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Original Research Article

ANALYSIS OF ANTIMICROBIAL ACTIVITY OF *CALENDULA ARVENSIS* AGAINST BACTERIAL PATHOGENS

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ABSTRACT:
There are several evidences of beneficial health effects of extracts from many types of plants. Calendula arvensis is a well known medicinal plant in our country.The purpose of this study was to examine the effectiveness of Calendula arvensis for control of growth and survival of microorganisms. Inhibition of growth was tested by the paper disc agar diffusion method. Antibiotic susceptibility discs were used as control. Minimum Inhibitory concentration (MIC) was determined by the tube dilution method. Extraction from Calendula arvensis was evaluated. Calendula arvensis leaf extract showed inhibition (MIC, inhibitory) to Escherichia coli, Klebsiela Phneumonia, Bacillus subtilis, Sarcina lutea and to the other bacterial strain tested. In this study, antimicrobial effects of leaves extract of Calendula arvensis on some microorganisms including pathogens were investigated. In this purpose extract of Calendula arvensis leaves, which were prepared, were tested on bacterial cultures such as Bacillus subtilis, Sarcina lutea, Escherichia coli and Klebsiela Phneumonia by the paper disc agar diffusion method. This study demonstrates that the potentiality of Calendula as a source of antimicrobials that could be harness for use in the Health care delivery process.Minimum inhibitory concentration was found for 2 µg/ml of leaf extract of Calendula arvensis in chloroform against Klebsiela Phneumonia and Escherichia coli and in Petroleum ether against Escherichia coli. Largest inhibitory zone was created by 512 mg/ml chloroform extract against Escherichia coli. Leaf extract of Calendula arvensis in Petroleum ether was proved as better for antibacterial activity.

Key Words: Calendula arvensis, Minimum Inhibitory Concentretion, Leaf extract, Disc agar diffusion method.

INTRODUCTION:

The use of plant materials for medicines has a long and bright history, since only a century back all drugs were obtained from natural sources. Nature is a 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 diseases all over the world. Plants produce a diverse range of bioactive molecules. Higher plants as source of medicinal compounds to play a dominant role in the maintenance of human health since ancient times [1]**.** The World Health Organization (WHO) estimates that 80% of world population use herbal medicine for some aspects of primary health care [2]. According to one estimate, more than 700 mono and poly-herbal preparations in the form of decoction, tincture, tablets and capsules from more than 100 plants are in clinical use. There are 4,22,127 plant species growing on earth, about 35,000 to 70,000 plants species are used as medicinal plants [3]. The practice of traditional medicine is widespread in China, India, Japan, Pakistan, Sri Lanka and Thailand [3]. The suitable weather and fertile soil have made Bangladesh a great source of medicinal plants. About five thousand herbs, shrubs, trees, aromatic and aquatic plants are scattered throughout the forests, jungles, hills, cornfields, plain fields, roadsides, gardens, marshy lands and watery places of Bangladesh. These medicinal plants which

ISSN 2278 – 5701

contain flavonolglycosides, triterpeneoligoglycosides, oleanane-typetriterpene,glycosides, saponins, and a sesquiterpene glucoside sreroids, alkaloids Which are most useful medicinal agents. The socio economic condition has far reaching effects on health condition of the Bangladesh population. Existing health care system is not very impressive. Though there is steady increase of facilities, the situation has not improved very much. Till now only 30% of the entire population has been brought under primary health care. Twenty percent of the populations of our country have accesses to the western medicines and rest 75-80% of the rural population still receives health care services from the indigenous traditional Ethno medicine practitioners. They play a significant role in providing primary health care services to rural people. They serve as important therapeutic agents as well as imported raw materials for the manufacture of traditional and modern medicines. *Calendula* is used in ayurveda for the treatment of fever and cancer [4]. It is used because of the broad area of biological activities like anti-inflammatory, anti-mutagenic, diuretic, antispasmodic activities. Knowledge of the various biological activities and or 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 material [5]. AIDS, cancer, dengue, fever, and so many diseases are the serious threatening to the present world. The scientists have given most priority in search of appropriate to combat the diseases. Natural products have been a major source of new drugs [6]. The potential for developing antibacterial into medicine appears rewarding, from both the perspective of drug development and the perspective of phyto-medicines [7].

Calendula arvensis is a species of flowering plant in the daisy family known by the common name field marigold. It is native to central and southern Europe, and it is known across the globe as an introduced species. Calendula (Calendula *arvensis* L.) or field marigold has been suggested to benefit in minor wounds, skin infections, burns, bee stings, sunburn, warts, and cancer. Some animal studies support its wound-healing claims. Calendula *arvensis* reported to contain sugars, carotenoids, phenolic acids, sterols, saponins, flavonoids, resins, sterins, quinones, mucilages, vitamins, polyprenylquinones, and essential oils. *Calendula arvensis* L. (Family Compositae) is an annual plant up to 1.5 feet tall. It is also used for gastrointestinal, gynecological, eye diseases, skin injuries and in some cases of burns. The present study was to identify bioactive chemical compounds from Leaves of *Calendula arvensis*, their antimicrobial activity and setting up the standards specification.

MATERIALS AND METHODS

Collection of Calendula arvensis plant material and Extraction

Healthy, disease free, mature *Calendula arvensis* plants was identified and selected for the collection of leaves from Kushtia Municipal garden, Bangladesh. After collection, the calendula (*Calendula arvensis*) leaves were cleaned, washed and dried under shade. Leaves were separated and pulverized into a coarse powder. This powder was used for the preparation of different solvents extracts by sequential extraction. Petroleum Ether and Chloroform were used to extract. 20 grams of leaves powder were transferred to soxhlet apparatus in 100 ml petroleum ether for 24 hours. And another 20 grams were transferred to soxhlet apparatus in 100 ml Chloroform for 24 hours with vigorous shaking. Then the filtrated extraction was evaporated using rotary evaporator to become semi dry material. Dark brown and green colored, oily extracts were utilized.

Serial dilution, Disk diffusion methods and Antimicrobial activity analysis

Extracts were diluted to 512µg/ml. and serially diluted to 256µg / ml, 128µg / ml, 64µg / ml, 32µg / ml, 16µg / ml, 8µg / ml, 4µg / ml, 2µg / ml by serial dilution methods [8]. 512 g of ground samples of calendula leaves were soaked in 10 ml of chloroform for 6 h. During this period the mixture was agitated every 15 min intervals and following filtration. The extract was filtrated and kept the solution in a screw cap. This is used as a dose level was 512 mg / ml. In analysis, dark brown and green colored, oily extracts were used without any dilution. Sample extracts were kept in freezer (+4 ^{o}C) until analysis were concluded. 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 121ºC temperature. 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 oven for 1 hour at 37 ºC. 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. 1 ml of distilled water was taken into the screw capped tube and pure colony of freshly

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cultured bacteria was added in to the tube and vortexed. The OD (optical density) was measured with the colorimeter and microbial population was confirmed to be within tube. This suspension was used as inoculums. Minimum inhibitory concentrations (MIC) of the most active Chloroform and Petroleum ether extracts were determined using serial dilutions of 512 mg/ml and 512 µg/ml to 2µg/ml in Chloroform and petroleum ether solvent against both strains of *E. coli* in Agar well diffusion method. 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.

All strains used in the study were inoculated to nutrient agar and incubated at 35±0.1 ^{o}C for 24 h and were allowed to grow until they reach 10^{8}-10^{9} cfu/ml. The 0.1 ml of inoculum from the prepared culture was transferred to medium. The inoculum was spread to surface of plates with a sterile spreader in laminar air flow. Paper discs embedded within a plant extract were placed on previously inoculated plates and were incubated at 35±0.1^{o} C for 24 h. After incubation the zones of growth inhibition around disks were measured in mm. Antibacterial activity studies were carried out for each test strains in duplicate and average measurement were calculated. Four organisms (0ne gm-negative *i.e. Escherichia coli others Bacillus subtilis,Sarcina lutea and Klebsiella pneumonia*) were tested in this study to determine the antibacterial effect of crude extracts(petroleum ether and Chloroform) *Calendula arvensis* . Antibacterial activity of extracts and crude of *Calendula arvensis* was observed. In antibacterial screening, nutrient agar (High media, India) was used as culture media. Then in vitro antibacterial activities of the extracts were measured by employing standard agar disc diffusion method. In the disc diffusion method, the discs were placed aseptically over the bacterial culture on nutrient agar plates & incubated at 37 ºC for 24 hours. After incubation for 24 hrs, the zone of inhibition around the discs was measured accurately. Discs were impregnated with each treatment and control was assayed on duplicate agar medium plate for *Escherichia coli, Bacillus subtilis, Sarcina lutea and Klebsiella pneumonia*. The experiment was replicated two times to confirm the reproducible results. Sterile, blank paper discs was impregnated with only sterile solvent (petroleum ether and Chloroform) and used as negative control each time. Standard Neomycin (30µg) was used as positive control for comparison of the antibacterial activity.

Statistical analysis

The data was analyzed using SPSS software (release 10.0) to find out significant differences in the antibacterial effects.

RESULT

Petroleum ether extract of Calendula arvensis

Petroleum ether extract of *Calendula arvensis* exhibit antibacterial activity against *Bacillus Subtilis, E. coli, Sarcina lutea and Klebsiella Pneumoniae*. Different concentration of petroleum ether extract of calendula (512 mg/ml) produced zone of inhibition of 1.7 cm against *Bacillus Subtilis,* 1.9 cm against *E coli*, 1.4 cm against *Sarcina lutea* and 1.5 cm against *Klebsiella Pneumoniae* (Table 1). Another concentration of petroleum ether extract of calendula (512 µg/ml) Produced zone of inhibition of 1.3 cm against *Bacillus Subtilis,* 1.4 cm against *E. coli*, 1.2 cm against *Sarcina lutea* ,1.2 cm against *Klebsiella Pneumoniae* (Table 1). And another concentration of petroleum ether extract of calendula (256 µg/ml) produced zone of inhibition of 1.0 cm against *Bacillus Subtilis,* 1.2 cm against *E. coli*, 1.0 cm against *Sarcina lutea,* 1.0 cm against *Klebsiella Pneumoniae* (Table 1). Similarly concentration of petroleum ether extract of calendula (128µg/ml) produced zone of inhibition of 0.9 cm against *Bacillus Subtilis* , 1.0 cm against *E. coli* , 0.9 cm against *Sarcina lutea* ,1.0 cm against *Klebsiella Pneumoniae* (Table 1). The concentration of petroleum ether extract of calendula (64 µg/ml) produced zone of inhibition of 0.8 cm against *Bacillus Subtilis,* 0.9 cm against *E. coli*, 0.9 cm against *Sarcina lutea,* 0.9 cm against *Klebsiella pneumoniae* (Table 1). The concentration of petroleum ether extract of calendula (32 µg/ml) produced zone of inhibition of 0.6 cm against *Bacillus Subtilis* , 0.9 cm against *E coli*, 0.6 cm against *Sarcina lutea* ,0.8 cm against *Klebsiella Pneumoniae* (Table 1). The concentration of petroleum ether extract of calendula (16 µg/ml) produced zone of inhibition of 0.6 cm against *Bacillus Subtilis* , 0.8 cm against *E coli* , 0.6 cm against *Sarcina lutea* and 0.7 cm against *Klebsiella Pneumoniae* (Table 1). The concentration of petroleum ether extract of calendula (8

ISSN 2278 – 5701

µg/ml) produced zone of inhibition of 0.4 cm against *Bacillus Subtilis,* 0.6 cm against *E. coli*, no zone against *Sarcina lutea* and 0.7 cm against *Klebsiella Pneumoniae* (Table 1).

The concentration of petroleum ether extract of calendula (4 µg/ml) produced zone of inhibition of 0.0 cm against *Bacillus Subtilis,* 0.4 cm against *E. coli*, 0.4 cm against *Sarcina lutea,* 0.7 cm against *Klebsiella Pneumoniae* (Table 1). In addition, MIC value was also (0.3 cm) determined. The MIC value against *E. coli* was 2 µg/ml.

Chloroform extract of Calendula arvensis

Chloroform extract of *Calendula arvensis* exhibit antibacterial activity against *Bacillus Subtilis, E. coli, Sarcina lutea, Klebsiella Pneumoniae.* Different concentration of chloroform extract of calendula (512 mg/ml) produced zone of inhibition of 1.6 cm against *Bacillus Subtilis,* 1.8 cm against *E. coli*, 1.6 cm against *Sarcina lutea,* 1.5 cm against *Klebsiella Pneumoniae* (Table 2). Another concentration of chloroform extract of calendula (512 µg/ml) produced zone of inhibition of 1.4 cm against *Bacillus Subtilis,* 1.5 cm against *E. coli*, 1.4 cm against *Sarcina lutea,* 1.3 cm against *Klebsiella Pneumoniae* (Table 2). Another concentration of chloroform extract of calendula (256 µg/ml) produced zone of inhibition of 1.3 cm against *Bacillus Subtilis,* 1.2 cm against *E. coli*, 1.2 cm against *Sarcina lutea,* 1.2 cm against *Klebsiella Pneumoniae* (Table 2). And another concentration of chloroform extract of calendula (128 µg/ml) produced zone of inhibition of 1.2 cm against *Bacillus Subtilis,* 1.1 cm against *E. coli*, 1.0 cm against *Sarcina lutea,* 1.0 cm against *Klebsiella Pneumoniae* (Table 2). The concentration of chloroform extract of Calendula (64 µg/ml) produced zone of inhibition of 0.9 cm against *Bacillus Subtilis,* 0.9 cm against *E. coli*, 0.8 cm against *Sarcina lutea,* 0.9 cm against *Klebsiella Pneumoniae* (Table 2). The concentration of chloroform extract of calendula (32 µg/ml) produced zone of inhibition of 0.6 cm against *Bacillus Subtilis,* 0.8 cm against *E. coli*, 0.6 cm against *Sarcina lutea* and 0.9 cm against *Klebsiella Pneumoniae* (Table 2).

The concentration of chloroform extract of calendula (16 µg/ml) produced zone of inhibition of 0.6 cm against *Bacillus Subtilis,* 0.7 cm against *E. coli*, 0.6 cm against *Sarcina lutea,* 0.7 cm against *Klebsiella Pneumoniae* (Table 2). The concentration of chloroform extract of calendulav(8 µg/ml) produced zone of inhibition of 0.5 cm against *Bacillus Subtilis,* 0.6 cm against *E. coli*, no zone against *Sarcina lutea* and 0.5 cm against *Klebsiella Pneumoniae* (Table 2). The concentration of chloroform extract of calendula (4 µg/ml) produced zone of inhibition of 0.4 cm against *Bacillus Subtilis,* 0.5 cm against *E. coli,* 0.5 cm against *Sarcina lutea,* 0.4 cm against *Klebsiella Pneumoniae* (Table 2). In addition, MIC value was also (0.4cm) determined. The MIC values against *E. coli* and *Klebsiella Pneumoniae* was 2 µg/ml.

DISCUSSION

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 this study, the purpose was to examine the inhibitory effects of *Calendula (calendula arvensis)* leaves extract against some pathogenic bacteria.. For this purpose, the Petroleum ether, Chloroform extracts of *Calendula* Leaves were tested on *Bacillus subtilis, Escherichia coli, Sarcina lutea, Klebsiella pneumonia* with disc diffusion method as in vitro.

In this study, extract of leaves displayed a variable degree of antimicrobial activity on different microorganisms. *Escherichia coli* and *Klebsiella pneumonia* was found to be more sensitive strain than the others. On the other hand *Bacillus subtilis* and *Sarcina lutea* were found to be more resistant bacteria against the *Calendula* leaves. The widest inhibition zone was formed against *Escherichia coli* and *Klebsiella pneumonia*. The least inhibitory effects were observed against *E. coli*. Some investigators noted that sensitivity of microorganisms to chemotherapeutics differs according to type of strain. Similiar results have been observed in our study. The extract of the *Calendula arvensis* leaves which was prepared using Petroleum ether and Chloroform, has a strong inhibitory activity on some pathogens.

Microorganisms are the concealed enemies to the mankind. They are small but cause a very profound damage in human body. The agents which have the capacity to kill the microbes or arrest the multiplication are called the antibacterial agents or drugs. There are a lot of antibacterial drugs of which some are discovered or established and some are hidden in the nature. Members of this family have attracted continuous phyto-chemical interest due to their considerable importance as a natural species or as medicinal plants. In the present work, the antibiotic potential of the extracts of *calendula arvensis* has been

ISSN 2278 – 5701

determined against the four pathogenic bacteria namely; *E coli, Klebsiella pneumonia,Bacillus subtillis and Sarcina lutea.* All of the extracts of calendula showed inhibition on all of these bacteria. For comparison of the activity of plant extracts, 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 *Calendula* showed the highest antibacterial activity against *Escherichia coli and Klebsiella pneumonia.* So, further microbiological investigation was confined only on Petroleum ether and chloroform fraction. However, gm negative organisms are more sensitive to the extract of *Calendula*. It may be used as a constituent of a drug. *Calendula arvensis* is used for the treatment of skin disorders and pain, and as a bactericide, antiseptic and anti-inflammatory.

Table 1: Comparison of Antibacterial activity and minimum inhibitory concentration (MIC) values of leaf extract of *Calendula arvensis* in Petroleum ether by Inhibition zone.

Test strains	Petroleum ether extract of *Calendula arvensis* leaf (Dose µg/ml)											
	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	Positive control	Negative control
B. Subtilis	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	-
E. Coli	1.9 cm	1.4 cm	1.2 cm	1.0 cm	0.9 cm	0.9 cm	0.8 cm	0.6 cm	0.4 cm	0.3 cm	2.5 cm	-
S. lutea	1.4 cm	1.2 cm	1.0 cm	0.9 cm	0.9 cm	0.6 cm	0.6 cm	-	0.4 cm	-	3.o cm	-
K. Pneumonia	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	-

Table 2: Comparison of Antibacterial activity and minimum inhibitory concentration (MIC) values of leaf extract of *Calendula arvensis* in Chloroform by Inhibition zone

Test strains	Chloroform extract of *Calendula arvensis* leaf (Dose µg/ml)											
	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	Positive control	Negative control
B. Subtilis	1.6 cm	1.4 cm	1.3 cm	1.1 cm	0.9 cm	0.6 cm	0.6 cm	0.5 cm	0.4 cm	-	2.6 cm	-
E. Coli	1.8 cm	1.5 cm	1.2 cm	1.2 cm	0.9 cm	0.8 cm	0.7 cm	0.6 cm	0.5 cm	0.4 cm	2.2 cm	-
S. lutea	1.6 cm	1.4 cm	1.2 cm	1.0 cm	0.8 cm	0.6 cm	0.6 cm	-	0.5 cm	-	2.5	-
K. Pneumonia	1.5 cm	1.3 cm	1.2 cm	1.0 cm	0.9 cm	0.9 cm	0.7 cm	0.5 cm	0.4 cm	0.4 cm	2.3	-

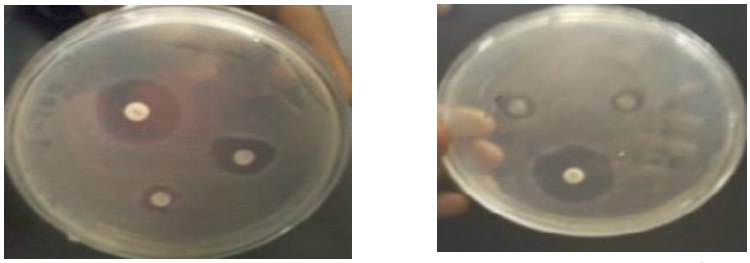

Figure 1: Antibacterial activity and minimum inhibitory concentration (MIC) values of Petroleum ether extract of leaf of *Calendula arvensis*.

The petals and pollen contain triterpenoid esters (an anti-inflammatory) and the carotenoids flavoxanthin and auroxanthin (antioxidants, and the source of the yellow-orange coloration). *Calendula arvensis* have been used as medicine for centuries. Traditionally, *Calendula* have been used to treat conjunctivitis, hepatitis, eczema, gastritis, minor burns including sunburns, warts, and minor injuries such as sprains and wounds. It has also been used to treat cramps, coughs, and snake bites. Research continues into the healing properties of *Calendula*. Historically, *Calendula* flowers have been considered beneficial in reducing inflammation, promoting wound healing, and used as an antiseptic. *Calendula* has been used to treat a variety of skin diseases and has been seen effective in the treatment of skin ulcerations and eczema. Taken internally through tea, it has been used for treatment of stomach ulcers, and inflammation. A sterile tea has been used to treat infections of the eye, like conjunctivitis, however, this practice is not recommended. *Calendula* today is being investigated for its anticancer properties. In conjunction with other herbs such as Echinacea purpurea, Scorzonera humilis L., and Aconitum moldavicum, there has been evidence of success in treating certain cancers (Heren's carcinoma) according to the Fedkovich Chernivtsi State University in the Ukraine. *Calendula* has been effective in treating juvenile acne and dry phthiriasis. Improvement has been seen in as little as 3-4 days of treatment according to the Universitatea de Medicina si Farmacie. Western Australia has been investigating *Calendula* for control of the Redlegged earth mite. Halotydeus destructor - Redlegged earth mite - is a major pest of pastures and crops in Australia. In some cases, the crops had better growth and production when Calendula was planted as a decoy crop. The Calendula was heavily attacked while the damage to crops was less.

The Petroleum ether and chloroform extracts of Calendula showed antibacterial activity against *Escherichia coli and Bacillus subtilis*. So, further microbiological investigation was confined only on Petroleum ether and chloroform fraction. In present study, *Calendula arvensis* was very effective in inhibiting the growth of *E. coli*. The effect of *Calendula arvensis* on *E. coli* was less than that to Neomycin (30ug). The extract of *Calendula arvensis* has been reported to possess antibacterial activity, however gram negative bacteria are more susceptible to the action of the oil, where as gram negative organisms are more sensitive of the leaves extract [9]. In current study, Minimum inhibitory concentration 2 µg/ml of leaf extract of *Calendula arvensis* in chloroform against *Klebsiela Phneumonia* and *Escherichia coli and in* Petroleum ether against *Escherichia coli*. The largest inhibitory zone was created by 512 mg/ml chloroform extract against *Escherichia coli.* The reason might be due to the impurities of solvent or the disc paper was not completely soaked. It has been proved from this experiment that traditional use of *Calendula arvensis*. has scientific basic. Further investigation is necessary to confirm the bioactive principles of the *Calendula arvensis* in Bangladesh.

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