

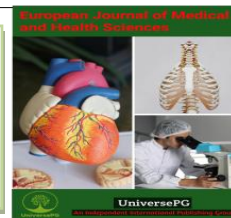


Publisher homepage: www.universepg.com, ISSN: 2663-7529 (Online) & 2663-7510 (Print)

<https://doi.org/10.34104/ejms.0190109>

European Journal of Medical and Health Sciences

Journal homepage: www.universepg.com/journal/ejms



Effect of Antibiotic Susceptibility and Inhibitory Activity for the Control of Growth and Survival of Microorganisms of Extracts of *Calendula officinalis*

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ABSTRACT

Extracts from various types of plants represent several evidences of beneficial health effects in the living system. *Calendula officinalis* is a most popular medicinal plant in our country. The purpose of the study was to determine the effectiveness of *C. officinalis* for the control of growth, and survival of microorganisms. Inhibition of growth was tested by the paper disc agar diffusion method. Minimum inhibitory concentration (MIC) was determined by the tube dilution method. *C. officinalis* leaf extract showed inhibition (MIC, inhibitory) to *Escherichia coli*, *Klebsiella pneumoniae*, and *Bacillus subtilis* and to the other bacteria tested. Antimicrobial effects of leaf extract of *C. officinalis* on some microorganisms including pathogens were analyzed. The extract of *C. officinalis* leaf which is prepared was tested on bacterial cultures such as *B. subtilis*, *S. lutea*, *E. coli*, *K. pneumoniae*, and also investigated that minimum inhibitory concentration (MIC) 4µg/ml of leaf extract of *C. officinalis* in petroleum ether against *K. pneumoniae* and largest inhibitory zone are created by 512mg/ml chloroform extract against *E. coli* leaf extract of *C. officinalis* in petroleum ether has showed better for antibacterial activity. The present study demonstrates that the potentiality of *C. officinalis* as a source of antimicrobials that could be harness for use in the health care delivery process.

Keywords: *Calendula officinalis*, Inhibitory agents, Leaf extract, and Disc agar diffusion method.

INTRODUCTION:

Nature is the potential source of medicinal agents and these agents have been used for thousands of years and numbers of modern drugs have been isolated from natural sources. Various medicinal plants have been used for years in daily life to

treat various diseases all over the world. Approximately, amongst 1500 identified medicinal plants 500 are commonly in use (Chidambaram *et al.*, 2014). Plants produce a diverse range of bioactive molecules (Uddin *et al.*, 2017). Higher plants as a major source of medicinal compounds to play a

dominant role in the maintenance of human health since ancient times (Farombi *et al.*, 2003). The WHO estimates that 80% of the world population use herbal medicine for some aspects of primary health care purposes (Shinwari *et al.*, 2009). There are 4,22,127 plant species growing on planet earth, about 35,000 to 70,000 plants species are used as medicinal plants (Hasan *et al.*, 2007). The practice of traditional medicine is widespread in China, India, Japan, Pakistan, Sri Lanka and Thailand (Hasan *et al.*, 2007). *Calendula* is used in ayurveda for the treatment of fever, diarrhea, and cancer (Krag *et al.*, 2013).

Medicinal plants are one of the necessary and valuable resources in a wide range of natural resources that they can have an important role in health. *C. officinalis* is globally known for its medicinal importance containing various photochemical activities. Knowledge of the various biological activities and chemical constituents of medicinal plants are desirable not only for the discovery of new therapeutic agents but also for information in discovering new sources of other economic materials (Khaleqzaman *et al.*, 2002). Natural products have been a major source of the new drugs (Vuorelaa *et al.*, 2004). The potential for developing antibacterial into medicine appears rewarding, from both the perspective of drug development and the perspective of phytomedicines (Rahaman *et al.*, 2004). It possesses cytotoxic as well as abnormal cell growth reducing potential. Traditionally, *C. officinalis* was used as anti-inflammatory, diaphoretic, analgesic, anti-

septic and in jaundice treatment (Chakraborty *et al.*, 2010). It is also used as a mouthwash after tooth extractions (Mukesh *et al.*, 2011).

Phytopharmacological studies of different *Calendula* extracts have shown anti-viral activity, anti-HIV properties of therapeutic interest, and anti-genotoxic properties (Perez-Carreón *et al.*, 2002). *Calendula* extract-containing creams and gels are commonly used to treat skin irritation, diabetes, inflammation, and burns, especially after radiotherapy, and to aid wound healing (Edwards *et al.*, 2015). *C. officinalis* is widely cultivated as an herb and can be grown easily in sunny locations in most kinds of soils. *C. officinalis* are considered by many gardening experts as one of the most versatile flowers to grow in a garden, especially since they are easy to grow, and tolerate most soils. Pot marigolds typically bloom quickly from seed (in under two months) in bright yellows, golds, and oranges. Some scientists indicate the high antimicrobial activity of *Calendula* stems (Goyal *et al.*, 2011). *C. officinalis* was very effective in inhibiting the growth of the zone of inhibition *E. coli* and *B. subtilis* of Petroleum ether and chloroform was found in cm respectively (Md *et al.*, 2014). However, gm negative organisms are more sensitive to the extract of *Calendula*. It may be used as a constituent of a drug (Happy *et al.*, 2018). The aim of the study was to identify bio-active chemical compounds from the flower of *C. officinalis*, their antimicrobial activity and setting up the standards specification.

MATERIALS AND METHODS:

Collection, Processing and Preservation of *Calendula officinalis* plant material - Healthy, disease free, mature *C. officinalis* plants was identified and selected for the collection of leaves from the garden of Kushtia (23°54'37.1"N 89°07'20.9"E) and Jhenidah (23°32'40.1"N 89°10'27.8"E) Pouroshova, Bangladesh. After cleaning the waste

materials of the leaf then plant material was air dried in room temperature. After 7 days the dried plant was grinded to form fine powder from the blender machine. This powder was used for the preparation of different solvents extracts by sequential extraction. The Good Agricultural and Field Collection Practices (GACP) of medicinal

plants of World Health Organization (WHO) were followed strictly.

Solvents and Chemical used - In this experiment, only a few selected solvents are used such as petroleum ether and chloroform. Petroleum ether, chloroform, etc are used in this experiment.

Disk Preparation - The filter paper was punched with the punching machine and disc was made. The disc paper was taken into the test tubes & sterilized in an autoclave for 15 minutes with 15 psi and 121 °C temperatures.

Medium preparation - In this study, nutrient agar medium was used for antibacterial screening. For the test 2.8gm of the nutrient agar media was taken into 500 ml autoclave conical flask. The media properly dissolved with the distilled water then sterilized in an autoclaved for 15minutes with 121°C. After autoclaving, the media was cooled for some time and poured into the autoclaved Petri dishes in the laminar airflow cabinet.

Inoculum preparation - 1ml of distilled water was taken into the screw-capped tube and the pure colony of freshly cultured bacteria was added into the tube and vortexes. The OD was measured with the colorimeter and microbial population was confirmed to be within tube. This suspension was used as inoculums.

Determination of minimum inhibitory concentration (MIC) of *Calendula officinalis* - MIC of the most active chloroform and petroleum ether extracts were determined using serial dilutions of 512mg/ml, 512µg/ml to 2µg/ml in chloroform and petroleum ether solvent against both strains of *E. coli* in Agar well diffusion method as mentioned earlier. The lowest concentration of the extract required to inhibit the growth of the organism *in vitro* is MIC. In the present study, it was determined following the serial dilution technique.

Preparation of sample solution - Stock working solution of the plant extracts were prepared by dissolving 10gm of the dried extracts in 1ml each of petroleum ether and chloroform solvent into two separate flasks. From these solutions, 1ml solution was added to 9ml petroleum ether. Then from this 2nd solution 8532µl was added to 1468µl chloroform. Therefore, the final concentration was reached to 512µg/ml. From these solutions, the 1 ml solution was added to 9ml petroleum ether. Then from this 2nd solution 5688µl was added to 4312µl petroleum ether. Therefore, the final concentration was reached to 512µg/ml. 6hours extraction of 5.12grams seed powder was added to the 10ml chloroform and petroleum ether; therefore get mother solutions which were used without dilutions.

Serial dilution - For preparing 512µg/ml to 2µg/ml, 1ml of solvent was added to each of nine screw-capped test tubes. 1ml of the sample having 512µg/ml extracts was added to the first test tube containing 1ml of respective solvent and mixed well in the vortex and then 1ml of this solvent was transferred to the second test tube containing 1ml of the same solvent. After mixing well, 1ml of this mixture was transferred to the third test tube. This process of serial dilution was continued up to the last test tube. Finally, the concentration of the last test tube was 2µg/ml.

Preparation of disc - The disc paper was soaked with each concentration of extracts and placed at room temperature for air dry for 15 hours. Then dried disc paper was placed in the oven for 1 hour at 37 °C. After completion of oven dry, the disc paper was labeled according to different concentration and finally, the labeled disc paper was taken into the vial and it was ready for antibacterial activity.

Antimicrobial activity test - All strains used in the study were inoculated to nutrient agar and incubated at 35±0.1°C for 24h and were allowed to

grow until they reach 10^8 - 10^9 cfu/ml. Antibacterial activity studies were carried out for each test strains in duplicate and average measurement was calculated. Four organisms, (three gm-negative i.e. *E. coli*, *Salmonella spp*, *Shigella spp*, and *Staphylococcus spp*) were tested in this study to determine the antibacterial effect of crude extracts (Petroleum ether and Chloroform) *C. officinalis*.

Antibacterial activity of extracts and crude of *C. officinalis* was observed. Then *in vitro* antibacterial activities of the extracts were measured by employing standard agar disc diffusion method. Discs were impregnated with each treatment and control was assayed on duplicate agar medium

RESULT AND DISCUSSION:

Petroleum ether extract of *Calendula officinalis*

- Petroleum ether extract of *C. officinalis* exhibit antibacterial activity against *B. subtilis*, *E. coil*, *S. lutea*, *K. pneumoniae*. Different concentration of petroleum ether extract of *Calendula* (512 mg/ml) produced a zone of inhibition 1.7cm against *B. subtilis*, 1.4cm against *E. coil*, 1.6cm against *S. lutea*, 1.5 cm against *K. pneumoniae* (**Table 1**).

Another concentration of petroleum ether extract of *Calendula* (512 μ g/ml) produced a zone of inhibition 1.3cm against *B. subtilis*, 1.1cm against *E. coil*, 1.3cm against *S. lutea*, 1.2cm against *K. pneumoniae* (**Table 1**). And another concentration of petroleum ether extract of *Calendula* (256 μ g/ml) produced a zone of inhibition 1.0cm against *B. subtilis*, 1.0cm against *E. coil*, 1.2cm against *S. lutea*, 1.0cm against *K. pneumoniae* (**Table 1**).

Similarly, concentration of petroleum ether extract of *Calendula* (128 μ g/ml) produced a zone of inhibition 0.9cm against *B. subtilis*, 1.0cm against *E. coil*, 1.0cm against *S. lutea*, 1.0cm against *K. pneumoniae* (**Table 1**).

plate for *E. coli*, *Shigella spp*, *Staphylococcus spp*, and *Salmonella spp*. The experiment was replicated two times to confirm the reproducible results. Sterile, blank paper discs were impregnated with only sterile solvent (Petroleum ether and Chloroform) and used as negative control each time. Standard Kanamycin (30 μ g) and Strepto-mycin (5 μ g) were used as a positive control for comparison of the antibacterial activity.

Statistical analysis - The Statistical analysis was performed by using SPSS software (release 10.0) to find out significant differences in the antibacterial effects.

The concentration of petroleum ether extract of *C. officinalis* (64 μ g/ml) produced a zone of inhibition 0.8cm against *B. subtilis*, 0.9cm against *E. coil*, 0.8cm against *S. lutea*, 0.9cm against *K. pneumoniae* (**Table 1**). The concentration of petroleum ether extract of *Calendula* (32 μ g/ml) produced zone of inhibition 0.6cm against *B. subtilis*, 0.9cm against *E. coil*, 0.6cm against *S. lutea*, 0.8cm against *K. pneumoniae* (**Table 1**).

The concentration of petroleum ether extract of *Calendula* (16 μ g/ml) produced zone of inhibition 0.6 cm against *B. subtilis*, 0.8cm against *E. coil*, 0.5cm against *S. lutea*, 0.7cm against *K. pneumoniae* (**Table 1**).

The concentration of petroleum ether extract of *Calendula* (8 μ g/ml) produced zone of inhibition 0.4cm against *B. subtilis*, 0.4cm against *E. coil*, no zone against *S. lutea* and 0.7cm against *K. pneumoniae* (**Table 1**).

Table 1: Comparison of antibacterial activity and MIC values of leaf extract of *C. officinalis* in petroleum ether by inhibition zone.

Test strains	Petroleum ether extract of <i>Calendula officinalis</i> leaf (Dose µg/ml)											Positive	Negative
	512 mg/ml	512 µg/ml	256 µg/ml	128 µg/ml	64 µg/ml	32 µg/ml	16 µg/ml	8 µg/ml	4 µg/ml	2 µg/ml			
<i>B. subtilis</i>	1.7 cm	1.3 cm	1.0 cm	0.9 cm	0.8 cm	0.6 cm	0.6 cm	0.4 cm	-	-	2.3 cm	-	
<i>E. coli</i>	1.4 cm	1.1 cm	1.0 cm	1.0 cm	0.9 cm	0.9 cm	0.8 cm	-	-	-	2.5 cm	-	
<i>S. lutea</i>	1.6 cm	1.3 cm	1.2 cm	1.0 cm	0.8 cm	0.6 cm	0.5 cm	0.4 cm	-	-	2.5 cm	-	
<i>K. pneumoniae</i>	1.5 cm	1.2 cm	1.0 cm	1.0 cm	0.9 cm	0.8 cm	0.7 cm	0.7 cm	0.7 cm	-	2.5 cm	-	

The concentration of petroleum ether extract of *Calendula* (4µg/ml) produced a zone of inhibition only 0.7cm against *K. pneumoniae* (Table 1). In

addition, the MIC value was also (0.3cm) determined. The MIC values against *K. pneumoniae* were 4ugml-1.

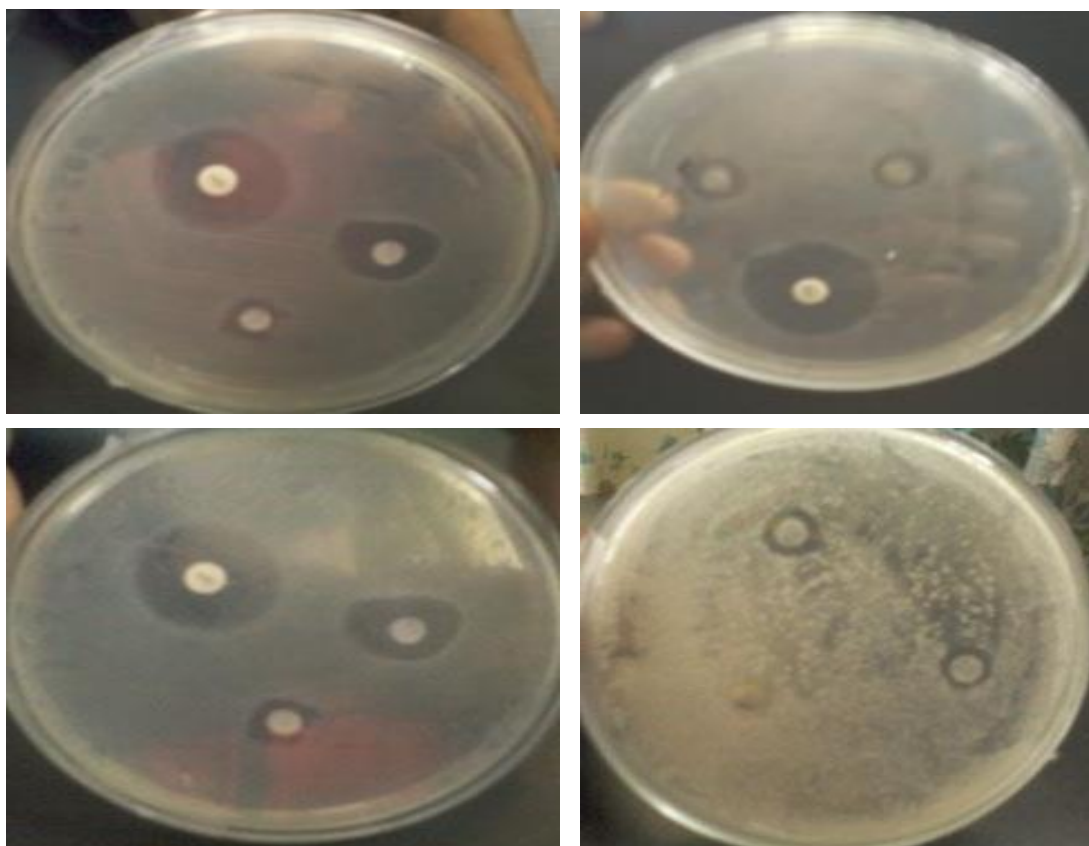


Fig 1: Comparison of Antibacterial activity and MIC values of leaf extract of *C. officinalis* in petroleum ether by inhibition zone.

Photochemical analysis of Solvents and Chemical - Photochemical analysis showed that almost all the solvents are responsible for extracting different components from the plants. Plant pharmacological studies have suggested that *Calendula* extracts have anti-viral, anti-genotoxic and anti-inflammatory properties (Eva Jimenez-Medina et al., 2006).

The Chloroform extract of *Calendula officinalis* - Chloroform extract of *C. officinalis* exhibit antibacterial activity against *B. subtilis*, *E. coli*, *S. lutea*, *K. pneumoniae*. Different concentration of chloroform extract of *Calendula* (512 mg/ml) produced a zone of inhibition 1.7 cm against *B. subtilis*, 1.8cm against *E. coli*, 1.6 cm against *S. lutea*, 1.7cm against *K. pneumoniae* (**Table 2**).

Table 2: Comparison of antibacterial activity and MIC values of leaf extract of *C. officinalis* in chloroform by inhibition zone.

Test strains	Chloroform extract of <i>Calendula officinalis</i> leaf (Dose µg/ml)											Positive control	Negative
	512 mg/ml	512 µg/ml	256 µg/ml	128 µg/ml	64 µg/ml	32 µg/ml	16 µg/ml	8 µg/ml	4 µg/ml	2 µg/ml			
<i>B. subtilis</i>	1.7 cm	1.5 cm	1.2 cm	0.8 cm	0.7 cm	-	-	-	-	-	-	2.3 cm	-
<i>E. coli</i>	1.8 cm	1.6 cm	1.2 cm	0.9 cm	0.7 cm	-	-	-	-	-	-	2.5 cm	-
<i>S. lutea</i>	1.6 cm	1.4 cm	1.2 cm	1.0 cm	0.8 cm	0.6 cm	0.6 cm	0.5 cm	0.5 cm	-	-	2.5 cm	-
<i>K. pneumoniae</i>	1.7 cm	1.5 cm	1.3 cm	1.1 cm	0.8 cm	0.7 cm	0.5 cm	-	-	-	-	2.5 cm	-

Another concentration of chloroform extract of *Calendula* (512µg/ml) produced zone of inhibition 1.5cm against *B. subtilis*, 1.6cm against *E. coli*, 1.4cm against *S. lutea*, 1.5cm against *K. pneumoniae* (**Table 2**). And another concentration of chloroform extract of *Calendula* (256µg/ml) produced a zone of inhibition 1.2cm against *B. subtilis*, 1.2cm against *E. coli*, 1.2cm against *S. lutea*, 1.3cm against *K. pneumoniae* (**Table 2**). The concentration of chloroform extract of *Calendula* (128µg/ml) Produced zone of inhibition 0.8cm against *B. subtilis*, 0.9cm against *E. coli*, 1.0cm against *S. lutea*, 1.1cm against *K. pneumoniae* (**Table 2**).

The concentration of chloroform extract of *Calendula* (64µg/ml) produced zone of inhibition 0.7cm against *B. subtilis*, 0.7cm against *E. coli*,

0.8cm against *S. lutea*, 0.8cm against *K. pneumoniae* (**Table 2**). The concentration of chloroform extract of *Calendula* (16µg/ml) produced zone of inhibition 0.0cm against *B. subtilis*, 0.0cm against *E. coli*, 0.6cm against *S. lutea*, 0.5cm against *K. pneumoniae* (**Table 2**).

The concentration of chloroform extract of *Calendula* (8µg/ml) produced zone of inhibition 0.5cm against *S. lutea* (**Table 2**). The concentration of chloroform extract of *Calendula* (32µg/ml) produced zone of inhibition 0.0cm against *B. subtilis*, 0.0cm against *E. coli*, 0.6cm against *S. lutea*, 0.7cm against *K. pneumoniae* (**Table 2**). The concentration of chloroform extract of *Calendula* (4µg/ml) produced zone of inhibition 0.5cm only against *S. lutea* (**Table 2**).

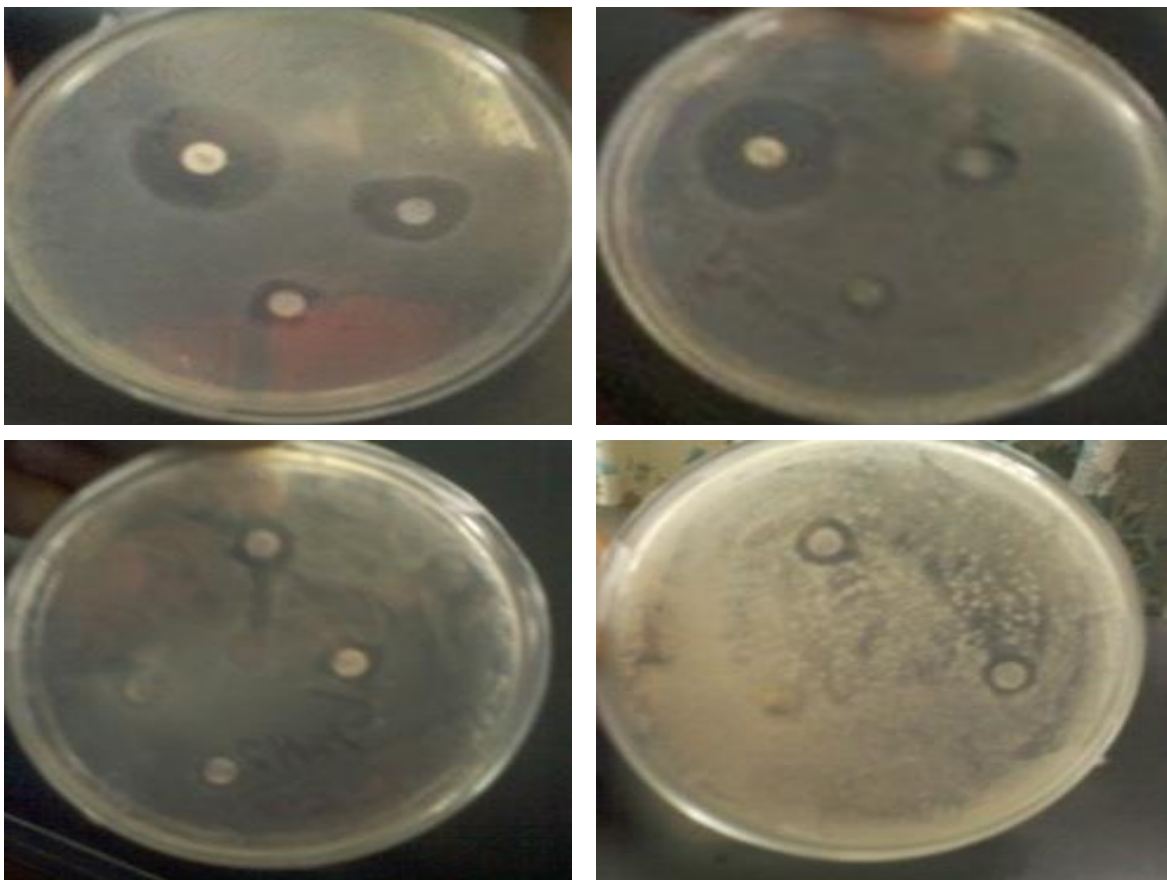


Fig 2: Comparison of Antibacterial activity and MIC values of leaf extract of *C. officinalis* in chloroform by inhibition zone.

In addition, the MIC value was also (0.5cm) determined. The MIC values against *S. lutea* were 4 ug/ml-1. For the comparison of the plant extracts activity positive control (different type of antibiotic disc) and negative control (only solvent absorbing disc) was used. The negative control showed no activity against all tested bacteria. The positive control showed significant antibacterial activity against all bacteria.

The petroleum ether and chloroform extracts of *C. officinalis* showed the highest antibacterial activity against *E. coli* and *K. pneumoniae*. In the present study, *C. officinalis* was very effective in inhibiting the growth of *E. coli* the zone of inhibition of petroleum ether and chloroform was

found. The inhibition of the effect of *C. officinalis* on *E. coli* was less than that of Neomycin (30ug) and also investigated that minimum inhibitory concentration 2µg/ml of leaf extract of *C. officinalis* in chloroform against *K. pneumoniae* and *E. coli* and in petroleum ether against *E. coli*.

The largest inhibitory zone is created by 512 mg/ml chloroform extract against *E. coli*. The extract of *C. officinalis* has been reported to possess antibacterial activity; however, gram-negative bacteria are more susceptible to the action of the oil, whereas gram-negative organisms are more sensitive of the leaves extract (Ali and Blunden *et al.*, 2003).

CONCLUSION:

The beneficial health effects of extracts from many types of plants that have been used for years in daily life to treat diseases all over the world. In the present study, the extract of leaves displayed a variable degree of anti-microbial activity on different microorganisms. *E. coli* and *K. pneumoniae* were found to be more sensitive strain than the others. On the other hand, *B. subtilis* and *S. lutea* were found to be more resistant bacteria against the *C. officinalis* leaves examining findings, the widest inhibition zone was formed *E. coli* and *K. pneumonia* around. The least inhibitory effects were observed for *E. coli*. The petroleum ether and

chloroform extracts of *Calendula* showed anti-bacterial activity against *E. coli* and *B. subtilis*. In conclusion, this result indicated that extracts of the *C. officinalis* leaves which were prepared using petroleum ether and chloroform have a strong inhibitory activity on some pathogens. The purpose was to examine the inhibitory effects of *C. officinalis* leaves extract, some bacteria causing poisoning and harmful for humans. So, the further microbiological investigation was confined only on petroleum ether and chloroform fraction and also investigation is necessary to confirm the bioactive principles of the *C. officinalis* in Bangladesh.

ACKNOWLEDGEMENT:

The research project was financially supported with proper guidance and help for data analysis in the Dept. of Biotechnology and Genetic Engineering, Islamic University, Bangladesh. Many thanks, to the co-workers supporting for successful completion of the research work.

CONFLICTS OF INTEREST:

The authors declared no potential conflicts of the interest with respect to the research, authorship and/or publication of this article.

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Citation: Shahen MZ, Mahmud S, Rony MH, Sohana SN, Imran MAS, Al Maruf MA, Azim MAA, Islam MM, Islam MR, Uddin ME, and Alam MS. (2019). Effect of antibiotic susceptibility and inhibitory activity for the control of growth and survival of microorganisms of extracts of *Calendula officinalis*. *Eur. J. Med. Health Sci.*, 1(3), 1-9. <https://doi.org/10.34104/ejmhs.0190109> 