ISSN (Print): 2476-8316
ISSN (Online): 2635-3490
Dutse Journal of Pure and Applied Sciences (DUJOPAS), *Vol. 7 No. 1March 2021*

Phytochemical Constituent and Antifungal Potentials of *Mentha piperita* (Pepper Mint) Leaf Extract on Urophathogenic *Candida albicans*

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Abstract

Mentha piperita (Lamiaceae),the pepper mint plant is an aromatic perennial herb 50-90cm high, cultivated in most part of the world,have traditionally been used in folk medicine.A total of 130 urine samples were collected from patients examined for urinary tract infection (UTIs) using standard microbiological procedures. The collected samples were subjected to isolation on specific medium using Cysteine Lactose Electrolyte Deficient (CLED) and Saboraud dextrose agar. The purified isolates were confirmed using biochemical tests, agar well diffusion test, minimum inhibitory concentration(MIC) and minimum fungicidal concentrations (MFC) were used to assess the antifungal activity of Methanolic, n- Hexene and aqueous extract of Menthapiperita, ketoconazole was used as positive control. The results showed that highest zone of inhibition was recorded for aqueous extract followed by methanol and least zone diameter was recorded in n-Hexene extract at a range of, 21.33±1.00, 16.00±1.00 and 8.33±0.57respectively. The phytochemical screening revealed the presence of secondary metabolites such as: reducing sugar, saponins, phenols, flavonoids, alkaloids and tannins. The minimum inhibitory concentration ranged from 6.25mg/ml in methanolto n-Hexene with 3.13mg/ml. The minimum fungicidal concentrations ranged from 3.13mg/ml in methanol and aqueous 0.78mg/ml while n-Hexene at 6.25mg/ml. Mentha piperitais recommended to be used as treatment of urinary associated diseases due to its high activities shown when compared with positive control. The ability of this plant extracts to inhibit the UTI pathogens indicated its potentials to be used as alternative source of synthetic drugs

Keywords: Antifungal activity, *Candida albicans, Mentha piperita,* phytochemicals

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Introduction

Diseases due to pathogenic fungi represent a critical problem to human health and is one of the main causes of morbidity and mortality worldwide (WHO, 1998). Fungi capable of developing resistance to drugs used as therapeutic agents, this resistance is a growing problem, and the future of antimicrobial treatments remains uncertain (Carreto *et al.,* 2010). Current antifungal drugs have demonstrated limitations such as low potency, low solubility, toxicity and the development of resistance by fungal strains. Thus, the investigation of natural products against *Candida* sp. has increased significantly over the last decade (Carreto *et al.,* 2010). *Candida* species are opportunistic fungal pathogens that can cause local or systemic infections in humans where prolonged treatment with antibiotics give them chance to become resistance to drugs (Duarte *et al.,* 2005).

Wide use of synthetic and semi-synthetic antimicrobial drugs, their advantages and disadvantages have been highlighted over the years, including the spread of drug-resistant pathogens, and research has focused on the use of natural products as useful antimicrobial tools (Wińska *et al.,* 2019), because it is presumed that drugs developed from plant sources may have minimal and very slow to induce drug resistance among the pathogens (Pramila *et al.,* 2011). The investigation of the efficacy of plant-based drugs used in traditional medicine have been paid great attention because they are cheap and have little side effects (Dharmasiri *et al*., 2003).Plants have been reported to contain many bioactive compounds making them sources of different substances of medicinal uses. These substances include alkaloids, flavonoids, steroids, resins, fatty acid tannins and phenols (Nair *et al.,* 2005; Sheeba, 2010; Balasaheb and Yogini, 2015). Bax *et al.* (2000) reported that global antimicrobial resistance is becoming an increasing public health problem and the pharmaceutical industries and fledgling biotechnology companies are intensifying efforts to discover novel antimicrobial in attempt to overcome microbial resistance and therefore, the search for new and more effective antimicrobial agents is a continuous process.

Mentha piperita (Lamiaceae),the pepper mint plant is an aromatic perennial herb 50-90cm high, cultivated in most part of the world,have traditionally been used in folk medicine. Leaves of mint plant are frequently used as herbal tea and for culinary purpose to add flavour and aroma. The distinctive smell and flavour, a characteristic feature of Mentha species, is due to the naturally occuring cyclic terpene alcohol called menthol. Menthol is prescribed as a medication for gastrointestinal disorders,common cold and muscular skeletal pain (Patil *et al*.,2007).The mint plants are rich sources of iron and magnesium, which play important roles in human nutrition (Arzani *et al*., 2007).

Urinary tract infections are serious health problem affecting million of people every year (Harkins, 2000; Griffiths, 2003). Urinary Tract Infections can be manifested as symptomatic or asyptomatic infection based on the presence or absence of the symptoms and hence symptoms enchance the diagnosis process among young healthy humans (Kunin,1997). Haris *et al.,* (2017) reported urinary tract infections as more common in women than men because they have a shorter urethra. This research work aimed to evaluate the phytochemical constituents and establish the antifungal effects of *Mentha piperita* leaf (Plate 1) extract on urophatogenic *C. albicans*.

Plate 1: Mentha piperita plant

Materials and Methods

Collection and extraction of plant materials

The plant was collected from Sharada Garden along Dan'agundi, Kano, Nigeria, using the protocol of Haris *et al.* (2017). The collected plant was brought to the Herbarium section of Bayero University Kano in a polythene bag for taxonomic identification. A voucher specimen number: BUKHAN0337 was given to the specimen. The plant material was brought to the microbiology laboratory, Bayero University Kano, rinsed with water to remove foreign materials and dried at a temperature of 40 ^{0}C before examination (Ahmed *et al.,* 2010; Ogugu *et al.,* 2012; Lalisan *et al.,* 2014; Haris *et al.,* 2017). The dried leaves were pulverized into powder using sterile electric blender (Haris *et al.,* 2017). The powdered leaves were divided into three different bottles and separately extracted using water, methanol and n-Hexane. Percolation method was carried out for over a period of seven days (Parra *et al.,* 2001). The extract was filtered using filter paper (Whatman No.1) and the filtrate was concentrated to dryness under reduced pressure using rotary evaporator at 40-50 ^{0}C. The aqueous extract was dried by gentle heating at 45^{0}C using water bath (Mayorga *et al.,* 2010). The dried extracts were then transferred into an air-tight container for further analysis.

Phytochemical Tests

Mentha piperita extract was subjected to qualitative phytochemical screening according to standard procedures to test for the presence of the following secondary metabolites; alkaloids, flavonoids, saponins, tannins, Cardiac glycosides and steroids as described byOdebiyi and Sofowora, (1978); Sofowora, (1984); Ogukwe *et al.,* (2004); Hassan *et al.,* (2005); El-mahmood and Doughari, (2008); William *et al.*,(2015).

Microbiological Analysis

Collection of urine samples

A total number of 130 urine samples from UTIs suspected patients were collected from Muhammad Abdullahi Wase Specialist Hospital Kano, Nigeria, and were transported immediately to microbiology laboratory, Bayero University Kano in an ice box maintained at 4 ^{0}C (Haris *et al.,* 2017).

Isolation of Fungal Specie

The urine samples were streaked onto sterile Cystine Lactose Electrolyte Deficient (CLED) agar and Saboroud Dextrose agar (SDA), then incubated at 37^{0}C for 24hrs (Haris *et al.,*

2017).The colonies that appear as pasty, opaque and off-white resembling bacterial colonies with a sweet smell like that of a ripe apple on CLED agar and later transferred to Saboroud Dextrose agar SDA. The isolates were identified microscopically according to their morphology as filamentous extension from yeast cells using germ tube test (Chessbrough, 2006; Ochei and Kolhatkar, 2007), the identified isolates were preserved in SDA broth and kept in refrigerator for further analysis.

Standardization of fungal inoculum

The fungal isolates were sub-cultured onto sterile Saboroud agar plates incubated at 37^0C for 24hrs; the sub-cultured isolates were inoculated into 5ml of sterile normal saline and compared with 0.5 Macfarlands turbidity standard that marched with 0.1×10^8cfu/ml (Cheesbrough, 2006).

Preparation of Concentrations

Di-methyl Sulfoxide (DMSO) was used for Methanolic and n-Hexane extracts while sterile distilled water was used for aqueous extract, double-fold dilutions were made for the four concentrations; 100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml (Haris *et al.*, 2017).

Antifungal Susceptibility Assay

One (1) ml of standardized inoculums of *C. albicans* were spread onto a prepared solid medium of SDA using a sterile glass rod (El-Mahmood and Ameh, 2007). The content was allowed to solidify. Using sterile cork borer, 5 wells approximately 6mm in diameter and 2.5mm deep were bored on the agar medium surfaces. Then 0.1ml of reconstituted extract at 12.5mg/ml, 25mg/ml, 50mg/ml and 100mg/ml were pipetted each into different wells, 0.1ml of pure respective solvents into another well as negative control, then 0.1ml of 12.5 mg/ml ketoconazole concentration was used for comparison as positive control (Abubakar, 2009; Haris *et al.*, 2017). These were allowed to stand for one hour on the laboratory bench for proper diffusion of the extracts into the media (Boardi *et al.*,2015).The plates were incubated at 37^0C for 24hrs and were observed for zone of inhibition (Igbinosa, 2009).

Determination of Minimum Inhibitory Concentration (MIC)

The lowest concentration of the extract that inhibits the growth of the test organisms was determined at 12.5mg/ml, 6.25mg/ml, 3.125mg/ml, 1.5625mg/ml, 0.78125mg/ml respectively. The tubes were incubated aerobically at 37^0C for 24hrs; Clear suspension shown after incubation was regarded as positive results.

Determination of minimum fungicidal concentration (MFC)

This was carried out with some modifications according to Irkin and Koruhrogh, (2007); lajuyigbe and Afolayan, (2012) by using fresh Mueller Hinton agar, isolates were inoculated with one loop full of culture taken from each of the first three broth cultures that showed no growth in the MIC tubes. Minimum Fungicidal Concentration assay plates were incubated for 24hrs. After the incubation period, the lowest concentration of the extract that did not show any fungal growth on the solid medium was regarded as MFC results.

Thin-layer Chromatography

The thin layer chromatography (TLC) of the leaves of *Mentha piperita* was performed on glass slides coated with silica gel (0.2mm Kiesel-gel 60 F254,Merck).The plates were dried and activated at 110^0C for 30minutes in an oven. The crude leaf extracts were then applied in to the TLC plates using micro-capillaries, the plates were dried and developed using chloroform and ethanol(4:2),(Wagner and Bladt, 1996). In one plate, the separated compounds were visualized with UV light (365 and 254nm) and sprayed with sulphuric acid

in ethanol spray reagent followed by heating at110^{0}C for 15 minutes. The retention factor (Rf) measures were determined by triplicate analyses for each extract using the following equation.

$$Rf = \frac{\text{Distance Travelled by the Spot}}{\text{Distance Travelled by the Solvent}}$$

Bioautography Agar Overlay Techniques

Fungal suspensions were adjusted to 0.5 MacFarland standard then, nineteen (19) ml of muller Hinton medium was poured on TLC plates placed in petri dishes, microbial suspension was placed on the medium and incubated at 37^{0}C for 48hrs. Microbial growth inhibition was determined by measuring the area of the inhibition zones after being revealed with a tetrazolium chloride solution. *Candida albicans* reduced tetrazolium salt through dehydrogenase activity and produced intensely coloured formazan, as reported by Eloff, (1998). The inhibition area was observed and classified as 0, - + in line with the length area of the chromatographical spot. The identified compounds by TLC that showed antifungal activity in the bioautographic method were chosen for GC/MS analysis.

Gas Chromatography-Mass Spectrometry

This technique was used in this study to identify the phyto-components present in the extracts. The analysis was carried out at National Research Institute of Chemical Technology (NARICT), Zaria, Nigeria. The TLC fractions of *Mentha piperita* leaf extract were performed using a QP2010 PLUS SHIMADZU, JAPAN column Flow (1.58ml/min). Gas Chromatography oven was kept at 80^{0}C for 5min and oven temperature programmed 280.00^{0}C for 5min at the rate of 10.00^{0}C/min, kept at 280^{0}C for 3min and then programmed (MS) to 230.00^{0}C for 2.50min, the injection temperature was kept at 250.00^{0}C for 5min.Identification of individual components was done using the NIST05 library of crude constituents.

Identification of Components of *Mentha piperita*

The interpretation of mass spectrum (GC-MS) was conducted using the database of National Institute Standard and Technique (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The composition and relative percentages of the peppermint leaf were elucidated by the aid of GC and GC/MS analyses. As a result, 12 major compounds were identified.

Statistical Analysis

Sensitivity experiments were carried out in triplicates. Data obtained were subjected to statistical analysis using one way Analysis of Variance (ANOVA).Turkey-Kramer Multiple Comparisons Test was used to separate the means. $P<0.05$ was considered significant, with the aid of SPSS version 16.0.

Results

Table 1 shows the preliminary phytochemical analysis of saponins, phenols, flavonoids, tannins and reducing sugars were detected from methanol and aqueous leaves extract while only saponins were detected in n-hexane.

Table 1: Preliminary Phytochemical Screening of Extracts of *Mentha piperita*

Constituents	Methanol	n-Haxene	Aqueous
Reducing sugars	+	-	++
Glycosides	-	-	-
Flavonoids	-	-	+
Saponins	-	+	++
Tannins	+	-	+
Phenols	++	-	+
Alkaloids	-	-	+

Key: **+** = Positive (Slightly present**); ++** = Positive (Moderately); **-** = Negative (Absent).

Among the 130 clinical samples collected, 67 were positive and 63 were negative. From the 67 positive samples only 6 shows the presence of *Candida albicans* as shown (Table2)

Table 2: **Germ Tube Test for Identification of *Candida albicans***

Isolate	Morphology	Germ tube tests	Suspected organism
C007	Yeast cells	+	C.albicans
C011	Yeast cells	+	C. albicans
C023	Yeast cells	+	C.albicans
C032	Yeast cells	+	C. albicans
C100	Yeast cells	+	C.albicans
C106	Yeast cells	+	C. albicans

Key: **+** = Positive

Table 3 shows the interpretation of physical nature of the plant extracts as it appeared as semi- solid and liquid form with viscosity. Color, time taken for extraction was differed among the solvent used. The result showed that higher yield of 6.4g and 5.6g was found in Aqueous and Methanolic extracts

Table 3: The Physical Assessment of the Various Extracts of *Mentha piperita*

Solvents	Color	Texture	Weight (grm)
Methanol	Green	Jelly syrup	5.6g
n-Hexane	Light green	Oily paste	2.0g
Aqueous	Brown	Syrup	6.4g

Tables 4 to 6 present the evaluation of antifungal potentials by agar well diffusion method which indicated that all the *Candida albicans* tested showed growth inhibition towards the plant extracts, methanolic and aqueous extracts showed high antifungal activity of 21.33 ± 1.15 and 16.±0.00 mm against C007 and C100 respectively, but C100, C011, and C32, have showed moderate effect with n-hexane extract of 14.66±4.04mm and 9.00±0.000mm. The standard antifungal drug (ketoconazole) used as positive control showed the growth inhibition of 25.33 ± 0.577mm respectively.

Table 4: Antifungal Susceptibility of Test Organisms to Methanolic Extract of *Mentha piperita*

Isolate	Inhibition Pattern (mg/ml)				Positive Control
	100	50	25	12.5	
					25.33±0.57
C007	12.00±1.00	10.66±0.00	9.66±0.00	7.33±1.00	
C011	10.66±0.00	10.00±1.00	8.00±1.00	7.33±1.15	
C023	13.33±1.15	10.33±0.01	9.33±0.57	8.66±1.52	
C032	10.33±1.52	10.66±1.15	9.00±1.00	8.33±0.03	
C100	16.00±0.00	14.66±1.00	10.66±0.51	9.33±1.15	

Table 5: Antifungal Susceptibility of Test Organisms to n-Hexene Extract of *Mentha piperita*

Isolate	Inhibition pattern (mg/ml)				Positive Control
	100	50	25	12.5	
					25.33±0.57
C007	0.00±0.000	0.00±0.000	0.00±0.000	0.00±0.000	
C011	12.00±0.00	11.66±1.00	10.00±1.00	8.33±0.15	
C023	9.00±0.15	7.33±0.58	0.00±0.00	0.00±0.00	
C032	9.66±1.52	7.33±0.57	0.00±0.000	0.00±0.000	
C100	15.66±0.00	12.66±1.00	10.33±0.51	9.33±1.15	

Table 6: Antifungal Susceptibility of Test Organisms to Aqueous Extract of *Mentha piperita*

Isolate	Inhibition Pattern (mg/ml)				Positive Control
	100	50	25	12.5	
					25.33±0.57
C007	21.33±1.15	12.66±0.00	9.33±1.00	7.33±0.57	
C011	19.66±0.00	15.00±1.00	12.00±1.00	9.00±1.00	
C023	16.00±1.00	12.00±0.01	9.00±1.00	6.66±1.15	
C032	9.33±0.57	8.66±0.57	7.00±1.00	0.00±0.000	
C100	12.00±0.00	10.00±1.00	9.33±0.51	7.33±0.57	

Table 7-11 shows the results of minimum inhibitory concentration and fungicidal concentration of the plant extracts against the test organisms were 6.25mg/ml, and 3.13mg/ml with all the isolates but C032, and C007 have showed their MIC and MFC values of 1.57mg/ml, and 0.78mg/ml respectively. A wide range of MIC values were recorded depending on the fungal strains.

Table 7: Minimum Inhibitory Concentration of *Candida albicans* using Methanolic Extract

Isolate	Concentrations (mg/ml)				
	12.50	6.25	3.13	1.57	0.78
C007	-	β	+	+	+
C011	-	β	+	+	+
C023	-	-	β	+	+
C032	-	β	+	+	+
C100	-	β	+	+	+

Key: + = Turbidity observed, - = No Turbidity observed, β = MFC value

Table 8: Minimum Inhibitory Concentration of *Candida albicans* using n-Hexene Extract

Isolate	Concentrations (mg/ml)				
	12.50	6.25	3.13	1.57	0.78
C007	-	-	β	+	+
C011	-	-	β	+	+
C023	-	-	β	+	+
C032	-	-	-	β	+
C100	-	-	β	+	+

Key: + = Turbidity observed, - = No Turbidity observed, β = MFC value

Table 9: Minimum Inhibitory Concentration of *Candida albicans* using Aqueous Extract

Isolate	Concentrations (mg/ml)			
	12.50	6.25	3.13	1.57
C007	-	-	β	+
C011	-	β	+	+
C023	-	β	+	+
C032	-	β	+	+
C100	-	-	β	+

Key: + = Turbidity observed, - = No Turbidity observed, β = MFC value

Table 10: Minimum Fungicidal Concentration of *Candida albicans* using Methanolic Extract

Isolate	Concentrations (mg/ml)				
	12.50	6.25	3.13	1.57	0.78
C007	-	-	-	-	β
C011	-	β	+	+	+
C023	-	-	-	β	+
C032	-	-	β	+	+
C100	-	-	-	β	+

Key: + = Turbidity observed, - = No Turbidity observed, β = MFC value

Table 11: Minimum Fungicidal Concentration of *Candida albicans* using n-Hexexne Extract

Isolate	Concentrations (mg/ml)				
	12.50	6.25	3.13	1.57	0.78
C007	-	β	+	+	+
C011	-	β	+	+	+
C023	-	-	β	+	+
C032	-	-	-	β	+
C100	-	β	+	+	+

Key: + = Turbidity observed, - = No Turbidity observed, β = MFC value

Table 12: Minimum Fungicidal Concentration of *Candida albicans* using Aqueous Extract

Isolate	Concentrations (mg/ml)				
	12.50	6.25	3.13	1.57	0.78
C007	-	-	-	-	β
C011	-	-	-	β	+
C023	-	β	+	+	+
C032	-	-	β	+	+
C100	-	-	β	+	+

Key: + = Turbidity observed, - = No Turbidity observed, β = MFC value

Table 13 presents the result of bioautography which aided in the identification of the antifungal principle active component. This assay was applied to all extracts and the inhibitory activities are equal to chromatographic spot.

Table 13: TLC Spots Data by Bioautography Method

Extract	Colour Band	Solvent Ratio	Rf-value	Activity
Methanolic	Green	4:2(Chlo :Etha)	0.35	+
n-Hexane	Blue-green	4:2(Chlo :Etha)	0.44	-
Aqueous	Yellow	4:2(Chlo :Etha)	0.15	+

Key: (-); Lesser inhibition activity than chromatographic spot; **(+);** Inhibition activity equal to chromatographic spot, Chlo: chloroform and Etha: ethanol.

The main compounds of the leaf extracts of *M. piperita* studied are given in Table 14.The predominant components in *M. piperita* were 9,12-octadecadienoyl chloride (24.61%),Methyl trans,trans-9,12-octadecadieno (13.92%) and 9,12,15-octadecatrien-1-ol (11.13%).

Table 14: Major Components of *Mentha piperita* Fractions Identified by Gas Chromatography/Mass Spectroscopy

S/N	Name of Compounds Detected	Area (%)	R.I
1.	Methylpentadecanoate	5.15	1814
2.	Methyl trans,trans-9,12-octadecadieno	13.92	2093
3.	Methyl(11E)-11-octadecenoate	10.95	2085
4.	Methyl linolen	7.89	2101
5.	Methyl n-octadecanoate	5.41	2077
6.	1,2-Ethanediyl ester	7.49	0
7.	Z-17-Nonadecen-1-ol acetate	1.60	2284
8.	9,12-octadecadienoyl chloride	24.61	2139
9.	9,12,15-Octadecatrien-1-ol	11.13	2077
10.	Vinyl octadecyl ether	9.29	2075
11.	Trimethylsilyl ether	0.44	1705
12.	Glycerol 2-monooleate	2.11	2705

Discussion

The extraction of crude extracts from *Mentha piperita* leaves using polarity of different solvents was in agreement with the findings of Naiman and Mazharuddin (2016); Para *et al.*(2013); Paramila *et al.*(2012). The property of less activity to extract some secondary metabolites using less polar organic solvents such as n-hexane corroborate with the findings of Haris *et al.,* (2017). He also reported the inhibition of urinary tract infection pathogens using solvents of different polarity in the extraction process. Tannins and flavanoids have therapeutic uses due to their anti-inflammatory, antifungal, antioxidant and healing properties (Thiago *et al*., 2008).Thus, the antifungal activity of leaf extract of *Mentha piperita* can be deduced to be as a result of the presence of tannins and flavonoids metabolites as described by Thiago *et al*., (2008)

Isolation and identification of *C. albicans* from urine was in agreement with the result of Ochie and Kolhatar (2007). Methanolic and aqueous extracts of the plant exhibited higher antifungal effects than n-hexane extract. The various solvent extracts of the plant have shown good antifungal effects against the pathogens. There was a slight variation in the results obtained for the remaining solvent. The difference in the antifungal activity with the same source when extracted with different solvent has proven that not all phytochemicals that are responsible for antifungal activities soluble in a single solvent. Hence solvents of different polarity employed in the present study was in accordance with the report of Naiman and Khan, (2016). Two possibilities that account for this higher activity are the nature of biological active components (alkaloids, flavonoids. tannins. phenols e.t.c.), which was enhanced in the presence of methanol and aqueous solvents, and their stronger extraction capacity (polarity) that have yielded a greater number of active constituents responsible for antifungal activity, this was in agreement with the report of Gosh *et al*., (2008); Haris *et al.,* (2017). The activity of the extracts from the broth dilution assay at low concentration corroborate with findings of Pramila *et al.,*(2012).

The predominant compounds detected in *Mentha piperita* fractions were reported by several authors to have antimicrobial effect(Mckay and Blumberg, 2006; Bassole *et al*., 2010;

Mohaddese and Nastaran, 2014).The 12 compounds identified were in agreement with the result of Gökalp *et al*.(2002) that detected 12 compounds in their study using essential oil of *Mentha piperita.*

Conclusion

In conclusion, the extract of *Mentha piperita* leaves has showed both fungicidal and fungi static activities against isolated *Candida albicans* from Urinary Tract Infected patients. Hence, this potential of the extracts act as a non-antifungal alternative for preventing UTI. This could lead to the reduction of the amount of antifungal prescribed for the treatment of UTI and minimize drug resistance. The ability of this plant extracts to inhibit the growth of these UTIs pathogens indicates the presence entities capable of suppressing the growth of the test organisms.

Recommendations

Therefore, further studies are recommended to assess the presence of other bioactive compounds of chemotherapeutic potentials and also to ascertain the efficacy, toxicity and suitability of using the *Mentha piperita* extracts in vivo.

Acknowledgements

The authors acknowledge Mr. Idris Bala, Principal Technologist, Dept of Microbiology, Bayero University Kano, Nigeria, Dr. Mohammad M. Sani School of Technology Kano State Polytechnic, Dr. Isah Yunusa and Dr. Kamilu Ahmad for providing facilities for this research work.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Author's Contributions

Author HNG, design the study. Authors AAI, HNG, and ASB anchored the experiments. Authors HNG, AAI and SU revised the manuscript. Authors HNG and ASB manage the financial activities.