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In vitro antibacterial potential of acetone leaf extract of *Otostegia integrifolia Benth* against human selected bacterial pathogens

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ABSTRACT

Otostegia integrifolia Benth (O. integrifolia) is the endogenous medicinal plant of Ethiopia mostly used for the treatment of Stomach ache, tonsillitis, hypertension, malaria, ascariasis, and lung diseases. The current study was focused on phytochemical analysis and evaluation of the antibacterial activity of *O. integrifolia Benth* leave extracts against selected human bacterial pathogen by the agar well diffusion and microtube broth dilution method. Phytochemical investigation was carried out for the identification of secondary metabolites responsible for antibacterial activity. *In vitro* antibacterial potential of *O.integrifolia Benth* leaf organic extracts against human pathogenic gram-negative (*E. coli, K. pneumonia, V. cholera*) and positive bacteria (*B.subtilis, E. faecalis, S.aureus)* were assessed by agar well diffusion, and bacterial inhibitory concentration of effective plant extracts was determined by 96 well plate broth dilution assay. Among all the tested organic leaf extracts, the acetone leaf extract of *O.integrifolia Benth* exhibited a promising broad spectrum of in vitro antibacterial activity against all tested multiple drug-resistant bacteria pathogens with significant MIC values of *K.pneumoniae* (2.144μg/ml), *V. cholera* (2.025μg), *B.substilis* (2.604 μg), *and S.aureus* (3.028 μg), respectively. The significant antibacterial activity of acetone leaf extracts of *O. integrifolia Benth* was due to the existence of flavonoids and phenolic compounds. The current studies demonstrated that the broad-spectrum antibacterial activity of acetone leaf extracts of *O.integrifolia Benth* might be helpful for the isolation of novel potent antibacterial agents against infectious bacterial pathogens without any side effects.

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INTRODUCTION

Multidrug-resistant Enterobacteriaceae family such as *Escherichia coli, Klebsiella pneumonia, Enterococcus faecalisis, and Vibrio cholera* produces extended-spectrum β-lactamases(ESBLs) enzymes (EC-3.5.2.6). They have shown higher hydrolyzing activity against β-lactam ring containing cefotaxime and oxyimino-β-lactam substrates such as ceftazidime, ceftriaxone, and cefepime, causing multidrug-resistant nosocomial and community-acquired infections (Chovanová *et al.*,

2013; Addis *et al.*, 2001). Recent reports suggested that the ESBL-producing Entero bacteriaceae family was causing inflammatory and urinary tract infections (UTI) and bloodstream associated infections (Sharmeen *et al.*, 2012). *E.coli* is a gram-negative bacteria which, when inhabitated in the human intestine tract during immune suppression of host, causes lower urinary tract infection and septicemia (Gkogka *et al.*, 2013). *K. pneumonia* is a gram-negative bacteria belonging to the Enterobacteriaceae family is inhabitated as intestinal flora of humans. It is also found in water and soil food, and it causes hospital-acquired urinary tract and wound infections (Beveridge, 1992). *V. cholerae* is a gram-negative bacteria belonging to the Vibrionacea family. It inhabitates in brackish water producing both enterotoxin and cholera toxin (CT), causing severe diarrhoea (Baumann *et al.*, 1984). *Enterococcus faecalis* is a gram-positive bacteria belonging to the Enterococcaceae family. It exists as commensal as well as an opportunistic pathogen causing clinical infections such as urinary tract infection and meningitis (Murray, 1990). *Bacillus subtilis* is a gram-positive bacteria belonging to the Bacillaceae family. It inhabitates in soil and also in the digestive tract of humans and generally harmless as it might occasionally cause human eye infections (Fuerst, 1978).

The multiple drug resistance in human pathogenic bacterial species has been developed due to excessive intake of commercially available synthetic antibiotics for the treatment of bacterial infections. Since two decades acquiring and spreading of multiple drug resistance among different bacterial pathogenic species depends on the interaction between bacterial cellular components and chemical structure of synthetic and semi-synthetic antibiotics, the genetical ability of bacterial species to acquire and transmit resistance among the bacterial species and between host and bacterial species. Inappropriate usage of antibiotics, host pathogenic virulent characteristics, environmental factors, and antibiotics are allied with adverse side effects of the host, including immunosuppression and allergy (Dey *et al.*, 2010) .

In rural Ethiopia, 80% of human population and 90% of livestock majorly depends on medicinal plants for the treatment of communicable and non-communicable diseases (Addis *et al.*, 2001; Mesfin *et al.*, 2009; Yirga, 2010). The traditional usage of medicinal plants in Ethiopia is an integral part of the culture and lifestyle of Ethiopian people (Bekalo *et al.*, 2009).

Plants can synthesize the bioactive secondary metabolites such as alkaloids, flavonoids, tannins and phenolic compounds, saponins and glycosides (Okwu, 2004). Different researchers in research laboratories and pharmaceutical companies screen various medicinal plants against multiple drug-resistant bacterial and fungal species. (Afolayan, 2003). Consequently, these conditions have been obligatory for microbiologists to search for novel antimicrobial agents from various medicinal plants (Aboaba and Ezeh, 2011; Abiramasundari *et al.*, 2011)

O.integrifolia Benth belongs to the family Lamiaceae endemic plant of Ethiopia and popularly known as an Abyssinian rose in Ethiopia. The dried leaves of O. integrifolia Benth plant are being used as fire fumigate for the eradication of insects in rooms and cooking vessels (Kidane *et al.*, 2013). Traditionally, this plant is being used in all regions of Ethiopia for curing tonsillitis, malaria, and ascariasis (Andemariam, 2010). The roots and leaves are being used for treating respiratory diseases (Giday *et al.*, 2007) . The objective of the current study was to evaluate the in vitro antibacterial activity of O. integrifolia Benth leaf extracts against tested multi-drug resistant human bacterial strains.

MATERIALS AND METHODS

Plant material collection and authentication

The leaves leaves of *O. integrifolia Benth* was collected in the month of October 2017, Axum city, Central Zone of Tigray region. The plant material was identified and authenticated by the Department of Biology, Addis Ababa University, and voucher specimen number of KH 001 was deposited in the National Herbarium for future reference.

Preparation of extracts

The fresh leaf material was washed thoroughly with distilled water, and then was shade dried at room temperature for two weeks. The dried leaves were coarsely powdered with an electrical grinder, and the powdered leaf material (100 g) was extracted by using various solvents such as petroleum ether, chloroform, acetone, and ethanol in a soxhlet apparatus (Das *et al.*, 2010).

Phytochemical Analysis

The preliminary standard phytochemical screening was carried out for identifying phytoconstituents such as alkaloids, coumarins, flavonoids, phenolic compound, and terpenoids (Egwaikhide and Gimba, 2007; Edeoga *et al.*, 2005).

Test microorganisms and microbial culture

All tested bacterial strains such as gram-negative

Table 1: Phytochemical Screening of Petroleum ether, chloroform, acetone and ethanol extracts of *O. Integrifolia Benth* leaves.

S.No	Plant Constituent	Extract			
		Petroleum ether extract	Chloroform extract	Acetone extract	Ethanol extract
1	Alkaloids	+	+	+	-
2	Coumarins	+	+	+	+
3	Flavonoids	+	++	+++	++
4	Phenolics compound	+	++	+++	++
5	Terpenoids	+	+	++	++

NB: (+) score indicate slight positive reactionfor Secondary metabolites.
(++) score indicate definitive positive reaction for Secondary metabolites.
(+++) indicate significant reactions were obtained for Secondary metabolites.
(-)Absent

Table 2: Zone of inhibition (mm) of organic leaf extracts *O.integrifolia Benth* against Gram negative and positive bacteria

Plant Extract/ Standard	Concent ration(μg/ ml)	Zone of inhibition (mm) of organic leaf extract *O.integrifolia Benth* against Gram negative and positive bacteria					
		B.subtilis ATCC 3915	*E. faecalis* ATCC 29212	*S.aureus* ATCC 29213.	*E.coli* ATCC 25922	*K.Pneumo* ATCC 700603	*V. cholera* ATCC 39315
O.I.L.PE	100mg	NA	NA	10 mm	NA	NA	NA
	250mg	NA	NA	11 mm	NA	12mm	13 mm
	500mg	NA	NA	13 mm	NA	14mm	15 mm
O.I.L.CH	100mg	7mm	NA	8 mm	NA	NA	10mm
	250mg	9 mm	8 mm	10 mm	NA	NA	11mm
	500mg	13 mm	10mm	15 mm	NA	NA	13mm
O.I.L.AC	100mg	10 mm	NA	11 mm	NA	NA	10 mm
	250mg	15 mm	NA	15 mm	NA	10 mm	15 mm
	500mg	18 mm	NA	16 mm	NA	12 mm	18 mm
O.I.L. ET	100mg	NA	NA	11 mm	NA	10 mm	10 mm
	250mg	NA	NA	12 mm	NA	12 mm	12 mm
	500mg	NA	NA	13 mm	NA	15 mm	13 mm
Ciproflaxin	20mg	38 mm	38 mm	36 mm	26 mm	31 mm	35mm

a: Indicates the Zone size includes 6-mm well ; NA- No zone of inhibition.

bacteria [*E.coli* (ATCC 25922), *K.pneumonia* (ATCC 700603), *and V.cholera* (ATCC 39315)] and gram-positive bacteria [*B.subtilis* (ATCC 3915) *E.faecalis* (ATCC 29212), *S.aureus* (ATCC 29213)] were procured from Clinical Bacteriology Laboratory, Ethiopian Health and Nutrition Research Institute (EHNRI), Addis Ababa, Ethiopia. The collected bacterial strains were subcultured for in vitro antibacterial activity.

Preparation and standardization of inoculums

A loopful of bacterial strains from the stock culture was transferred into Mueller-Hinton broth (MHB) test tubes and allowed to incubate at 37 °C for 24 hrs. 1 ml of prepared bacterial culture was inoculated in 5mL of Mueller-Hinton broth (MHB) and incubated till it reached the turbidity equal to that of the standard 0.5 McFarland solution. The 0.5 McFarland turbidity standard was considered to be equiv-

Table 3: Minimum Inhibitory Concentration (MIC) of *O. integrifolia Benth* leaf extracts on tested bacterial strains

Plant Extract	MIC (μg/ml) of acetone leaf extracts of the *O. Integrifolia Benth* against tested multidrug resistant bacteria.			
	B.substilis ATCC 3915	*S.aureus* ATCC 29213	*K.pneumoniae* ATCC 700603	*V. cholera* ATCC 39315
Acetone extract	2.604	3.028	2.144	2.025

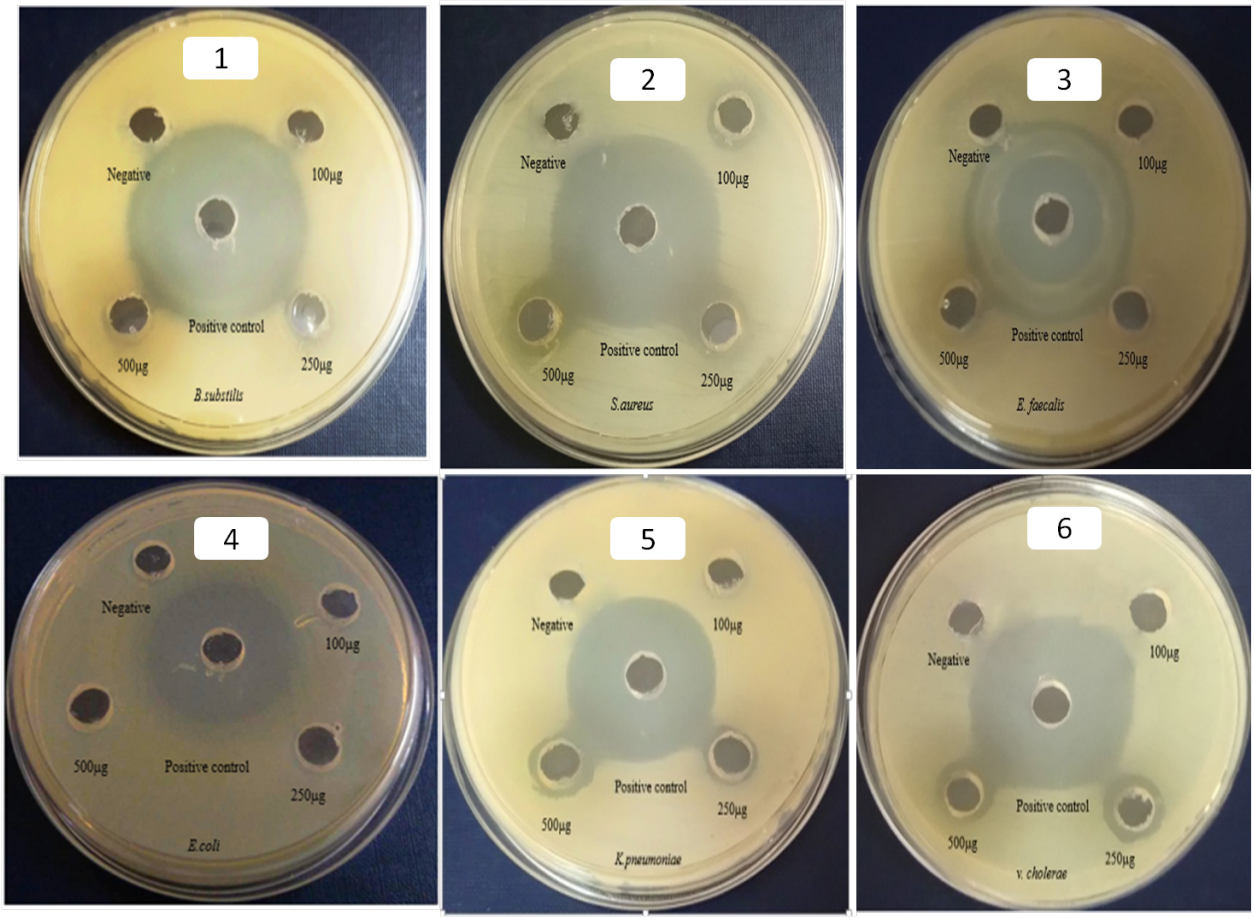

Figure 1: The Zone of inhibition of petroleum ether leaf extracts *O. integrifolia Benth* of against tested multiple drug-resistantbacteria by the agar well diffusion method. 1-3 Gram positive bacteria ; 4-6 Gram positive bacteria

alent to a cell density of 1 x 10^8CFU /mL (Hyder *et al.*, 2010).

In vitro antibacterial Assay

Agar well diffusion assay

The antibacterial activity of the prepared organic plant extracts was determined by agar well diffusion method (Bauer *et al.*, 1966). The selected bacterial pathogens from nutrient broth were spread on the Mueller-Hinton agar (MHA) plates. Five wells of 6mm in diameter were made with a sterile cork borer, and about 100 -150 μL of 10% of DMSO dissolved organic tested plant extracts (100 μg to 500 μg/mL) were filled in test wells and then allowed to be incubated at 37 °C for 24hrs. The stranded Ciproflaxin (20 μg) was used as a positive control for both gram-positive bacteria and gram-negative bacteria and DMSO was used as a negative control. The antibacterial activity was determined by measuring the zone of inhibition in millimeters. The experiments were conducted in triplicates and the results were represented as mean $\pm$ standard deviation.

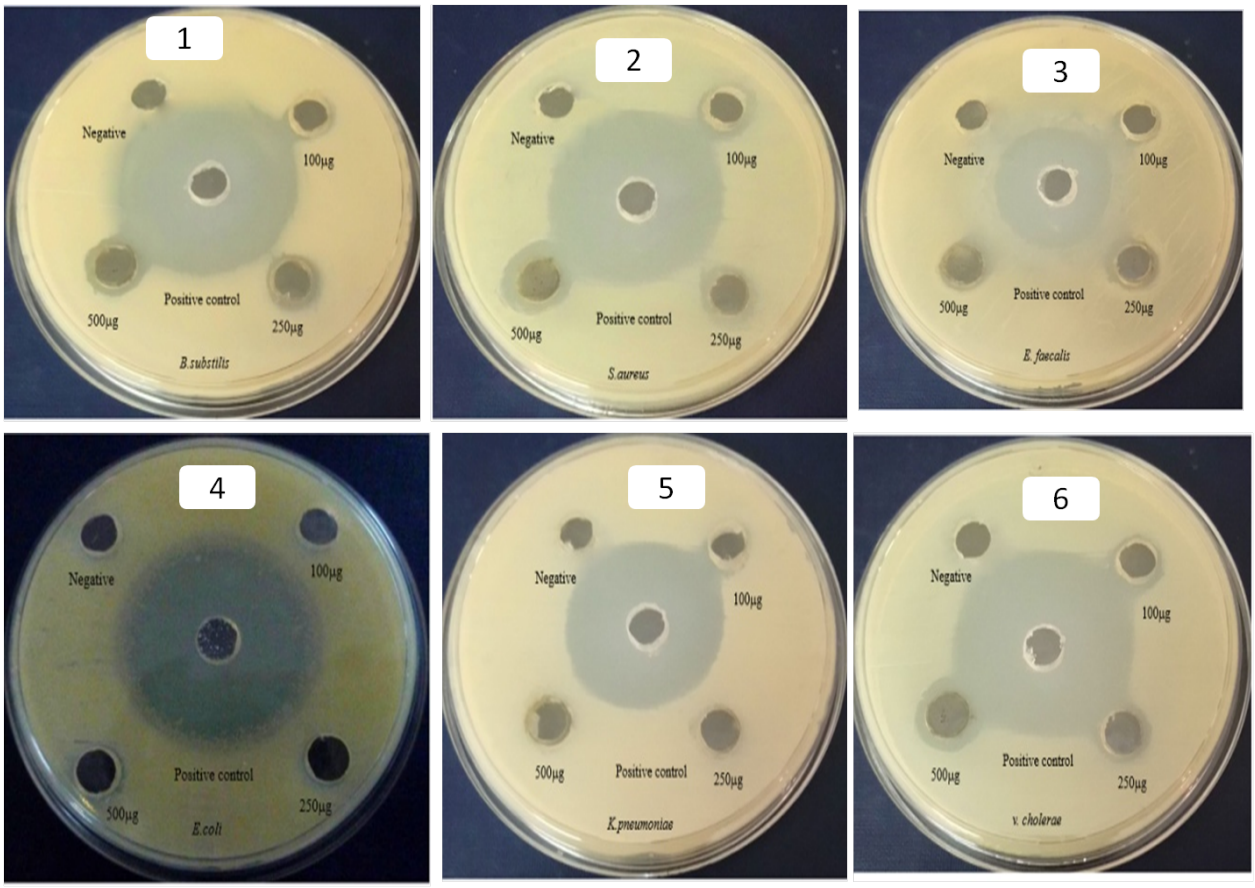

Figure 2: The zone of inhibition of chloroform leaf extracts *O. integrifolia Benth* of against tested multiple drug-resistant bacteria by the agar well diffusion method. 1-3Gram positive bacteria ; 4-6 Gram positive bacteria

MIC (Minimum Inhibitory Concentration)

The Minimum Inhibitory Concentration (MIC) of effective plant extracts, exhibiting significant antibacterial activity against tested gram-positive *B.substilis and S.aureus* and gram-negative *K.pneumoniae and V.cholera* bacterial pathogens, was determined by Mueller–Hinton broth microdilution method. Various concentrations of (50, 25, 12.5, 6.25, 3.15 and 1.562μg/ml) acetone leaf extract of O.integrifolia in 10% of DMSO solution were placed into the 96 well plates. Then, 100 μl bacterial cell suspensions containing 10^8 CFU ml-1 were inoculated in each well/plate (Wikler, 2009) and allowed to be incubated at 37 °C for 24 hrs. The culture intensity of each well was read at 600 nm and compared with the untreated control.

Statistical Analysis

The results were expressed as Mean ± SD and the statistical differences between the Mean ± SD of the control group and the experimental group were assessed by GraphPad Prism 6.0.

RESULTS AND DISCUSSION

Plants consist of a wide range of biologically active secondary metabolites responsible for different biological activities. Hence the presences of biologically active ingredients in the plant extracts are used for the treatment of both infectious and non-infectious diseases. These biologically active ingredients include alkaloids, coumarins, flavonoids, phenolic compounds, and terpenoids.

Phytochemical screening

The qualitative phytochemical analysis of various organic leaf extract of *O.integrifolia Benth*, showing the presence or absence of alkaloids, coumarins, flavonoids, tannins/phenols, and terpenoids, was investigated. As shown in the Table 1, petroleum ether leaf extract of *O.integrifolia Benth* showed the presence of an inadequate amount of secondary metabolites such as alkaloids, coumarins, flavonoids, tannins/phenols, and terpenoids.

The chloroform and ethanolic leaf extract of *O.integrifolia Benth* contained a significant number of secondary metabolites such as alkaloids, coumarins, flavonoids, tannins/phenols, and ter-

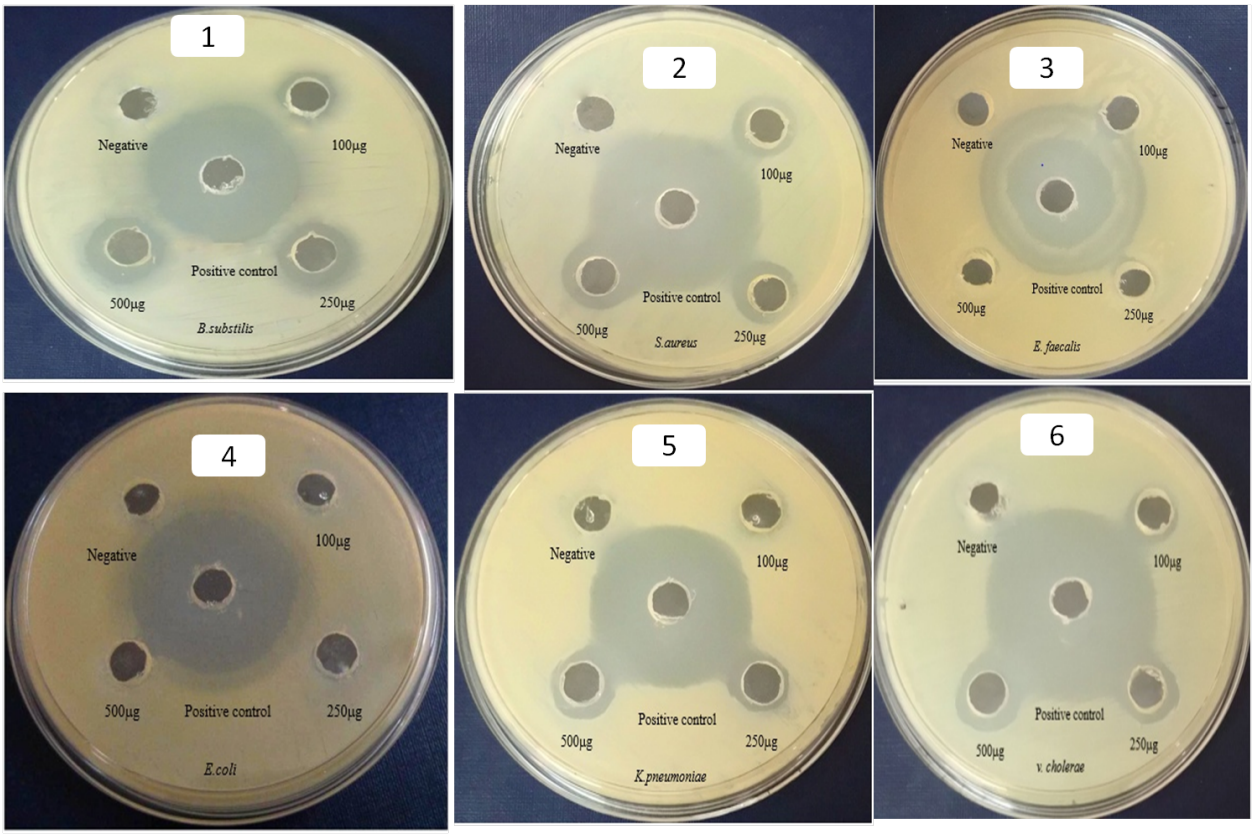

Figure 3: The zone of inhibition of acetone leaf extracts *O. integrifolia Benth* of against tested multiple drug-resistant bacteria by the agar well diffusion method. 1-3Gram positive bacteria ; 4-6 Gram positive bacteria

penoids. The acetone leaf extract of O.integrifolia Benth showed the presence of a maximum number of alkaloids, coumarins, flavonoids, tannins/phenols, and terpenoids. The highest secondary metabolites were found in acetone extracts of *O.* integrifolia Benth leaf which could be attributed to the promising broad spectrum of antibacterial activities. The secondary metabolites present in different organic extracts of *O.integrifolia Benth* leaves have therapeutic importance for curing infectious diseases. Our results also correlated with the reports of (Abyot, 2012). He reported that 80% of methanolic leaf extract of *O. integrifolia Benth* consists of phenolic compounds and flavonoids.

Antibacterial activity of leaf extract of *O. integrifolia Benth*

Nowadays, medicinal plants are widely used as an alternative source for novel antibacterial agents against multiple resistant bacteria (Munuswamy *et al.*, 2013). Many secondary metabolites of plants have shown significant antibacterial activity by targeting various drug-resistant pathways. The development of multidrug resistance in many pathogens against conventional antibiotics is a serious threat as it increases morbidity and mortality of both developing and developed countries (Mahato and Sharma, 2018).

Hence the current investigation has been aimed at the screening of secondary metabolites and evaluating the *in vitro* antibacterial activity of the leaf extract of *O.integrifolia Benth* against disease-causing, multidrug-resistant bacterial pathogens such as *E.coli* (responsible for gastroenteritis, urinary tract infections, and neonatal meningitis), *K.pneumonia* (causative organism of pneumonia), *V.cholerae* (characterized by severe diarrhoea), *B subtilis* (causes human eye infections), *S.aureus* (a wound infecting pathogen causes septicemia), *and E.faecalisis* (causes urinary tract infection, bacteremia, bacterial endocarditis, and meningitis).

Antibacterial activity of organic leaves extract of *O.integrifolia Benth*

The *in vitro* antibacterial activity of the leaf extract of *O.integrifolia Benth* at different concentrations (100, 250, and 500 μg/ml) and standard antibiotic Ciproflaxin (20 μg/ml) were shown in Table 2. The *O.integrifolia* leaf extract shows the zone of inhibition (*in vitro* antibacterial activity) ranging from 7 mm to 18 mm diameter (which included 6 mm of agar wells).

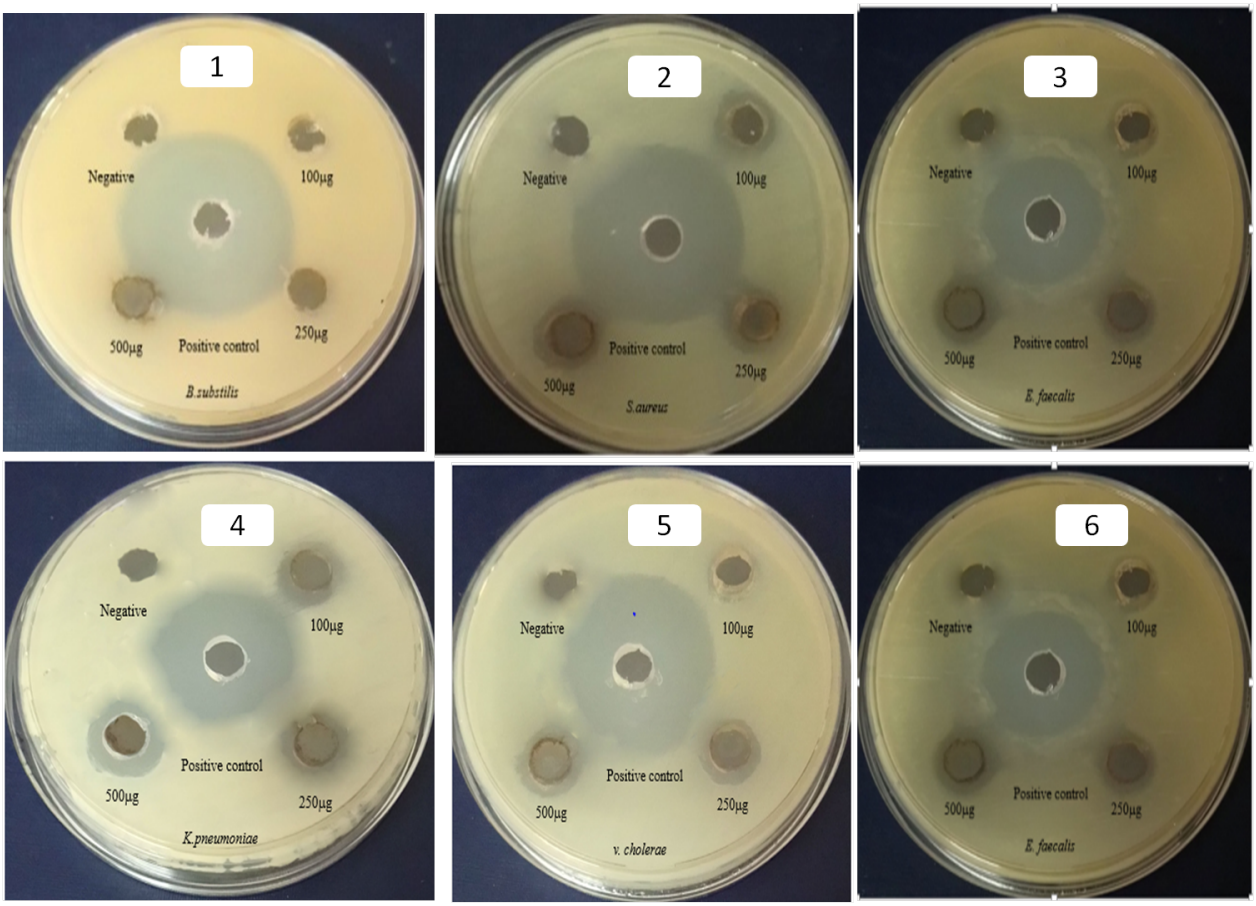

Figure 4: Zone of inhibition of Ethanol leaf extracts *O. integrifolia Benth* of against tested multiple drug-resistant bacteria by the agar well diffusion method. 1-3Gram positive bacteria ; 4-6 Gram positive bacteria

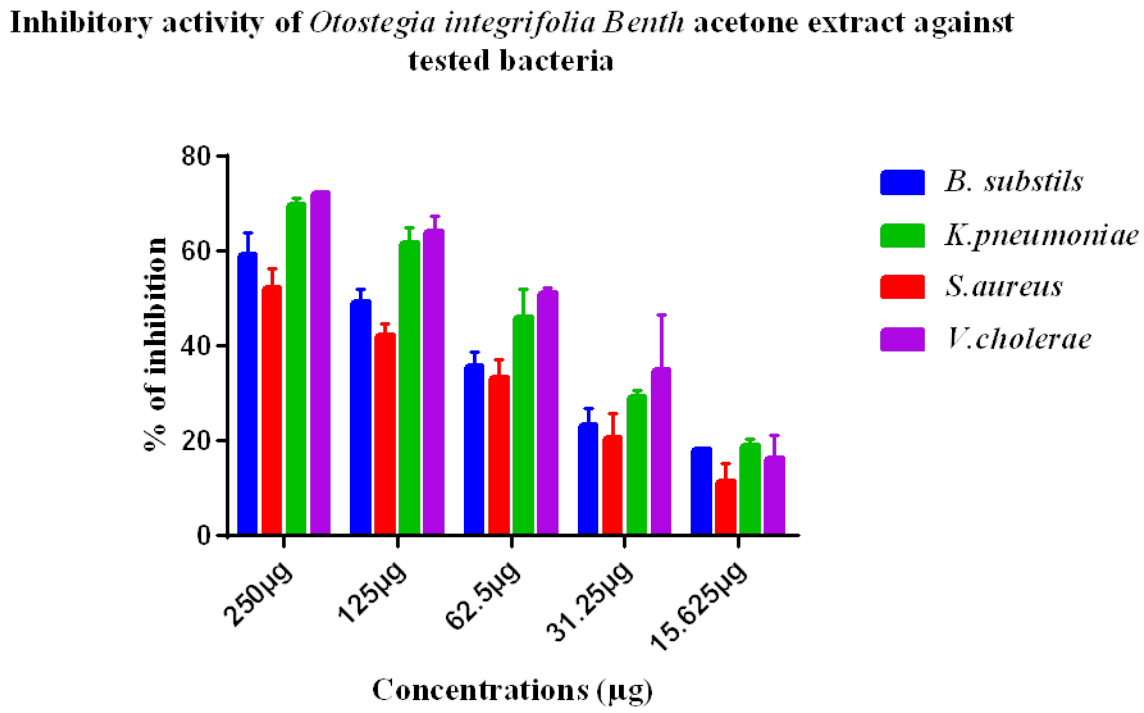

Figure 5: Percentage of inhibition of acetone leaf extract *O. integrifolia Benth* against tested Enterobacteriace family compared with Ciproflaxin standard

Significant results were shown by acetone leaf extract of *O.integrifolia* against tested gram-positive and gram-negative bacteria.

The antibacterial activity of petroleum ether leaf extract of *O.integrifolia Benth* (as shown in Table 2 and Figure 1), exhibited a moderate zone of inhibition against the tested, gram-negative bacteria [*V.cholera* in the range between (12mm-15 mm) *and K.pneumoniae* (12-14 mm) at a concentration of 250 μg/ml-500 μg/ml)] and gram-positive bacteria [(S.aureus (1-13 mm) at a concentration range of 100 μg/ml-500 μg/ml]. The *in vitro* antibacterial activity of petroleum ether leaf extract of *O.integrifolia Benth* showed effective narrow spectrum against the tested, gram-negative *K.pneumoniae and V. cholera* bacteria.

The antibacterial activity of chloroform leaf extracts of *O.integrifolia Benth* (as shown in Table 2 and Figure 2), exhibited significant zone of inhibition against the gram-positive bacteria [*S.aureus* (8-15 mm), *B.subtilis* (7-13 mm), *E.faecalis* (8-10 mm) at a concentration of 100 μg/ml-500 μg/ml] and moderate zone of inhibition against the gram-negative bacteria [*V.cholera* (10-13 mm) at a concentration of 100 μg/ml-500 μg/ml].

The antibacterial activity of acetone leaf extract of *O.integrifolia Benth* (as shown in Table 2 and Figure 3) exhibited prominent significant zone of inhibition against the tested gram-positive bacteria [*B.subtilis* (10- 18 mm) *S. aureus* (11-16 mm) at a concentration of 100 μg/ml-500 μg/ml] and moderate zone of inhibition against gram-negative bacteria] *K.pneumoniae* (10-12 mm) *and V.cholera* (10-15 mm) at a concentration of 100 μg/ml-500 μg/ml], but failed to inhibit the growth of gram-negative bacteria such *E.coli* and gram-positive bacteria such as *E.faecalis*. The acetone leaf extracts of *O.integrifolia Benth* showed an effective broad spectrum of *in vitro* antibacterial activity against both gram-positive and gram-negative bacteria.

The antibacterial activity of ethanolic leaf extracts of *O.integrifolia Benth* (as shown in Table 2 and Figure 4) had shown significant zone of inhibition against gram-positive bacteria [*B.subtilis* (10-15 mm), *S.aureus* (11-18 mm) at a concentration of 100-500 μg/ml] and moderate inhibition against gram-negative bacteria [*K.pneumoniae* (10-12 mm) at 250-500 μg/ml *and V.cholera* (10-13 mm) at 100 μg/ml-500 μg/ml.

The ethanolic leaf extracts of *O.integrifolia Benth* showed an effective broad spectrum of *in vitro* antibacterial activity against both gram-positive and gram-negative bacteria and did not show any activity against gram-negative bacteria such as *E.coli* and gram-positive bacteria such as *E.faecalis*.

MIC of the effective plant extracts

The Minimal inhibition concentration was carried out for the evaluation of the inhibitory potential of the plant extract against the most susceptible tested bacteria. Only the tested bacteria which were highly susceptible to the acetone leaf extracts of *O.integrifolia Benth* was taken for determining the MIC. As shown in Figure 5 and Table 3, the acetone leaf extracts of *O.integrifolia Benth* shows maximum zones of inhibition of 75%, 70%, 60%, and 55% against *V.cholera, K.pneumoniae, S.aureus, and B.substilis* with MIC values of 2.025 μg/ml, 2.144 μg/ml, 3.028 μg/ml, and 2.604 μg/ml.

CONCLUSIONS

The results of the current study revealed that the acetone leaf extract of *O.integrifolia Benth* showed a broad spectrum of *in vitro* antibacterial activity against tested gram-positive bacteria such as *B.subtilis, S.aureus,* and gram-negative bacteria such as *K. pneumonia and V.cholera*. The phytochemical analysis of acetone leaf extract of *O.integrifolia Benth* had shown the presence of alkaloids, coumarins, flavonoids, tannins and phenolics compounds, and terpenoids, which are responsible for potential *in vitro* antibacterial activity. This study suggests that the acetone leaf extract of *O.integrifolia Benth* has shown promising *in vitro* antibacterial activity and phytochemical constituent which are useful for further studies to untangle novel treatment strategies for diseases associated with a bacterial infection.

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Conflict of Interest

The authors confirm that this article content has no conflict of interest.

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