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EVALUATION OF ANTIBACTERIAL ACTIVITY OF METHANOLIC LEAVE EXTRACT AND FRACTIONS OF BALSAM APPLE (*Mormodica balsamina*) AGAINST *SALMONELLA* TYPHIMURIUM AND *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Microorganisms have evolved defence mechanisms against antibacterial agents and are resistant to some antibiotics. The purpose of this study was to evaluate the antibacterial activity of methanolic leaves extract and fractions of *M. balsamina* against *Salmonella* typhimurium and *Staphylococcus aureus.* Four different solvents (hexane, ethyl acetate, n-butanol and residual) fractions of methanolic extract of *M. balsamina* were tested for antibacterial activity using agar well diffusion method against *Salmonella* typhimurium and *Staphylococcus aureus*. Fractions were isolated using Column Chromatography and Fourier Transform Infra-red Spectroscopy (FTIR) was used to detect the characteristic peak values and their functional groups present in the specific fraction that exhibited potent antibacterial activity. The results highlighted that the n-butanol fraction was active against all isolates with maximum zone (19.50±2.12mm) at 100mg/ml while hexane, and ethyl acetate fractions were not active at all concentrations. Phytochemicals investigation showed that saponins and flavonoids were present in large quantity. Further assessment of minimum inhibitory concentration and minimum bactericidal concentration ranged between 3.125 to 50 mg/ml respectively. Fourier Transform Infra-red (FTIR) Spectra of fraction C revealed the presence of different functional groups ranging from carboxylic (R-C (O)-OH), alkanes (C-H), aldehydes (R-CH=O) and alkenes (RCH=CH_2), Amides (R-C(O)-NH_2), Alkyl halides(R-F), Alcohol (Ar-OH), Ethers (Ar-o-R), and Aromatic (C-H) respectively. The presence of bioactive secondary metabolites, low Minimum Inhibitory Concentration and low Minimum Bactericidal Concentration may justify the traditional uses of the leaves of *Mormodica balsamina* for therapeutic purposes. FTIR graphs provided characteristic peaks which represented the components responsible for antibacterial activity.

Keywords: *Mormodica balsamina*, Fractionation, Antibacterial, Minimum Inhibitory Concentration, Maximum Bactericidal Concentration and Furrier Transform Infra-red

INTRODUCTION

Bacteria that resist treatment with more than one antibiotic are called multidrug-resistant organisms (MDROs). Multidrug-resistant organisms are found mainly in hospitals and long-term care facilities (Centre for Disease Control and Prevention, 2013a). Anti-microbial agents are undeniably one of the most important therapeutic discoveries of the 20th century. However, with the 'antibiotic era' barely five decades old, mankind is now faced with the global problem of emerging resistance in virtually all pathogens (Peterson and Dalhoff, 2004). Herbal medicines serve the health needs of about 80% of the world's population, especially for millions of people in the vast rural areas of developing countries and more than 65% of the global population uses medicinal plants as a primary health care modality (WHO, 2001). Considering the high costs of synthetic drugs and their various side effects, the search for alternative products from plants used in folklore medicine is further justified (Abebe *et al.*, 2003). It is believed that plants which are rich in a wide variety of secondary metabolites belonging to chemical classes such as sterols, alkaloids, glycosides, saponins, flavonoids, tannins, and carbohydrates generally possess in their anti-microbial activities (Vieira *et al*., 2008).

Plant derived compounds (phytochemicals) have been attracting much interest as natural alternatives to synthetic compounds (Kalimuthu *et al*., 2010). Treatment and control of diseases by the use of available medicinal plants will continue to play a significant role in medical health care systems in many countries (Ekundayo *et al*., 2011). *Mormodica balsamina* also known as 'Balsam apple' is an important medicinal and nutritional plant of the family Cucurbitaceae. It is an annual to perennial tendril-bearing herb, native to tropical regions of Africa. The leaves, fruits, seeds, and bark of this plant is reported to have various medicinal and nutritional properties (Tommasi *et al.*, 1995; Karumi *et al.*, 2003; Matawalli *et al.*, 2004; Hassan and Umar, 2006; Benoit-Vical *et al.*, 2006; Bot *et al.*, 2007). The aim of this study was to investigate the antibacterial activity of methanolic extract and fractions of *M. balsamina* against *Salmonella* typhimurium and *Staphylococcus aureus*.

MATERIALS AND METHODS

Sample collection and identification

Fresh leaves of *M. balsamina* were obtained from Birnin Yero village of Igabi Local Government Area, Kaduna State, Nigeria. The sample was authenticated by comparism with herbarium specimen with voucher number 081 at the herbarium of the Department of Biological Sciences, Ahmadu Bello University, Zaria. The leaves of *M. balsamina* were washed with distilled water and dried at room temperature for one month and were grinded using a mechanical grinder.

Preparation of plant extract

The dried powdered sample was extracted in a soxhlet apparatus with methanol. The solution of extract was gently evaporated to dryness in a water bath at 40^0C. The extract was stored in a refrigerator at 4^0C until when required.

Phytochemistry

Total flavonoids, tannins, cyanogenic glycosides, oxalate, phytates and saponins were quantitatively determined from the methanolic leaves extract of *M. balsamina* using a method described by Mir *et al.,* (2013).

Screening of methanolic extract for antibacterial activity

The antibacterial activity of the methanolic extract was determined using agar well diffusion test described by Mahalngam *et al.,* (2011). The antibacterial activity of the methanolic extract was tested on two clinical isolates; *Staphylococcus aureus*, and *Salmonella* typhimurium. *Staphylococcus aureus* represented gram positive bacteria while *Salmonella* typhimurium represented gram negative bacteria.

Determination of minimum inhibitory concentration (MIC)

The MIC was evaluated on isolated fractions which showed activity on the test organisms. The method used was the tube dilution method (Adeniyi *et al.*, 1996).

D**etermination of minimum bactericidal concentration (MBC)**

The MBC was determined by collecting 1ml of broth culture from the tubes used for the MIC determination and subculturing onto fresh solid nutrient agar plates. The plates were incubated at 37^0C for 24 hours. The least concentration that did not show any growth after incubation was regarded as the MBC (Adesokan *et al*., 2007).

Fractionation using separation funnel

The methanolic extract was suspended in warm water at 40^0C to dissolve completely. The prepared solution was then partitioned successively using the following solvents of increasing polarity; n-hexane, ethyl acetate and n-butanol. After shaking the apparatus, the mixture separated into two different layers of liquid and were all collected separately to obtain the purified organic product left behind. All the fractions were evaporated to dryness using rotary evaporator at a temperature of 40^0C and kept in air tight container for further analysis.

Column chromatographic separation of n-butanol fraction

About a 100g of silica gel G (60- 120µm mesh size) was carefully packed using wet method in a heavy walled glass tube leaving sufficient head space. The column was allowed to settle for some hours to allow the silica gel (stationary phase) settle sufficiently. Four grams of the saturated extract pre-adsorbed on silica gel was loaded onto the packed adsorbent and allowed to stabilize for 3 hours before elution begins. Gradient elution of the solvents (mobile phase) started with methanol (100%) as the first or initial eluent. Subsequently, the polarity was increased, methanol and chloroform was added gradiently as methanol: chloroform (19:1), (9:1), (8:2), (7:3), and (6:4). Fractions of 20 ml each were collected and allowed to evaporate at room temperature. Each fraction was numbered in accordance with how they were collected respectively. The column fractions were monitored on TLC, and visualized with U.V light, P-anisaldehyde and 10% sulphuric acid were the general detecting reagents used. Fractions that showed same spot, colour and at the same Rf value were combined together in one beaker.

Functional groups identification

The sample was scanned using infrared in the range of 4000-400cm^{-1} using Fourier Transform Infrared Spectrometer Nicolet Model – 6700 (Thermo Scientific, USA). The spectral data obtained were compared with the reference chart to identify the functional groups present in the sample.

Statistical analysis

The results were expressed as mean ± standard deviation and analyzed using SPSS windows version 20. Analysis was performed using one-way ANOVA; Duncan multiple range test was used to compare significant differences within the groups. P-value with $p \leq 0.05$ was considered as significant.

RESULTS

Table 1: Quantitative Phytochemical Analysis of the Methanolic Leaves Extract of *M. balsamina*

Phytochemical compounds	**Quantitative Values (%)**	
Tannins	1.42±0.03	
Flavonoids	4.64±0.05	
Saponins	19.66±0.05	
Cyanogenic glycosides	0.45±0.02	
Phytic acids	1.74±0.01	Mean ± SD of Triplicate Determinations
Oxalates	0.07±0.00	

Table 2: Antibacterial Activity of Methanolic Extract of *M. balsamina* against *Salmonella* typhimurium and *Staphylococcus aureus*

Concentration of extract (mg/ml)/ Standard drug (µg/ml)	*Staphylococcus aureus*	*Salmonella* typhimurium
100	19.00±1.41^{c}	22.00±0.00^{d}
50	15.50±0.71^{b}	18.50±0.71^{c}
25	13.50±0.71^{b}	14.50±0.71^{b}
12.5	10.50±0.71^{a}	11.00±1.41^{a}
Cipro. 10	34.00±1.41^{d}	35.00±0.71^{e}

Values are mean±SD of triplicate determinations, values with different superscripts down the column are significantly different ($P<0.05$). Cipro. = Ciprofloxacin.

Table 3: Antibacterial Activity of N-Butanol Fraction of Methanolic Extract of *M. balsamina* against *Salmonella* typhimurium and *Staphylococcus aureus*

Concentration of extract (mg/ml)/ Standard drug (µg/ml)	***Staphylococcus aureus***	***Salmonella* typhimurium**
100	16.50±0.71[c]	19.50±2.12[c]
50	13.50±0.71[b]	16.00±0.00[b]
25	10.50±0.71[a]	12.50±0.71[a]
12.5	0.00	0.00
Cipro. 10	34.00±1.41[d]	35.50±0.71[d]

Values are mean±SD of triplicate determinations, values with different superscripts down the column are significantly different (P<0.05). Cipro. = Ciprofloxaci

Table 4 Fractions Obtained from Column Chromatographic Separation of N-Butanol Fraction.

Number	Fractions	Eluting solvent*
A	1-9	Absolute cloroform
B	10-22	19:1
C	23-37	9:1
D	38-47	8:2
E	48-70	7:3
F	71-88	6:4

*****Eluting solvent; cloroform:methanol

Table 5 Antibacterial Activity of Column Chromatographic Fractions of Methanolic Extract of *M. balsamina* against *Staphylococcus aureus*

	ZONE OF INHIBITION (mm)					
Conc. (mg/ml)/ Std. (µg/ml)	A	B	C	D	E	F
100	25.00±1.41^{b}	14.00±1.41^{a}	29.00±1.41^{c}	11.50±0.71^{a}	25.50±0.71^{c}	23.00±1.41^{d}
50	15.50±0.71^{a}	12.00±0.00^{a}	28.50±0.71^{c}	10.00±0.00^{a}	23.50±0.71^{c}	19.50±0.71^{c}
25	13.00±1.41^{a}	0.00	24.50±0.71^{b}	0.00	16.50±0.71^{b}	14.00±1.41^{b}
12.5	0.00	0.00	16.50±2.12^{a}	0.00	12.00±0.00^{a}	10.00±0.00^{a}
Cipro 10	34.00±1.41^{c}	34.00±1.41^{b}	34.00±0.71^{d}	34.00±1.41^{b}	34.00±1.41^{d}	34.00±1.41^{e}

Values are mean±SD of triplicate determinations, values with different superscripts down the column are significantly different ($P<0.05$). Conc. = Concentration. Std. = Standard. Cipro. = Ciprofloxacin. A, B, C, D, E and F = n-butanol Fractions of *M. balsamina*

Table 6 Antibacterial Activity of Column Chromatographic Fractions of Methanolic Extract of *M. balsamina* against *Salmonella* typhimurium

Conc. (mg/ml)/ Std. (µg/ml)	ZONE OF INHIBITION (mm)					
	A	B	C	D	E	F
100	26.50±2.12^{c}	18.50±2.12^{b}	31.00±1.41^{cd}	13.00±1.41^{b}	27.50±0.71^{c}	24.00±1.41^{d}
50	21.50±2.12^{b}	12.50±2.12^{a}	28.50±0.71^{c}	11.00±0.00^{a}	24.00±1.41^{b}	21.50±0.71^{c}
25	17.00±1.41^{ab}	0.00	22.00±0.00^{b}	0.00	16.50±2.12^{a}	15.00±0.00^{b}
12.5	12.50±2.12^{a}	0.00	15.00±1.41^{a}	0.00	13.50±0.71^{a}	11.00±0.00^{a}
Cipro 10	35.50±0.71^{d}	35.50±0.71^{c}	35.50±0.71^{d}	35.50±0.71^{c}	35.50±0.71^{d}	35.50±0.71^{e}

Values are mean±SD of triplicate determinations, values with different superscripts down the column are significantly different (P<0.05).Conc. = Concentration. Std. = Standard. Cipro. = Ciprofloxacin. A, B, C, D, E and F = n-butanol Fractions of *M. balsamin*

Table 7 Minimum Inhibitory Concentration (MIC) of Column Chromatographic Fractions of Methanolic Extract of *M. balsamina*

	MIC of fractions (mg/ml)					
	A	B	C	D	E	F
Staphylococcus aureus	25	50	3.125	50	6.25	25
Salmonella typhimurium	25	50	3.125	50	6.25	25

A, B, C, D, E and F = column chromatographic fractions

Table 8 Minimum Bactericidal Concentration (MBC) of Column Chromatographic Fractions of Methanolic Extract of *M. balsamina*

	MBC OF FRACTIONS (mg/ml)					
	A	B	C	D	E	F
Staphylococcus aureus	50	-	6.25	-	12.5	50
Salmonella typhimurium	50	-	3.125	-	12.5	50

- = No MBC. A, B, C, D, E and F = column chromatographic fractions.

Table 9 FTIR Peak Values and Functional Groups of Fraction C

S/No.	Peak values (cm^{-1})	Intensity	Bond	Functional Groups
1	702.48	m-s, s	RCH=CHR, C-H bend	Alkenes, Aromatic
2	1029.88	m-s	=C-O-C sym.	Ethers
3	1225.40	m-s, m-s	asym. Stretch, C-O stretch	Ethers, Alcohols
4	1314.55	v-s	C-F stretch	Alkyl halides
5	1653.04	s, w-m	C=O stretch, C-C stretch	Amides, Alkenes
6	1734.00	s	C-O-C stretch	Aldehydes
7	2916.99	broad, s	O-H stretch, C-H stretch	Carboxylic, Alkanes
8	3410.24	w-m, s	N-H stretch, O-H stretch	Amides, Carboxylic

Intensity abbreviations: vw = very weak, w = weak, m = medium, s = strong, vs = very strong.

Plate 1: Chromatogram of n-butanol Fraction of Methanolic Extract of *M. balsamina* in Chloroform-Methanol (8:2) detected with p-anisaldehyde in H_2SO_4

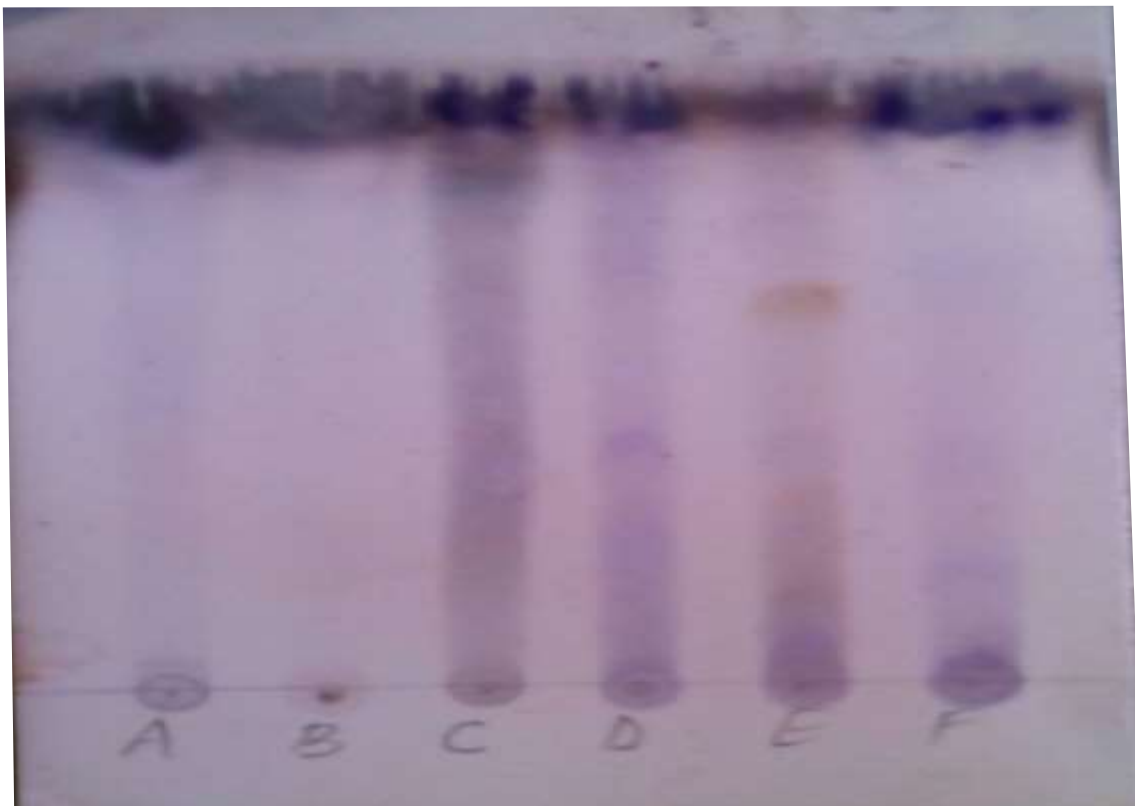

Plate 2: Chromatogram of Fractions obtained from Column Chromatographic Separation of n-butanol Fraction of *M. balsamina* in Chloroform-Methanol (8:2) detected with p-anisaldehyde in H_2SO_4

DISCUSSION

The phytochemical analysis of *M. balsamina* showed the presence of saponins, tannins, cyanogenic glycosides, phytic acids, oxalates and flavonoids. The presence of these biologically active chemicals may have been responsible for the antibacterial activity of these plant extract. Saponins are surface active agents which alter the permeability of the cell wall of organisms thus facilitating the entry of toxic materials or leakage of vital constituents from the cell (Daniyan *et al*., 2010). Tannins are polyphenols known to exhibit antibacterial, antiviral and anti-tumor activities. It was also reported that certain tannins are known to inhibit HIV replication selectively and is also used as diuretic (Evans, 2002). Flavonoids have been referred to as nature's biological response modifiers because of strong experimental evidence of their inherent ability to modify the body's reaction to allergen, virus and carcinogens. Some flavonoids have also been reported to act like some cumarins in the inhibition of giant cell formation in HIV infected cell cultures (Evans, 2002). The inhibitory activity exhibited by the secondary metabolites tends to agree with the reports of Junaid *et al.*, (2006); Aiylaagbe *et al*., (2007) and Ashish *et al*., (2013) both linked the antibacterial activity of plants to the presence of secondary metabolites.

According to Chemical and Laboratory Standard Institute CLSI (2005), any plant material should be considered an effective therapeutic agent if it extract produces zones of inhibition ≥10.00 mm on the target pathogenic organism. The methanol leaves extract showed wide range of antibacterial activity which agreed with similar reports documented by Umeh *et al.,* (2005) and Yagana *et al.,* (2012). That the antimicrobial constituents of plants are preferentially concentrated in the leaves. The inability to inhibit *Staphylococcus aureus* and *Salmonella* typhimurium by the ethyl acetate, residual and hexane fractions of methanolic extract of *M. balsamina* may be that the active component was not eluted in the respective solvents. Fraction C exhibited MIC at lowest concentration against *Staphylococcus aureus* and *Salmonella* typhimurium. Fraction C also exhibited the lowest MBC against *Staphylococcus aureus* and *Salmonella* typhimurium. The low MIC and MBC exhibited by the fractions against *Staphylococcus aureus* and *Salmonella* typhimurium are of great significance since it could be used as an alternative to orthodox antibiotic in the treatment of infections caused by these bacterial pathogens, especially as they frequently developed resistance to known antibiotics.

The FTIR spectra analysis was utilized to identify the functional group of the active ingredients on the basis of peak value in the vicinity of infrared radiation. IR-spectrum shows strong absorption peaks at 3410.24 (broad), 2916.99 (s), 1734.00 (s) and 1653.20 (w-m), 1314.55 (v-s), 1225.40 (m-s), 1029.88 (m-s) and 702.48 (s) which correspond to the presence of

Carboxylic (R-C (O)-OH), Alkanes (C-H), Aldehydes (R-CH=O), Alkenes ($RCH=CH_2$), Alkyl halides (R-F), Alcohol (Ar-O-H), Ethers (Ar-O-R) and Aromatic (C-H) functional groups, respectively. FTIR analysis of the fraction C shows a strong presence of hydroxyl group which is common in all phenolic compounds. All the absorption bands were attributed to (OH) stretching vibrations from phenols, a group of compounds (chemical) containing hydroxyl functional groups (-OH) attached to an aromatic hydrocarbon. Phenolic compounds from natural resources displayed antibacterial activity (Soundararajan *et al*., 2012). The location site(s) and the amount of hydroxyl groups found in the phenols are related to their relative toxicity towards microorganisms, with evidence that increased hydroxylation is directly proportion to toxicity (Geissman, 1963). Also, carboxylic acids were found to be linked with many antibacterial and activities which are found to exist in various plant metabolite molecular structures such as ursolic acid, which had been reported as a strong antibacterial agent Sultana *et al*., (2010). Many active compounds were produced by plants which contained these active groups (secondary metabolites).

CONCLUSION

The results of the study showed that methanolic of *M. balsamina* was effective against the pathogenic bacteria, *Staphylococcus aureus*, and *Salmonella* typhimurium. Fourier Transform Infra-red spectra of fraction C revealed the presence of different functional groups ranging from carboxylic, alkanes, aldehydes, alkenes, Amides, Alkyl halides, Alcohol, Ethers and Aromatic respectively. The presence of bioactive secondary metabolites, low minimum inhibitory concentration and low minimum bactericidal concentration may justify the traditional uses of the leaves of *M. balsamina* for therapeutic purposes.

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