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ANTIMICROBIAL ACTIVITIES AND PRELIMINARY PHYTOCHEMICAL SCREENING ON THE CRUDE EXTRACTS OF THE LEAVES OF *CINERARIA ABYSSINICA* SCH. BIP. EXA. RICH

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ABSTRACT

In Ethiopian traditional medicine, the aqueous decoction of the leaves of *Cineraria abyssinica* Sch. Bip. exA. Rich (Asteraceae) is used for treatments of various ailments including diarrhea, however, to date, there appear to have been no reports on the phytochemistry and the antimicrobial activity of the plant. The main aim of this study was, therefore, to carry out preliminary phytochemical screening and antimicrobial activities on leaf extracts of *C. abyssinica*. The *in vitro* antimicrobial activities of the aqueous and 80% methanolic crude extracts of the leaves of *C. abyssinica* were investigated against Gram positive and Gram negative bacteria by agar disc diffusion method. Both the aqueous and 80% methanolic extracts showed various degrees of potent antibacterial activities comparable to the standard drug ciprofloxacin against all of the bacteria tested except *Bacillus* species. Preliminary phytochemical screening of the plant revealed the presence of polyphenols, flavonoids, coumarins, saponins, terpenoids and phytosterols. The acute toxicity studies showed the nontoxic nature of the plant up to 3 g/kg. Therefore, the present study revealed for the first time the presence of antimicrobial phytochemicals in the leaves of *C. abyssinica* that scientifically validated the traditional use and its great potential to be used for treatment of infectious diarrhea.

INTRODUCTION: Infectious diseases have continued to be leading causes of morbidity, disability and mortality in the world. Approximately 15 million people die each year due to infectious diseases - nearly all live in developing countries. Their control is a constant challenge that faces health workers and public health officials in both industrialized and developing countries [1-2]. Indiscriminate and injudicious use of antibiotics has led to increased microbial resistance.

Recent surveys conducted in various parts of Ethiopia have shown that there have been rapid incidences of drugs resistance [3-8]. Drugs that once seemed unconquerable are losing their effectiveness for a wide range of infections. Consequently, newer agents have been introduced at increased economic costs to the patient but they too have become inefficacious in due course and pose a great threat to human health in world.

So, noble emerging and re-emerging infectious diseases as well as the undesirable side effects of certain antibiotics have triggered huge interest in the search for new cost-effective and safer antimicrobial drugs of plant origin in both industrialized and developing countries [2, 4]. Plant derived antimicrobials represent a vast untapped source for medicines and further exploration of plant antimicrobials is needed [9].

Traditional medicines continue to provide front-line pharmacotherapy for many millions of people worldwide. According to the WHO, 80% of the world's population primarily those of developing countries rely on plant-derived medicines to meet their primary healthcare [10, 11]. A large proportion of these plants are used for treatment of infectious diseases including bacteria, virus and fungi, which account for approximately one-half the death in tropical countries [12].

The biodiversity of the Ethiopian flora offers great possibilities in the search for natural products with novel structures that have a range of biological activities including treatment of various infectious diseases. However, many of the plant species used in Ethiopian traditional medicine have not been investigated either from the point of view of their chemical composition or in terms of their pharmacological activities [13, 14]. Therefore, screening these plants may provide safer, more effective and chipper antimicrobial drugs that will increase the therapeutic arsenal for treatment of various infectious diseases.

Cineraria abyssinica Sch. Bip. exA. Rich (Asteraceae) locally known as 'Esemefirh' in Amharic [15] and 'Baluketel or Fatu kitel' in Harari and Oromifa, is an erect or scrambling, annual or perennial herb that can grow up to 20-100 cm high. It has repeatedly branched stem, with alternate, simple to lyrately pinnatified petiolate leaves and radiate capitula with yellow florets. It extends from Ethiopia into Yemen and Saudi Arabia [15].

Based on the information provided by the local community from Harar, eastern part of Ethiopia, the aqueous decoction of the leaves of *C. abyssinica* is employed for treatments of various ailments such as hypertension, cancer, diabetes, liver and kidney diseases and diarrhea. Despite its wider use locally, there are no prior reports on the phytochemistry and pharmacological effects of this plant. The present research was therefore, undertaken to examine the possible antibacterial effects of the plant and to correlate these activities with the traditional use.

MATERIALS AND METHODS:

Plant material: The leaves of *C. abyssinica* were collected from and around the town of Harar in the Harari People Region, 525 km east of Addis Ababa, Ethiopia in September 2008. The plant was authenticated by Ato Melaku Wondafrash of the National Herbarium, Addis Ababa University, Biology Department and a voucher specimen has been deposited at the National Herbarium, Addis Ababa University (Collection number, B 01).

Chemicals and instruments: All the chemicals and reagents used for the experiments are analytical grade.

Bacterial strains: The antibacterial activity of the plant was carried out on the following Gram-negative bacterial strains *E. coli* K99, *E. coli* K88, *E. coli* 306, *E. coli* LT37, *E. coli* 872, *E. coli* ROW 7/12, *E. coli* 3:37C, *E. coli* CD/99/1, *Salmonella typhi Ty2, Shigella dysentery 1, S. dysentery* 8, *S. soneii* 1, *S. boydii* D13629, *S. flexneri* Type 6, *Vibrio cholerae* 1313, *V. cholerae* 293, *V. cholerae 1315* and Gram-positive bacteria *Bacillus pumilus* 82, *B. subtilis* ATCC 6633 and *Staphylococcus aureus* ML 267. All the bacterial strains were procured from the Department of Pharmaceutical Technology, Jadavapur University, Kolkata, the Central Drug Laboratory, Kolkata and the Institute of Microbial Technology, Chandigarh, India. The strains were first checked for purity on the basis of standard microbiological, cultural and biochemical tests and then used for their sensitivity on test samples.

Preparation of Crude Extracts: Two different extractions were carried out. The leaves of *C. abyssinica* were shade dried and powdered. The powder (300 g) was extracted exhaustively at room temperature by maceration with 5 L of 80% methanol for 72 h with occasional shaking (3x) and the other by aqueous decoction for 30 min. The combined methanolic filtrates were then dried in a Rotary vacuum evaporator (< 40°C) and the aqueous extract was lyophilized.

The yields of the extracts were 20% and 17% for 80% methanol extract and for the aqueous extract respectively.

Antibacterial Assay: One loopfull (loop diameter: 3 mm) of an overnight grown nutrient broth culture of the test organisms was added by checker board technique to the marked quadrant of the sterile 100 mm petridishes containing various concentrations (5, 10, 25, 50, 100, 200, 400, and 800 µg/ml of the aqueous and 80% methanolic crude extracts. The spot inoculated plates were incubated at 37°C for 24 h to determine minimum inhibitory activity (MIC) of the extract against the test microorganisms [16]. The lowest concentration that inhibited growth of the bacteria was taken as the MIC value for the test substance.

The antibacterial activities of the aqueous, and 80% methanol crude extracts, of *C. abyssinica* leaves were also determined by agar disc diffusion method. The sterile nutrient agar was cooled to 48-50°C and then seeded with the test organisms and poured into sterilized petridishes. When the agar solidified, the total surface of the agar was divided into six quadrants and six filter paper discs (6 mm diameter) containing 200 µg/ml of the tested extracts, in triplicate, standard drug (ciprofloxacin 200 µg/ml) and one control (10% dimethylsulfoxide (DMSO)), were placed in the middle of each zone. All the plates were incubated at 37°C for 48 h and the zone of inhibition was calculated by measuring the diameter of zone of no bacterial growth around the filter paper disc. For each set of data, the average of three determinations was recorded [17, 18].

The 80% methanol extracts or the powdered plant material where ever required was used for testing the presence or absence of secondary metabolites such as phytosterols, polyphenols (tannins, flavonoids and coumarins), saponins, alkaloids and anthraquinones following standard procedures [19].

Acute Toxicity Tests: Acute toxicity studies were carried out on the aqueous and 80% methanolic leaf extracts of *C. abyssinica* according to [20]. Ethical clearance to conduct the study on experimental animals was obtained from Mekelle University, College of Health Sciences, Research & Communication Service Office. Normal healthy male mice weighing 20 to 25 g fasted for 12 h were randomly divided into drug-treated 'test' groups and vehicle-treated 'control' group, totally making up 7 groups of 6 mice per cage. 0.5, 2.0 and 3.0 g of each of the extracts suspended in 1% carboxyl methyl cellulose (CMC)) was separately administered orally to the mice in each of the test groups. The mice in the control group were treated with vehicle alone (1% CMC). 2 h after treatment, the mice in both the test and control groups were allowed access to food and water, and behavioral changes were observed over a period of 24 h for sign of acute toxicity. The mortality caused by the extract within this period of time was observed.

RESULTS AND DISCUSSION: The MIC values obtained from agar disc diffusion technique (**Table 1**) showed that the aqueous and 80% methanolic crude extracts displayed various degrees of considerable broad spectrum antibacterial activities against Gram-negative and Gram-positive bacteria except *Bacillus* species. Compared to the 80% methanol crude extract, the aqueous extract showed higher antibacterial activity against most of the bacteria tested except against *Bacillus* species where the 80% methanol extract was found to more potent (MIC = 400 µg/ml). The aqueous extract showed maximum activity against *Salmonella* species and *Staphylococcus aureus (MIC = 10* µg/ml*)*, *Shigella* species (10-50 µg/ml), *Vibrio* species (25 µg/ml) and moderate activity against *E. coli* strains (50 µg/ml), but least activity against *Bacillus* species (800 µg/ml).

Comparison of the antibacterial activities of 200 µg/ml of the aqueous and 80% methanolic crude extracts with the standard drug ciprofloxacin (200 µg/ml) (**Table 4**) showed that the aqueous extract displayed zone of inhibition comparable to that of ciprofloxacin against all the bacteria except for *Bacillus* species for which the test extract was less active. The activity of the test substances against *S. thyphi* is particularly worth mentioning. While the 80% methanol extract and ciprofloxacin showed the same diameter of zone of inhibition (15.0 mm), the zone of inhibitions against *S. thyphi* by the aqueous extract (16.5 mm) was higher than ciprofloxacin (**Table 2**). Typhoid fever caused *S. thyphi* is a major health problem in developing countries, particularly in Sub-Saharan Africa countries and hence the considerable anti-salmonella activity shown by the test extracts shows the great potential of the plant for treatment of typhoid fever.

TABLE 1: MINIMUM INHIBITORY CONCENTRATIONS OF THE AQUEOUS AND 80% METHANOLIC LEAF EXTRACTS OF *CINERARIA ABYSSINICA* AGAINST BACTERIA

Name of Bacteria	MIC (µg/ml)	
	Aqueous	80% Methanol
Gram positive		
Bacillus pumilus 82	800	400
Bacillus subtilis ATCC 6633	800	400
Staphylococcus aureus ML 267	10	200
Gram negative		
E.coli K99	50	200
E.coli K88	50	200
E.coli 306	50	200
E.coli LT37	50	200
E.coli 872	50	200
E.coli ROW 7/12	50	400
E.coli 3:37C	50	400
E.coli CD/99/1	50	200
Salmonella typhi Ty2	10	50
Shigella dysentery 1	10	25
Shigella dysentery 8	10	25
Shigella soneii 1	10	25
Shigella boydii D13629	50	25
Shigella flexneri Type 6	25	25
Vibrio cholerae 1313	25	100
Vibrio cholerae 293	25	100
Vibrio cholerae 1315	25	100
Vibrio cholerae 85	25	50

TABLE 2: COMPARISON OF ZONES OF INHIBITION OF THE AQUEOUS AND 80% METHANOLIC LEAF EXTRACTS OF CINERARIA ABYSSINICA WITH THAT OF STANDARD ANTIBACTERIAL AGENT CIPROFLOXACIN AT 200µg/ml

Name of Bacteria	Aqueous	80% Methanol	Ciprofloxacin
Gram positive			
Bacillus pumilus 82	9.0	10.0	18.5
Bacillus subtilis ATCC 6633	9.0	10.0	18.0
Staphylococcus aureus ML 267	17.0	11.0	18.0
Gram negative			
E.coli K99	14.5	13.0	16.0
E.coli K88	15.5	13.5	16.5
E.coli 306	15.5	13.5	16.5
E.coli LT37	15.0	13.0	16.0
E.coli 872	15.5	13.0	16.0
E.coli ROW 7/12	15.0	12.0	16.5
E.coli 3:37C	14.5	12.5	15.5
E.coli CD/99/1	15.5	13.0	17.0
Salmonella typhi Ty2	16.5	15.0	15.0
Shigella dysentery 1	18.0	15.5	20.5
Shigella dysentery 8	18.5	15.5	21.0
Shigella soneii 1	18.0	16.0	19.5
Shigella boydii D13629	17.5	16.5	20.0
Shigella flexneri Type 6	19.0	15.0	20.5
Vibrio cholerae 1313	16.5	14.0	17.0
Vibrio cholerae 293	16.5	14.5	17.5
Vibrio cholerae 1315	17.0	14.0	18.0
Vibrio cholerae 85	17.0	14.5	18.0

The major causes of infectious diarrhoea are enteropathogenic bacteria such as *E. coli*, *S. typhi*, *Shigella* species, *V. colera*. Abera and Biadglegne have recently showed that S. *typhi* and *Shigella* species are the most common bacteria in stool culture in Northern Ethiopia [7, 20]. In the present investigation, the extracts displayed considerable antibacterial activities against theses multidrug resistant enteropathogenic bacteria thereby by justifying the traditional use of the plant for treatment of diarrhoea and its immense potential as a source of natural antibacterial drugs and/or an alternative cheaper herbal drug.

As shown in **Table 3**, preliminary phytochemical screening of the 80% methanol leaf extract of *C. abyssinica* revealed the presence of polyphenoles, flavonoids, coumarins, saponins, terpenoids and phytosterols while alkaloids, anthraquinones, cardiac glycosides and tannins were absent. Many literatures have shown the antibacterial activities of these phytochemicals [22-28] and therefore, the presence of one or more of these secondary metabolites may be responsible for the different antibacterial activities of the plant.

TABLE 3: PRELIMINARY PHYTOCHEMICAL SCREENING ON THE 80% METHANOLIC LEAF EXTRACT OF CINERARIA ABYSSINICA

2nd metabolites tested	Reagents used	Result
Alkaloids	Dragendorff's reagent	Absent
	Mayer's reagent	Absent
Anthraquinones	Bontrager's reagent	Absent
Cardiac glycosides	Kedde reagent Keller-Kiliani Libermann-Bruchard's reagent Salkowski test	Absent
Polyphenols	1 % $FeCl_3$ and 1 % $KFe(CN)_6$	Present
Flavonoids	Shinoda reagent Lead acetate	Present
Coumarins	Ammonia fluorescence test	Present
Saponins	Froth test 10% sodium nitrate and conc. H_2SO_4	Present
Tannins	Gelatine test	Absent
Phytosterols	Libermann-Bruchard's Salkowski test	Present

The acute toxicity study revealed the non-toxic nature of the aqueous and 80% methanolic leaf extracts of *C. abyssinica* up to 3 g/kg. No mortality was observed in the extract-treated mice and the behavior of the treated mice also appeared normal.

Thus, the promising *in vitro* antibacterial activity by the plant merits further research that involve, sub-acute and chronic toxicity. Bioactivity-guided fractionation of the plant may also generate safer, more effective and cheaper antibacterial drugs that increase the therapeutic arsenal for different infectious diseases.

CONCLUSION: In conclusion, the present study revealed for the first time the pronounced broad spectrum antibacterial activities of the leaves of *C. abyssinica* against enteropathogenic bacteria. The presences of polyphenols, flavonoids, coumarins, saponins, terpenoids and phytosterols may be responsible for the potent antibacterial activity of the plant. Acute toxicity study revealed the nontoxic nature of the plant up to 3 g/kg and hence, the present study justifies the traditional claim of the plant and its immense potential to be used in the treatment of infectious diarrhoea.

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