		27	3.5
K. pneumoniae UPCC 1360	'Lunas-bagon' extract	27	27	28	27.33	1.7
	Chloramphenicol		38		38	5.3
P. aeruginosa UPCC 1244	'Lunas-bagon' extract	17	17	18	17.33	0.7
	Chloramphenicol		15		15	1.5
S. typhimurium UPCC 1368	'Lunas-bagon' extract	25	25	25	1.5	1.5
	Chloramphenicol		30		30	4.0
Gram-positive bacteria						
B. subtilis UPCC 1295	'Lunas-bagon' extract	23[a]	23[a]	23[a]	23[a]	1.3
	Chloramphenicol		20		20	2.3
S. aureus UPCC 1143	'Lunas-bagon' extract	24	24	24	24	1.4
	Chloramphenicol		33		33	4.5

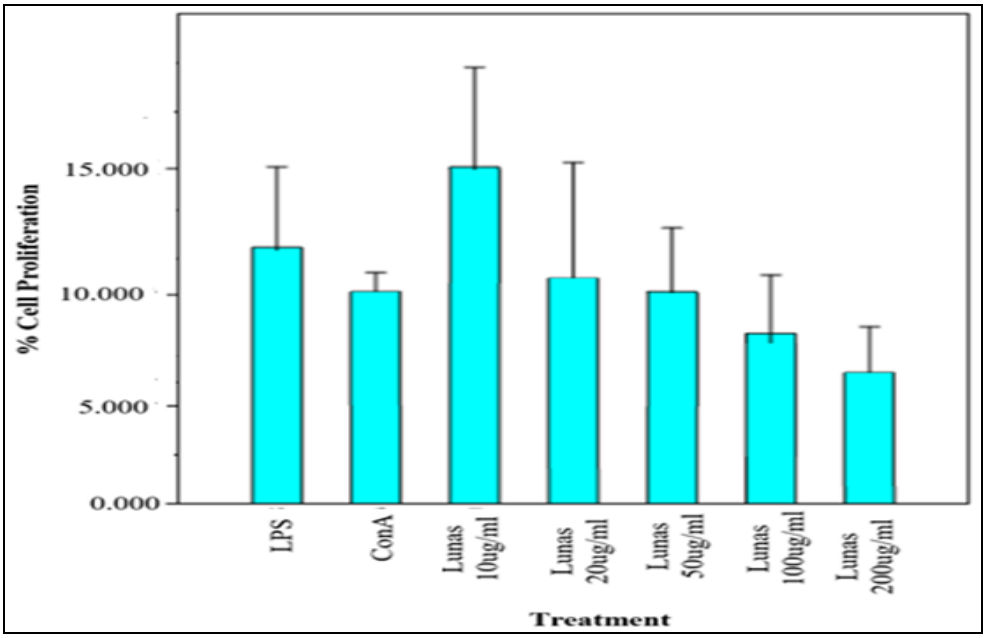
[a] Irregular inhibition zone, R – Replicates

The quantitative analysis of the results of the MTT assay on BALB/c showed that there is no significant differences between the bark ethanolic extracts and the untreated controls (**Table 3**). However, qualitative inspection of the data (**Fig. 3**) showed a reduction in the metabolism of the cells of MTT when the concentrations of the extracts were increased to 200.0 ug/ml. It can be argued that there is an optimum amount of the compounds in the extract to not affect cellular metabolism but increasing them may have induced apoptosis or inhibited proliferation of the cells. However, the reported MTT cytotoxic activity of *L. amara* wood extract against two human cancer cell lines, cervical cancer cells (HeLa) and breast cancer cells (T47D) showed potential inhibitory activity using ethyl acetate as the most effective solvent as compared to methanol and n-hexane [37].

TABLE 3: RESULT OF THE ONE-WAY ANOVA FOR SIGNIFICANT DIFFERENCE WITHIN AND AMONG DIFFERENT TREATMENTS

	Sum of Squares	df	Mean Square	F	p
Between groups	206.051	6	34.3416	0.8356	0.5636
Within groups	534.146	13	41.0882		
Total	740.197	20			

p<0.05, significant; Levene´s test for homogeneity of variance, from means, p = 0.1021



LPS – Lipopolysaccharide, Con A (Concanavalin A)

FIG. 3: PERCENT CELL PROLIFERATION OF BAL B/C MICE SPLENOCYTES USING MTT CYTOTOXICITY ASSAY

Priliminary biochemical tests revealed the presence of flavonoids, steroids, tannins, fatty acids, alkaloids and saponins but absence of cynogenetic glycosides and anthroquinones (**Table 4**).

TABLE 4: RESULTS OF PHYTOCHEMICAL SCREENING OF THE VINE BARK ETHANOLIC EXTRACT OF LUNASIA SP.

Alkaloids	Saponins	Flavonoids	Steroids	Tannins	Cyanogenic glycosides	Anthraquinones	Fatty acids
++	++	+++	+++	+++	-	-	+++

(+) indicates present: +turbid, ++moderate, +++heavy; (-) indicates absent

The presence of these phytochemicals may explain the medicinal (antibacterial, antiviral and antitoxin) and therapeutic (stimulant, anticancer and analgesic) properties of the plant. The absence of cyanogenic glycosides and anthraquinones of Lunasia sp. vine bark extract may indicate no or less toxic effect as these compounds, cyanogenic glycosides may cause food poisoning resulting in gastric irritation and damage [38] while anthraquinones and its derivatives may cause nausea, vomiting, abdominal cramps and diarrhea with both therapeutic dose and overdose [39].

Related studies on *L. amara* show the essential biological activities of Lunasia sp. can be attributed to the presence of quinoline alkaloids for antibacterial activity [24]. Alkaloids like graveolinine, 4-methoxy-2-phenylquinoline and kokusagine were found to have antituberculosis activity [40]. Quinoline alkaloids are the active principle such as the lunacrine and lunamarine for the central nervous system activity. Another alkaloid, lunacridine and its trifluoroacetyl derivative were found to be effective for DNA intercalation, topoisomerase II decatenation, cytotoxicity and caspase activation activities [15]. The presence of a fully aromatic ring and 4-methoxyl group of quinoline alkaloids were attributed to be the active principle in the antitubercular activity of the extract against *M. tuberculosis* multidrug resistant strains [40, 22].

Further analysis of the extract using GC-MS showed six peaks indicated the presence of six phytochemical constituents (**Fig. 4**). When the

compounds were characterized and quantified on the comparison of the mass spectra of the constituents with the National Institute of Standards and Technology (NIST) library, three compounds with over 80% similarity and other three with less similarity but high quantities were identified (**Table 5**).

TABLE 5: PHYTOCOMPONENTS IDENTIFIED IN THE BARK ETHANOLIC EXTRACT OF LUNASIA SP. VINE VARIETY USING GC - MS

Reference Compounds with the NIST library	Retention Time	Similarity of the compounds in the extract compared with the mass spectra of the constituents with the NIST library	%
α-copaene*	13.855	97	11.054
α- Gurjunene*	14.308	99	29.099
β-caryophyllene*	14.460	93	14.540
β-selinene	14.136	68	6.510
calamenene	16.026	64	30.932
globulol	15.518	53	7.865

*considered compounds having over 80% similarity with the standard

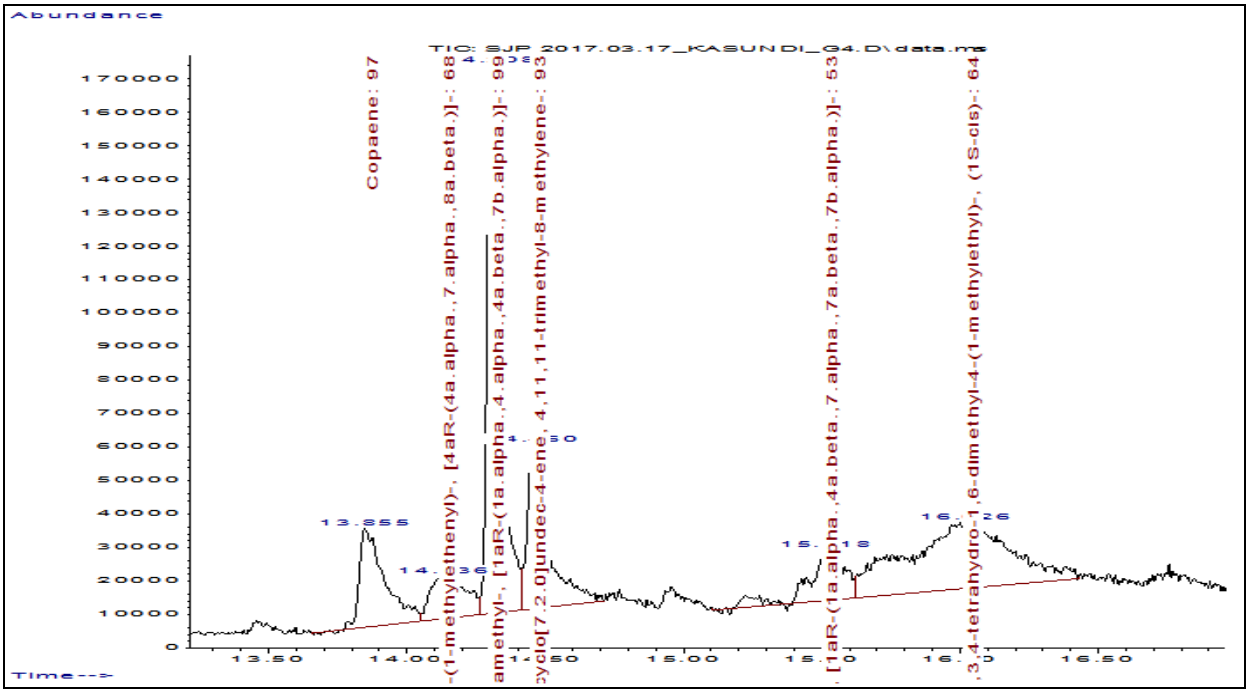


FIG. 4: GC-MS CHROMATOGRAM PEAK TIME AND ABUNDANCE OF LUNASIA SP. VINE BARK ETHANOLIC EXTRACT

Of the six essential oils identified, α-copaene, α-gurjunene and β-caryophyllene were determined with over 90% similarity to sesquiterpenes. These three compounds of essential oils were earlier reported to have medicinal and therapeutic properties, thus could also be the same compounds which contributed to the antibacterial activity and the folk medical uses of Lunasia sp. vine variety. It was shown that the high amount of α-copaene in the essential oil of inner bark of *Kielmeyera coriacea* Mart. and Zucc. enables the oil to exhibit significant antimicrobial activity against the anaerobic bacteria *Prevotella nigercens* but not animal cells [41]. Also, the cytotoxicity results of α-copaene against Vero cells (kidney fibroblasts, African green monkey) revealed low toxicity [41]. The compound α-gurjunene was reported to be a potent antimicrobial and antibacterial agent [42]. Likewise, β-caryophyllene compound was also reported to show antibiotic effects [43].

It is also important to note that these three compounds were not only effective antimicrobials but were also reported to be effective chemotherapeutics. A Study revealed the anti-proliferative, antioxidant, anti-genotoxic and cytotoxic activities on rat neuron and N2a neuroblastoma cell lines indicating that α-copaene

exhibited mild cytotoxic effect on N2a neuroblastoma cell [44]. The β-caryophyllene compound was reported to act as analgesic, anti-inflammatory, antioxidant, anticarcinogenic and local anesthetic activities [43]. It was also shown in a studythat the β-caryophyllene's anxiolytic and anti-depressant effects explain its use for the treatment of anxiety and depressive disorders [45]. The other three constituents in the extract were having low similarity were β-selinene, calamenene and globulol. As the similarity was less than 80% it could be suggested that these three compounds are probably derivatives with the similar antimicrobial and cytotoxic properties of β -selinene, calamenene and globulol. PubChem bioassay database in NCBI recorded that the bioactive β-selinene is used as anti-acne, antibacterial, antiviral, antimycotics, analgesic, antipyretic, anti-inflammatory and antineoplastic and the synthesized β-selinene drugs were used for joint disorders or arthritis and treatments for dermatological disorders.

The bioactive β-selinene was also found to have anti-spasmodic action relieving flatulence, colic pain, vomiting and calming the digestive system from gastric disorders [46].

However, calamenene-rich essential oils containing 7-hydroxycalamenene were reported to have a more effective antimicrobial activity against methicillin resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecalis*, *Mycobacterium tuberculosis*, *M. smegmatis*, *Mucor circinelloides* and *Rhizopus oryzae*a side from being a potent source of antioxidant [47]. Finally, the bioactive globulol was reported as anti-infective (antibiotics, antiseptics and chemotherapeutics), antipyretic, anti-inflammatory, anti-asthmatic, antipsychotic, anxiolytic, antidepressant, analgesic and synthesized as drug for dermatological and nervous system disorders as shown in the NCBI Pubcem Bioassay Database. The presence of these six compounds detected in the ethanolic extracts of Lunasia sp. may provide scientific bases for the traditional use of this plant for the treatment not only for microbial infections but also for other health-related concerns.

CONCLUSION: The results of this study show the antimicrobial properties of the bark ethanolic extracts of the vine variety 'Lunas-bagon' of Lunasia sp. against Gram-negative and Gram-positive bacteria. The extracts also did not significantly affect cellular metabolism of splenocytes, thus it is nontoxic. The absence of cyanogenic glycosides and anthraquinones may also explain the absence of toxic compounds in the ethanolic extracts, thus it will not inhibit cell proliferation or induce apoptosis in normal cells. The identification of some bioactive compounds in *Lunasia* sp. through phytochemical screening and GC-MS analysis demonstrated that these were having biomedical applications other than their uses as antimicrobials. The information generated clearly indicates that the the folk medical uses of Lunasia sp. vine bark have biomedical and biochemical bases.

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