Antibacterial potential of the 80% methanol and chloroform extracts of *Clematis hirsuta*

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The leaves and roots of *Clematis hirsuta* Perr and Guill is traditionally applied to heal respiratory tract and for the treatment of various animal diseases in different regions of Ethiopia. The objective of this work is to evaluate the antibacterial activities of *C. hirsuta*. The leaves were collected and air dried in shade at room temperature, made into powder and was soaked in 80% methanol and chloroforms (1 g: 10 ml). The powder was placed in a shaker for 72 h at room temperature. The extract was prepared in 3% Tween 80 for antibacterial test. The antibacterial activities and the minimum inhibitory concentration (MIC) test were determined by paper disk diffusion and agar dilution methods respectively. The 80% methanol and chloroform extract of the leaves of *C. hirsuta* showed significantly higher inhibition zone than the negative control on some pathogenic bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Shigela boydii* and *Salmonella thyphi*), but the extracts had significantly lower inhibition zone than the standard drugs (chloramphenicol and ampicillin). The chloroform crude extract of the leaves *C. hirsuta* showed the best inhibition zone (12.33±0.50) on *P. aeruginosa* at 200 mg/ml concentration. The chloroform extract of *C. hirsuta* had the lowest MIC and minimum bactericidal concentration (MBC) at 3.125 and 6.25 mg/ml on *P. aeruginosa*, respectively. The 80% methanol *C. hirsuta* leaf extract was not toxic at 500, 1000 and 2000 mg/kg body weight to albino mice.

**Keywords:** Inhibition zone, acute toxicity, minimum inhibitory concentration, minimum bactericidal concentration.

**INTRODUCTION**

Medicinal plants are used in spiritual therapies, manual techniques and applied to treat, diagnose or prevent illness (WHO, 2013). In Ethiopia, more than 80% of the people use traditional medicines to apply them in various types of infections and disorders (Bekele, 2007). It is reported about 200,000 natural products are known, but most of drugs are isolated from higher plants and microorganisms (Ramawat and Merillon, 2007). However, traditional practitioners in Ethiopia use the plants without scientific dose optimization. It has reported that different parts of the plant are used traditionally to treat different types of infections (Ogbulie et al., 2007). *Clematis hirsuta* belongs to family Ranunculaceae. It is perennial woody climber and measures up to 1 to 4 m long. It bears oppositely arranged compound leaves. Flower is borne in panicles with white or yellow color. *Clematis* species are known to have secondary metabolites including glycoside ranunculin, saponins, tritrepenoid, alkaloids and...
cyanogenic glycosides (Trease and Evans, 1996). Thus the *Clematis* species are used as medicinal plants. Mainly *C. hirsuta* are used in folk medicine worldwide especially in Asia as anti-inflammatory, analgesics and anti-rheumatics (Al-Taweel et al., 2007). The petroleum ether and butanol extracts of *C. hirsuta* revealed the presence of sterols and triterpenes and has anti-inflammatory on rat model (Abdel-Kader et al., 2008). In the other studies, the methanolic and ethanolic extracts of the related species (*C. ispanhana* and *C. orientalis*) shows antibacterial activity (Raei et al., 2013).

In Ethiopia, *C. hirsuta* with local name “Azo tero” is used in folk medicine as analgesics and anti-rheumatics. Roots of *C. hirsuta* and *Sida schimperiana* (Malvaceae) are crushed, powdered and mixed with water for oral and nasal administration to treat blackleg and *C. hirsuta* accounts 36.73% of all medicinal plant considered in the study for the treatment of various animal ailments in Bale Districts (Yineger et al., 2007). *C. hirsuta* is also used to heal respiratory tract problem and cataract in Meinit ethnic group, southern part of Ethiopia (Giday et al., 2009). Thus, the study aimed at evaluating the antibacterial activities of the 80% methanol and chloroform extracts of leaves of on *C. hirsuta* some pathogenic bacteria.

**MATERIALS AND METHODS**

Methanol (Reagent chemical Services Ltd., United Kingdom), chloroform (Merck KGAa, 64271, Darmstadt, Germany), nutrient agar (Oxoid LTD., Basingstoke, Hampshire, England), Muller-Hinton agar (Oxoid LTD., Basingstoke, Hampshire, England), barium chloride sulfuric acid (SDFCL Fine Chemical Ltd., Mumbai, India), Tween-80 (Uni-Chem Chemical Reagents ), sodium chloride (Nikko Chemical, India), ampicillin (Oxoid LTD., United Kingdom), chloramphenicol (Oxoid Ltd., United Kingdom), barium chloride (BDH Chemicals Ltd. Poole, England) were used.

**Plant collection and identification**

The fresh leaves of *C. hirsuta* were collected from natural vegetation in Dejen district, Eastern Gojjam Zone, Amhara Regional State, Ethiopain in June 2014. The specimens were identified and authenticated by Mr. Melaku Wondafrash at National Herbarium, Department of Biology, Addis Ababa University. The voucher specimens were deposited in the herbarium and named with *C. hirsuta* (ah004).

**Preparation of solvent extraction**

The fresh leaves of *C. hirsuta* were washed three times with tap water and once with sterile distilled water. After wash, the plant materials were air dried in shade at room temperature (25 to 30°C) for two weeks until it became completely dry. Following the drying process, about 0.3 kg leaves was powdered and sieved through a fine mesh (Canadian Series sieves with 5×10⁻⁴ opening) and stored in dry bottle at temperature for further use as described in Subbarayan et al. (2010). About 0.05 kg of powder of *C. hirsuta* was soaked into 0.5 L of chloroform and 0.5 l 80% methanol in separate flasks. The mixtures in the erlenmeyer flask were placed on a platform shaker of 120 rpm for 72 h at room temperature (Mohana et al., 2009). Then, the solutions were filtered by Whatman no. 1 filter papers and the solvent extract was concentrated separately under reduced pressure using rotary flash evaporator at 45°C. After complete evaporation of the solvent each the extract was weighed and in dissolved in 3% Tween-80 for biological assay.

**Anti bacterial susceptibility test determination of leaf extracts of *C. hirsuta***

*S. aureus*, *E. coli*, *P. aeruginosa*, *S. boydii* and *S. typhi* were taken from Microbiology Department, Ethiopian Public Health Institute (EPHI), Addis Ababa. The standard bacteria were screened for susceptibility at different doses (50, 100 and 200 mg/ml) of *C. hirsuta* extracts and standard antibiotics ampicillin (30 µg/disk) and chloramphenicol (30 µg/disk). Bacterial broth culture was prepared at a density of 10⁴ cells ml⁻¹ which approximately equals to 0.5 McFarland standards. The test microorganisms were uniformly swabbed on the Mueller Hinton Agar (MHA) using the cotton swab. The paper disc diffusion technique was applied to determine the antimicrobial activities of the tested plant extracts. Sterile paper discs (6 mm in diameter) immersed in stock solutions containing 50, 100 and 200 mg/ml prepared in 3% Tween-80 of plant extracts and allowed to dry for 15 min to allow it to diffuse and paper disks were placed on uniformly swabbed agar plate using sterile forceps. The plates were then incubated for 24 h at 37°C and diameters of the inhibition zones were recorded. All tests were carried out in triplicates.

**Minimum inhibitory concentration (MIC) determination by agar dilution method**

Minimum inhibitory concentration (MIC) was determined by agar dilution methods as described by European society of clinical microbial and infectious diseases (ESCMID, 2000). Nineteen millimeter of molten Mueller Hinton agar (MHA) and 1 ml of extracts from each plant at different concentration (50, 25, 12.5, 6.75 and 3.125 mg/ml) were mixed thoroughly and poured on Petri dish. Petri dishes then were allowed to dry to avoid drops of mixture. The bacterial suspensions in 0.85% saline contains about 1.5×10⁸ cells ml⁻¹ colony forming unit (CFU) which were standardized with 0.5 McFarland. 1 µl of bacterial suspension (approximately 1×10⁴ CFU) was inoculated on plate having Muller Hinton agar and extracts as described above. After drying the inoculums spot, the plates were incubated at 37°C 24 h. The MIC was determined by observing the growth bacteria with our naked eyes.

**Minimum bactericidal concentration (MBC) determination**

Some portions of tests were taken from the (MIC) test plate and were sub-cultured on solid nutrient agar by making streaks on the surface of the agar. The plates were incubated at 37°C for 24 h and the MBCs were determined after 24 h. Plates that did not show growth were considered to be the MBC for the extract.

**Oral acute and sub-acute toxicity tests**

Oral acute and sub acute toxicity tests were done according to OECD guideline (2001). The Swiss albino mice which were obtained from Ethiopian public health institute (EPHI) and reared in animal house in department of biology, college of natural science, Addis Ababa University were used. The mice were starved for 3 h before the experiment began with only water allowed and 1 to 2 h
Table 1. Percentage extract yield from the leaves of chloroform C. hirsuta extracts.

<table>
<thead>
<tr>
<th>Solvents/plant extract</th>
<th>Dry powder (g)</th>
<th>Solvent (ml)</th>
<th>Ratio (w/v)</th>
<th>Yield (g)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>80% Methanol/ C. hirsuta</td>
<td>50</td>
<td>500</td>
<td>1:10</td>
<td>7.88</td>
<td>15.75</td>
</tr>
<tr>
<td>Chloroform/ C. hirsuta</td>
<td>50</td>
<td>500</td>
<td>1:10</td>
<td>4.94</td>
<td>9.9</td>
</tr>
</tbody>
</table>

Table 2. The bacterial inhibition zone of leaves extract of C. hirsuta on some bacteria (mg/ ml).

<table>
<thead>
<tr>
<th>Extract/solvent</th>
<th>Dose (mg/ml)</th>
<th>E. coli</th>
<th>P. aeruginosa</th>
<th>S. aureus</th>
<th>S. boydii</th>
<th>S. typhi</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.h/Me</td>
<td>50</td>
<td>a=6.67±0.01*</td>
<td>a=6.78±0.25*</td>
<td>a=6.37±0.17*</td>
<td>b=6.67±0.02*</td>
<td>b=6.67±0.02*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>a=7.5±0.05*</td>
<td>a=7.05±0.06*</td>
<td>a=7.47±0.15*</td>
<td>b=7.82±0.06*</td>
<td>b=7.33±0.05*</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>a=9.3±0.12*</td>
<td>a=8.5±0.50*</td>
<td>a=8.00±0.29*</td>
<td>b=9.00±0.06*</td>
<td>b=7.90±0.05*</td>
</tr>
<tr>
<td>C.h/Ch</td>
<td>50</td>
<td>a=7.04±0.04*</td>
<td>a=8.40±0.12*</td>
<td>a=7.23±0.02*</td>
<td>b=7.2±0.15*</td>
<td>b=7.33±0.04*</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>a=8.5±0.05*</td>
<td>a=9.3±0.1*</td>
<td>a=8.31±0.06*</td>
<td>b=8.33±0.02*</td>
<td>b=8.33±0.09*</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>a=10.7±0.14*</td>
<td>a=12.33±0.02*</td>
<td>a=10.26±0.16*</td>
<td>b=10.34±0.13*</td>
<td>b=11.60±0.12*</td>
</tr>
<tr>
<td>A</td>
<td>30 µg</td>
<td>12.5±0.29*</td>
<td>15.33±0.33*</td>
<td>18.4±0.29*</td>
<td>23.66±0.33*</td>
<td>26.83±0.6*</td>
</tr>
<tr>
<td>C</td>
<td>30 µg</td>
<td>0±0±0</td>
<td>0±0±0</td>
<td>0±0±0</td>
<td>0±0±0</td>
<td></td>
</tr>
</tbody>
</table>

C. h = Clematis hirsuta; Me = 80% methanol; Ch = chloroform; A = ampicillin; C = chloramphenicol; T80 = Tween 80; * = A inhibition; b = C inhibition; C = chloramphenicol; = is significantly higher than; = is significantly lower than; d = T80 inhibition.

after the extracts were given. Female Swiss albino mice, 5 in each group were administered orally with 500, 1000 and 2000 mg/kg body weight from each extract which was dissolved in 3% Tween 80 within 0.2 ml for test and 0.2 ml 3% Tween were administered as control. For acute toxicity studies; gross physical changes within 24 body and weight measured for 14 days. At end of the experiment the experimental mice were anesthesia with chloroform and discarded.

Data analysis

Data was analyzed by using window software; IBM SPSS, version 20. The results were presented as the Mean±Standard Error of the Mean (Mean±SEM) and statistical significance was considered as a 95% confidence interval (P<0.05). For toxicity test and bacterial sensitivity test was compared by using one way ANOVA and followed by Tukey’s test.

RESULTS

The crude extract percentage yield

The highest and minimum yield of the C. hirsuta extract obtained from 80% methanol and a chloroform 15.75 and 9.9%, respectively as shown in Table 1.

The bacterial inhibitions of chloroform and 80% methanol extract of C. hirsuta are presented as shown in Table 2. The highest inhibition zone (12.33±0.50 mm) was recorded on P. aeruginosa at the concentration (200 mg/ml) whereas; the lowest inhibition zone (6.37±0.17 mm) was recorded from methanol extract at the concentration (50 mg/ml) on S. aureus.

The minimum inhibitory concentration (MIC) of leaves of C. hirsuta was determined on five tested bacteria at concentration range of 50 to 1.56 mg/ml as shown in Table 3. The minimum bactericidal concentration is presented in Table 4. Most bacteria showed their MBC at 6.25 and 12.5 mg/ml.

Acute toxicity test of 80% methanol C. hirsuta leaf extract on albino mice

Since 80% methanol C. hirsuta leaf extract the highest yield and supposed to dissolve all metabolites dissolved by chloroform, only 80% methanol C. hirsuta leaf extract was used to determine oral acute toxicity.

The methanol extracts of the leaves of C. hirsuta was administered orally with a single dose of 500, 1000 and 2000 mg/kg body weight to mice.

There were no physical signs such as depression, decrease in feeding activities and hair erection after 80% crude extract administration of C. hirsuta. Similarly, there was no mortality in the 14 days follow up after crude extract administration. The weight of the mice increases significantly in each group from day 0 to day 7 and 14 in both extract and water administered mice as shown in
Gher weight he -

As -

aeruginosa highest inhibition zone (12.33±0.50 chloroform extract of the leaves (Traore et al., 2000).

Saponin is known to have antiplasmodial activities


maintain a healthy heart and urinary tract (Rice

radical scavengers, anti

used as antioxidant activity as hyd

2013).

DISCUSSION

Clematis species are known to have different pharmaceutical compounds including glycosides, saponins, alkaloids, xanthes and anthocyanidins (DaCheng et al., 2013). Anthocyanins is type of flavonoids which can be used as antioxidant activity as hydrogen donating free radical scavengers, anti-inflammatory properties and maintain a healthy heart and urinary tract (Rice-Evans et al., 1996, Prior and Cao, 2000). Researches revealed that alkaloids have pharmacological applied as anesthetics and CNS stimulants (Madziga et al., 2010). Saponin is known to have antiplasmoidal activities (Traore et al., 2000). In this research, the crude chloroform extract of the leaves of C. hirsuta showed the highest inhibition zone (12.33±0.50 mm) on P. aeruginosa at the concentration (200 mg/ml) and followed by S. typhi (11.60±0.12 mm), E. coli (10.7±0.14 mm), S. boydii (10.34±0.13 mm) and S. aureus (10.26±0.16 mm), respectively by agar disk diffusion methods. On the other hand, the methanol extract of C. hirsuta leaf at the concentration of 200 mg/ml had 8.00±0.29, 9.3±0.12, 8.5±0.50, 9.00±0.06 and 7.90±0.05 mm inhibition on S. aureus E. coli P. aeruginosa S. boydii and S. typhi, respectively. Thus, the chloroform extracts of the leaves had significantly higher inhibition zone than the 80% methanol at the same concentration (200 mg/ml). On the other hand, researches in Iran revealed the antimicrobial activities of C. orientalis on S. aureus, E. coli and P. aeruginosa with inhibition zone of 11±1.5, 10±1.52 and 9.6±0.57 mm, respectively (Raei et al., 2013). As concentration increases from 50 to 200 mg/ml in both extracts, bacterial inhibition zone increased. From this, it is suggested that the plant extracts had dose dependent antibacterial activities.

C. hirsuta leaves also showed a pronounced antifungal activity against the three dermatophytes tested on T. rubrum, M. canis, and E. floccosum and yeast C. albicans at the concentration of greater to equal to 46.9 mg/ml (Cos et al., 2002). The inhibition zone of the standards drug chloroamphenicol (30 μg) was interpreted in such a
way that its bacterial inhibition resistant (≤12 mm), intermediate (13-17 mm) and sensitive (≥18 mm) as described in Bauer et al. (1966). Based on these criteria, the chloroamphenicol inhibition on S. boydii (23.66±0.33 mm) and S. typhi (26.83±0.6 mm) in this research was more sensitive. In the contrary, the ampicillin inhibition on S. aureus E. coli P. aeruginosa was 18.4±0.29, 12.5±0.29 and 15.33±0.33, respectively. The chloroform extract of C. hirsuta on P. aeruginosa showed the minimum inhibition concentration and minimum bacterial concentration at 3.125 and 6.25, respectively.

C. hirsuta is used to heal respiratory tract problem and cataract in Meinit ethnic group (Giday et al., 2009). This research agreed with traditional healers in that the two plant extracts inhibit the growth of bacteria such as P. aeruginosa infecting and affecting the skin. The 80% methanol leaf of C. hirsuta in this research (2000 mg/kg) on mice did not reveal any sign symptom and mortality. This result is agreeable to Bhosale et al. (2010). The weight of the mice was also considered as a parameter in the determination of acute toxicity at 500, 1000 and 2000 mm/kg. For this, OECD guideline (2001) was used as a reference for testing the extracts. The weight of mice before treatment significantly increased in both extract and water administered mice at all concentrations on days 7 and 14. This data showed the extracts at or lower 2000 mg/kg are not toxic to mice.

Conclusion

C. hirsuta is one of the major herbal plants used in Ethiopia. Both 80% methanol and chloroform extracts of the leaf of C. hirsuta showed antibacterial properties on some pathogenic bacteria (E. coli, P. aeruginosa, S. aureus, S. boydii and S. typhi) at 50, 100 and 200 mg/ml. Furthermore, the 80% methanol extracts of C. hirsuta leaf was not toxic to albino mice at or less than 2000 mg/kg body weight. Thus, this plant is supposed to have antimicrobial properties therefore, further isolation and compound identification is recommended for biological studies.

CONFLICTING INTEREST

The authors declare that they have no conflict of interest.

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REFERENCES


