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Assessment of Antimicrobial Effects, Antioxidant Activity and Phytochemical Analysis of *Clematis simensis* Leaves Extract

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ABSTRACT: *Clematis simensis* is an important medicinal plant in Ethiopia. An experimental study was conducted on the crude extracts of the leaves of *C. simensis* to screen the major phytochemical constituents, evaluate the antibacterial activities and assess the antioxidant activities. The antibacterial activities of the extracts were evaluated using agar disc diffusion method, Minimum Inhibitory Concentration by broth dilution and Minimum Bacterial Concentration by plate counting agar. Antioxidant activity was determined by Phosphomolybdenum reduction assay, reducing power assays and hydroxyl radical scavenging activity. The phytochemical analysis indicated the presence of tannin, flavonoids, polyphenols, and terpenoids in the leaves of *C. simensis.* Among the test bacteria, *Staphylococcus aureus* was found to be the most susceptible bacteria followed by *Pseudomonas aeruginosae* with a mean zone of inhibition of 12 mm and 10 mm, respectively. Moreover, the antibacterial activities of the plant extract for each bacterial strain were statistically significant ($P<0.05$). According to Phosphomolybdate reduction assay, the highest antioxidant activity was ethyl acetate fraction at 0.99 µg AAE/mg with 500 µg/ml concentrations. In all tested bacterial strains, the inhibition zone agreed with its MIC and MBC values. Therefore, further investigation should be conducted on antimicrobial activities and identification of the antioxidant constituents of the plant using other veterinary important species and strains of bacteria.

Keywords: Antimicrobial, Antioxidants, *Clematis simensis*, phytochemical

INTRODUCTION

Ethiopia has a huge livestock population in Africa and owns about 59.5 million heads of cattle, 30.70 million sheep, 30.20 million goats, 2.16 million horses, 8.44 million donkeys, 0.14 million mules, 1.21 million camels and 56.53 million poultry (Central Statistical Agency, 2016/17). However, the production and productivity of livestock products remains poor as a result of a number of animal diseases that have a direct effect on economic developments of the country. Infectious diseases particularly microbes play an important role in the cause of morbidity and mortality among humans and animals, especially in developing countries (Food and Agricultural Organization, 2017).

The impact of infectious diseases both in human and animals are very high in developing countries like Ethiopia because of factors like limited access to modern drugs, which is again aggravated by less availability, high cost and to the emergence of drug resistant bacteria (Theuretzbacher, 2012). Moreover, in Ethiopia, the far existence of the majority of livestock raisers from veterinary clinics as well as the inadequate funding at the national level for the prevention and control of animal diseases adds to the burden, especially among pastoralists' areas of the country (Tekle, 2015). Due to these factors, livestock keepers in Ethiopia particularly in rural and pastoral areas are still frequently visiting traditional healers to get solutions for their unhealthy animals (Kalayou et al., 2012). Therefore, traditional medicine (TM) is important when livestock raisers have no other animal health care options (Endalew, 2007). The use of medicinal plants as sources of medicine in the form of TM has expanded globally and is gaining popularity (Lemma et al., 2002). It has continued to be used for primary health care for both humans and animals in developing countries, and also in countries where conventional medicine is predominant in the national health care system (Hawaze et al., 2012). Ethiopia contains more than 6,500 species of higher plants which have been used by traditional healers for the treatment of human as well as animal diseases in which above 80% of the population has been relying on traditional medicine and greater than 95% of medicinal preparations are of plant origin (Gidey, 2001). Despite its significant contribution to the society, TM has received very little attention in modern research and development

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and less effort has been paid to upgrade the traditional health practices in the country. However, the long history of use of medicinal plants in Ethiopia and its huge biotic riches can be of paramount importance in future research and drug discovery (Tadeg et al., 2005). Ethno-veterinary medicine has been very crucial for the animal health cares of most developing countries and researches are still going on aiming the production of low cost, resistance free and locally available antimicrobial drugs (Theuretzbacher, 2012). However, to the best of our knowledge, there was no published research findings conducted and reported on phytochemical and ethno-veterinary importance of C. *simensis* in the current study area that is having traditional therapeutic claims for the treatment of different infectious disease both in humans and animals in Takusa district, north Western Ethiopia. Therefore, the aim of this study was to investigate phytochemical compositions, to assess the antimicrobial effects of *C. simensis* to evaluate the antioxidant and free radical scavenging activities of solvent fractionation of the plant.

MATERIALS AND METHODS

Collection, identification and preparation of plant materials

Fresh leaves of *C. simensis*, used for this experiment, were collected from natural habitats in Takusa district around Lake Tana (646km northwest from the capital city Ethiopia), Central Gondar zone, Amhara region, Ethiopia, between October and December of 2017 using clean scalpel. The identity of the plant sample was identified by experienced botanist in University of Gondar, College of Computational and Natural Science, Department of Botany. Finally the voucher specimens were deposited for further references in University of Gondar, College Natural and Computational Science herbarium.

Plant extraction

After collection and authentication, the leaves of *C. simensis* were thoroughly washed with tap water to remove dirt and soil. The leaves of the plant were dried under shade and then, powdered into a coarse size by using Grindomix apparatus. Then after, the coarse powder of the plant was subjected to crude extraction, but for solvent fractionation, the crude extract was further powdered, sieved and used for fractionation purpose (Molla, 2015). Alcohol extraction crude extraction was conducted according to Hossain et al. (2013). Briefly, 150g of powdered sample were macerated in 400ml of 80% methanol alcohol for a period of 3 days with occasional shaking using a shaker at room temperature. The extract was filtered by using gauze and with Whatman No 3 filter paper. The residue was re-macerated for the second and third times with fresh solvent. Then the filtrate was dried by rotary vaporizer under reduced pressure at 50 to get the crude alcohol extract, the concentrated filtrate was frozen in a deep freezer and dried using lyophilization to remove its aqueous content. Finally, the dried extract has been packed in a closed vessel and stored in deep freezer until required for the experiment. Fractionation by non-polar solvents was conducted according to Hossain et al. (2013). Briefly, in 10g of the crude extract, 150ml distilled water was added into a sterile flask. Then 30 milliliters of non-polar solvent started from n-hexane were added to it and shaken by hands for some minutes. The mixture was transferred into separation funnel then allowed to stand until it separated and formed a layer. The upper layer was separated and collected in to sterile flask then after the same process will be repeated for DCM and ethyl acetate respectively by adding equal amount. Then fractions were collected and evaporated in water bath at 50^0c in room temperature for 3 to 5 days to obtain fractionate.

Phytochemical screening

Phytochemical analysis was carried out on methanol extracts of the plant materials by using standard procedures to identify the presence or absence of various chemical constituents such as Phenolic compounds, Terpenoids, Flavunoides, Tanins, Quinones and Coumarines. The presence of phenolic compounds was tested using the procedures indicated by Rohit (2015). Briefly, 5ml the extract was dissolved in 5ml of distilled water in sterile test tube. To this, 1ml of 1% Ferric chloride and 1ml of 1% potassium ferricyanide solution was added. Reddish blue color indicated the presence of phenolic compounds. Salkowski test was used to test terpenoids following the procedures indicated by Rohit (2015). Briefly, about 2ml of the plant extract was dissolved in 2ml chloroform in sterile test tube. Then after, 3ml of concentrated sulphuric acid was added to form a layer. Development of a reddish-brown color indicates the presence of terpenoids. Flavunoides were tested following the procedures indicated by Akrayi and Abdurrahman (2013). Briefly, 10ml ethyl acetate solution was added in test tube containing 1 ml of the extract, the test tube heated for three minutes, cooled and filtered then 4ml of the filtrate and 1ml dilute with 10% ammonium hydroxide. The formation of a yellow color was taken as a positive test for the presence of flavonoids. The presences of tannins were tested using the procedures indicated by Akrayi and Abdurrahman (2013).

Briefly, about 0.5ml of methanol extract fraction of the plant was mixed with 10ml in test tube, boils and filtered. A few drops of 0.1% ferric chloride were added to the test tube. The color changed to brownish blue- or blue-black color indicated the presence of tannin. Quinones were tested using the procedures followed by Suman-Kumar (2013). Briefly, about 2ml of the plant extracted was added to the test tube, and 1ml of ethanol was added to it. The mixture was treated with Sulphuric acid. The formation of red color indicates the presence of Quinones in the plant extract. Coumarines were tested using the procedures indicated by Suman-Kumar (2013). Briefly, about 2ml of the plant extracts was taken in a test tube. 3ml of 2 normality sodium hydroxide was added to the test tube results in yellow color formation. When 1ml of 5 normality Hydrochloric acid was added it becomes colorless. This indicates the presence of Coumarines.

Antimicrobial assay

Test organism. The microbial strains used for this experiment were obtained from National Animal Health Diagnostic and Investigation Center (NAHDIC) and Ethiopian Public Health Institution (EPHI). The bacterial strains used under this study include: *Escherichia coli* (*E. coli*)-(clinical isolate), *S. typhi-(*ATCC -6539), *Staphylococcus aureus* (*S. aureus*)-(ATCC- 29213), *K. pneumoniae* - (ATCC- 27853), Citrobacter (ATCC-43864) and *Pseudomonas aeruginosa* (*P. aeruginosa*) - (ATTC). These bacterial strains were selected based on availability and their antibiotic resistance capability.

Inoculum preparation. Nutrient agar and Muller Hinton agar (MHA) that used for culturing of bacteria strains were prepared following manufacturers protocol. After cooling the media, it was poured in to a sterile petridish aseptically and waited for some time until congealing of the agar. Then, standard and clinical isolate bacterial were sub-cultured with inoculated and spread by using inoculating wire loop following aseptic conditions in a safety cabinet and incubated at 37 for 24 hours. The bacterial turbidity of each bacterium was prepared and standardized by growth method, following the guideline of Clinical and Laboratory Standard Institute (CLSI) (CLSI, 2012). The bacterial suspension in broth was done after preparing Muller Hinton broth (MHB) and normal saline in distilled water following manufacturer protocols and 5ml of broth was transferred to pre-labeled test tube, then three to five single colonies of similar morphological type of each bacterium was picked up by wire loop from fresh agar plate of bacterial culture that was suspended in to test tube containing five ml of MHB and incubated at 37 until visibly turbid for 6-8 hours. The turbidity of the inoculum in a broth culture was then equilibrated and adjusted with 0.5 McFarland standards. The inoculum density will standardize to achieve a final concentration of $1x10^8$ CFU/ml by the growth method (Jorgensen and Turnidge, 2007). The turbidity of the inoculum in the tube was adjusted visually by either adding bacterial colonies or by adding sterile normal saline solution to the broth containing bacterial suspension, then again compared with 0.5 McFarland standards which assumed to be containing $1x10^8$ CFU/ml of bacterial concentration. The adjustment and comparison between turbidity of the inoculum and 0.5 McFarland standards by observing visually with naked eye against 0.5 McFarland turbidity equivalence standard card with a white background and contrasting black lines in the presence of adequate light.

Determination of inhibition zone diameter. Agar well diffusion assay for antimicrobial activities of methanol extracts of the plant *C.simensis* was conducted according to Teshome and Teshale (2013). All tests were performed in sterile Petridishes (90mm) containing 20ml of MHA. It was labeled as Ⅰ, Ⅱ, Ⅲ, Z, ○ and □ for 500 mg/ml, 250mg/ml, 125mg/ml, 62.5mg/ml, and Di-Methyl Sulfoxide (DMSO) and chloramphenicol respectively (annex:3). The standardized bacterial broth cultures prepared in section 2.4.2 were streaked evenly on sterile MHA with sterilized cotton swab. After thirty minutes on each plate, five equidistant wells were made with a 6mm diameter by using sterilized Corkborer. The labeled wells were filled with 100µl of 500mg/ml, 250mg/ml, 125mg/ml and 62.5mg/ml of the crude extract. The concentrations were determined based on the data obtained from the previous studies (Molla, 2015). In addition, the commercial antibiotic disc of ampicillin 0.01mg/disc (for *S.typhi*, Citrobacter and *P.aeruginosa*), chloramphenicol 10mg/dic (*E.coli*, *K.pneumoniae* and *S.aureus*) were used as a positive control, and those antibiotics was selected based on availability. Then after, the plates were left undisturbed for about 1 h at room temperature inside the safety cabinet. After incubated at 37°C for 24 h, the clear zone of inhibition was evaluated and measured by using vernier caliper in millimeters. The experiment was performed in three independent tests for each bacterial strain and the mean of zones of inhibition was calculated (CLSI, 2012).

Determination of Minimum inhibitory concentration. The Minimum Inhibitory Concentration (MIC) of crude extract of the plant *C. simensis* was determined by broth micro dilution method which subjected to serial 2, 3, 5-triphenyl tetrazolium chloride

(indicate bacterial growth) based microtitre dilution technique on 96 well plates (Shaaban et al., 2013). All the wells of microtitre plates were filled with sterilized 50µl of MHB (CLSI, 2012). Then the first column (A-H) of all rows of microtiter plate was filled with stock solution 50µl of 250mg/ml starting concentration of the crude extract. Two fold serial dilution of the crude extract (throughout the row) was carried out until the 10^{th} column and 50µl was discarded from 10^{th} column. Subsequently, the serial dilution procedures gave rise to a final concentration of the plant material ranging from 125 mg/ml to 0.224 mg/ml. The 11^{th} and 12^{th} column were used as the growth control for labeled bacterium in which, 50µl of 5% DMSO and chloramphenicol 10mg/disc was to 11^{th} and 12^{th} column as negative and positive control respectively (Rouis et al., 2013). After standardization within 15 min approximately 1.5×10^{6} CFU/mL with MHB, 50µl of bacterial suspension was added to each well equally except 7^{th} and 8^{th} column which is left for sterility and color contrast control respectively (Annex: 3). Finally, after wrapping each plate loosely with parafilm, the plates were incubated at 36°C for 24h. After incubation, 30µl of 2, 3, 5-triphenyl tetrazolium chloride was added to all wells and incubated for 15 minutes. Any color change observed from purple to pink was taken as positive for growth of bacteria by comparing with 8^{th} column. The MIC value was recorded as the lowest concentration of the plant extract for the last well where no change of colors (i.e. no bacterial growth) was observed. All the experiments were performed in triplicates for each bacterium, and the average value was taken for the MIC of test plant material (Eja et al., 2016).

Determination of Minimum Bactericidal Concentration. Minimum Bactericidal Concentration (MBC) was determined by a method described in CLSI, (2012). In this technique, the contents of all wells containing a concentration of test material of the MIC value from each triplicate, in the MIC determination test was used for MBC determination, 3µl from each rows of MIC value was streaked on plate counting agar with micropipette aseptically and incubated at 37°C for 24h. The lowest concentration of the extract which showed no bacterial growth after incubation was observed for each triplicate and noted as the MBC. The average value was taken for the MBC of test material against each tested bacterium.

Thin layer chromatography (TLC). The phytochemical profiles of the solvent fractions were determined using TLC by spotting 10µl of solution at a concentration of 10mg/ml. A solution of the sample containing a mixture of compounds is applied to the layer of adsorbent, 2cm far from the end of one edge; the next spot was putted at 1.5cm apart. The TLC plate is propped vertically in a closed container (developing chamber), with the edge to which the spot was applied down. The solvent, which is in the bottom of the container, travels up the layer of adsorbent by capillary action, passes over the spot and, as it continues up, moves the compounds in the mixture up the plate at different rates resulting in separation of the compounds (Choma and Grzelak, 2011). The TLC plate have been developed in a mobile phase comprising of n-hexane: ethyl acetate: acetic acid (30:15:5), benzene: ethanol: ammonia (18:2:0.2), tri-chloro methane (TCM): methanol: water (75:20:5). Approximately 15 to 20 minutes that allowed for the mobile phase to move up the TLC plate. The plates were removed from the developing tank and sprayed with vanillin/Sulphuric acid solution and heated at 100°C to allow color development, after which they read under UV light (254nm and 366nm) finally recorded their travel distance of different spots (Suliman, 2010).

Assessment of antioxidant activity

Phosphomolybdenum reduction assay, reducing power assay and hydroxyl radical scavenging activity assay (HRS) assay were to assess the antioxidant activities of the plant. The total antioxidant capacity of the methanol extract of leaves of *C.simnesis* was evaluated by the Phosphomolybdenum reduction assay method according to the procedure described by Narayanan et al. (2014). First, the cuvates absorbance was read at 695nm before adding the sample. Three cuvates were prepared and labeled for (10, 20, 40) µg/ml of plant extract. The extract was added to each cuvate, respectively then after, an equal amount of DMSO was added to the respective cuvates and 90µl distilled water was added equally to each cuvates. Finally equal amount of 1ml of reagent solution (0.588ml sulfuric acid, 0.049g sodium phosphate and 0.039g ammonium molybdate) were added to each covet and incubated at 95°C for 90min. The absorbance of the reaction mixture was measured at 695nm using a spectrophotometer in one minute interval for five minutes.

Reducing power was determined by the method prescribed by Narayanan et al. (2014). The sample in 1ml of methanol at various concentrations was mixed with a phosphate buffer (1ml, 0.2molality, pH 6.6) and potassium ferricyanide (1ml, 1%), and the mixture was incubated at 50°C for 20min. Next, 1 ml of TCM (10% w/v) were added to the reaction mixture, which was then centrifuged at 3000 revolution per minute. The upper layer of the solution

(1ml) was mixed with distilled water (1.5ml) and ferric chloride (150µl, 1%), and the absorbance was measured at 700nm. The increased in absorbance of the mixture was indicated increased reducing power. HRS assay activity of the extracts was determined according to the method reported by Sasikumar and Kalaisezhiyen, (2014). The reaction mixture contained 1.0ml of different concentration of extracts (125, 250, 500µg/ml), 1.0ml of iron-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5ml of 0.018% EDTA, 1.0ml of DMSO (0.85 % in 0.1molality phosphate buffer pH 7.4) and 0.5ml of 0.22% ascorbic acid. The tubes were capped tightly and heated in a water bath at 80-90ºC for 15min, the reaction was terminated by adding 1.0ml of ice-cold tri-chloro acetic acid (17.5 %). To the above reaction mixture 3.0ml of Nash reagent (75.0g of ammonium acetate, 3.0ml of glacial acetic acid and 2.0ml of acetyl acetone were mixed and distilled water was added to a total volume of 1L) was added and incubated at room temperature for 15min for color development. The intensity of the yellow color formed was measured at 412nm against a reagent blank. Ascorbic acid was used as standards.

% inhibition = [(Control- Test)/control] ×100 i.e. control is the absorbance Cuvates

Statistical analysis

The experimental data were analyzed by using the SPSS version 20 software. The statistical differences of the mean zone of inhibition, MIC and MBC of crude extract for individual bacterium was carried out by employing one way analysis of variance (ANOVA) followed by Tukey Post Hoc Multiple Comparison test at a significance level of P<0.05. The experimental data were expressed and reported as Mean±Standard error of the Mean (SEM). The MIC and MBC are analyzed using descriptive statistics using SPSS software.

RESULTS

The result of phytochemical screening test is shown in Table 1. Accordingly, tannins, flavonoids, polyphenols and terpenoids were detected on crude extract of leaves of *C. Simensis*, whereas, Quinones (Anthraquinones) and Coumarines were not detected.

The growth of test bacterial strains was inhibited by crude extract of leaves of *C. simensis* in concentration dependent manner (Tables 2 and 3). Among the test bacteria, *S. aureus* was the most susceptible bacterium at 500mg/ml of crude extract followed by *P.aeruginosa* with a mean zone of inhibition of 11mm and 10mm, respectively. Whereas, *K. peumoniae* and *citrobacter* not effective at all. Generally, the antibacterial activities of the plant extract for each bacterial strain were statistically significant at P values <0.05 between different concentrations of the crude extract for which it was show zone of inhibition.

Table 1. Results of the preliminary phytochemical screening of crude extracts of leaves of C. S*imensis* by using chemical test method

Plant	Major phytochemical constituents tested	Plant extract (crude extract)
C.Simensis	Polyphenols	+
	Terpenoids	+
	Flavonoids	+
	Tannins	+
	Quinones	-
	Coumarines	-

[+] indicates Presence of phytochemicals; [−] Indicates absence of phytochemicals in the crude extract

Table 2. Zone of inhibition (in mm) produced by different concentrations of crude extracts of leaves of *C. Simensis* against *E. coli* (clinical) and other (ATCC) tested bacteria

Concentration	Zone of inhibition for tested bacteria					
	E.coli	*K.pneumonaie*	*S.aureus*	Citrobacter	*S.typhi*	*P.aeroginosa*
500mg/ml	8.33±0.333^{a3}	NE	12.00±0.577^{a3c3}	NE	8.00±0.577^{a3}	11.00±0.577^{a3c3}
250mg/ml	NE	NE	8.00±0.577^{a3}	NE	NE	8.67±0.333^{a3d3}
125mg/ml	NE	NE	NE	NE	NE	NE
62.5mg/ml	NE	NE	NE	NE	NE	NE
Chlorampinicol	14.00±0.000	13.00±0.000	20.33±0.333	NT	NT	NT
Ampicillin	NT	NT	NT	11.33±0.333	13.67±0.333	19.67±0.333

Values are expressed as Mean±S.E.M (n=3), analysis was performed with One-Way ANOVA followed by Tukey Post Hoc Multiple Comparison test; [a] compared to positive control, [b] to 500 mg/ml, [c] to 250 mg/ml, [1]P<0.05, [2]P<0.01, [3]P<0.001. The negative control has shown no antibacterial activity *=positive control (Chlorampinicol, 10mg/dic), ATCC= standard bacterial strains whereas Clinic=clinically isolated strains. NT= Not effective (inhibition zone ≤ 7mm), NT= not tested

Table 3. The MIC (in mg/ml) and MBC (in mg/ml) of the crude extract of the leaves of *C. Simensis* against tested bacteria

Test bacterial strain	Crude extract of the plant	
	MIC	MBC
E.coli (clinical)	14.045±0.562	14.045±0.562
S.typhi (ATTC)	13.045±0.349	13.045±0.349
K.Pneumoniae (ATCC)	35.507±0.618	35.507±0.618
Citrobacter (ATCC	27.300±0.417	27.300±0.417
P.aeruginosa (ATCC)	6.159±0.613	6.159±0.613
S.aureus (ATCC)	4.485±0.289	4.485±0.289

Values are expressed as Mean±S.E.M (n=3), analysis was performed with One-Way ANOVA followed by Tukey Post Hoc Multiple Comparison test between each groups of tested bacterial strain for its level of significance $P < 0.05$.

The MIC and MBC of crude extract of are presented in the Table 4. The MIC value was in accordance to its inhibition zone, that is, the highest inhibition zone diameter is the bacterium, the lower is the concentration of the extract required for growth of inhibition in all tested bacteria. The maximum MIC (less diluted of the study plant) obtained was 35.507 mg/ml against *K. Pneumoniae*, and the minimum MIC (highest dilution) was 4.485 mg/ml against *S. aureus.*

The MBC is similar to MIC value, the maximum mean of MBC (least dilution of study plant) was 35.507 mg/ml against *K. pneumoniae* and the minimum mean of MBC (highest dilution) was 4.485 mg/ml against *S. aureus*. Generally, *E.coli* was not statistically significant (P=0.610) against *S. typhi.* However, statistically significant differences (P=0.001) were observed for other tested bacteria. Additionally, *S.auerus* also statistically not significant against *P.aeruginosa* (P=0.149).

As shown in the Table 5, TLC chromatogram of the solvent fractionation of crude extract was conducted to determine RF of phytochemicals present in the plant extract because preliminary phytochemical screening test did not done in solvent fractionation due to insufficient amount. In a mobile phase of n-hexane; ethyl acetate; acetic acid (30:5:5), n-hexane fraction (NHF) exhibited 3 visible zones and 2 visible zones when observed under UV-366 nm and UV-254nm respectively while one visible zones and no visible zones in TCM: methanol: water (75:20:5) under UV-360nm and UV-254nm with RF value range between 0.211 and 0.811cm. In DCM fraction (DCMF) there was 2 visible zones and one visible zone under UV-366nm and UV-254nm respectively with a mobile phase of n-hexane: ethyl acetate; acetic acid (30:5:5) but only one visible band in mobile phase of TCM: methanol: water (75:20:5) under UV-360nm with RF value ranges between 0.423 and 0.541. Whereas in ethyl acetate fraction (ETF) 4 visible zones and 2 visible zones under UV-360nm and UV-254nm respectively in n-hexane: ethyl acetate; acetic acid (30:5:5) and two visible bands. Whereas in TCM: methanol: water (75:20:5) two visible zone bands was observed under UV-366 while no visible bands observed in under UV-254 nm with RF value between 0.299 and 0.676 (Table 4). As shown in Figure1, the number of major bands that were identified for the plant extracts was ranged between one and three i.e. yellow bands in n-hexane, light yellow color bands in ethyl acetate fraction at the same distance and blue-black color bands ethyl acetate fraction.

Increased absorbance of the reaction mixture indicated increased reducing power of the extracts as in the standard curve shown in figure 3. The reducing power of all the extracts increased as the concentration also increased. At 500µgAAE/mg concentration of the plant, standard ascorbic acid absorbance obtained was 1.021. At the same concentration *C. simensis* of ETF was found to have the absorbance value 0.998 while all other extracts had low reducing values. NHF exhibited weak antioxidant properties than other extracts.

As it is shown in Figure 4 the hydroxyl radical scavenging activity of various leaf extracts of *C.simensis* was more or less good. The ETF exhibited highest hydroxyl radical scavenging activity compared to other extracts while lowest recorded in DCMF. The Inhibitory concentration at 50% (IC50) values of ascorbic acid, ETF, DCMF and NHF were 0.81, 0.97, 2.62 and 2.93 µgAAE/mg, respectively. The decreasing order of hydroxyl radical scavenging activity of the extracts was found to be ETF>DCMF>NHF. When added all the extracts of *C.simensis* to the reaction mixture, scavenge hydroxyl radicals activity is in a concentration dependent manner. It was also found that the free radical scavenging activity of ETF of this plant (IC50 = 0.81µgAAE/mg) was stronger than that of NHF (IC50 = 2.93µgAAE/mg). Total antioxidant capacity of *C.simensis* leaves extracts, expressed as equivalents of ascorbic acid (µgAAE/mg), is shown in Figures 2. The antioxidant capacity of *C.simensis* leaves extracts was found to decrease in the order ETF> DCMF>NHF.

All the extracts showed an increase in antioxidant capacity with an increase in dose. The highest antioxidant activity of ETF was found to be 0.99µgAAE/mg at 500µg/ml extract concentration and the lowest was at 125µg/ml, respectively. Among the four extracts DCMF was exhibited as the lowest antioxidant activities at 125µg/mg with antioxidant activities of 0.246µg of *ascorbic acid equivalents (AAE)* /mg.

Table 4. Retention Factor values (in cm) of solvent fractions of the plant *C. Simensis*

Solvent fractionation	Solution A	Solution B		
	UV-366nm	UV-254nm	UV-366nm	UV-254nm
N-hexane	0.243,0.473,0.881	0.338,0.378	0.284	NS
DCM	0.432,0.541	0.243	0.466	NS
Ethyl acetate	0.229,0.297,0.459,0.676	0.459,0.676	0.388,0.666	NS

Solution A. n-hexane: ethyl acetate: acetic acid (30:15:5) Solution B; TCM: methanol: water (75:20:5), NS: not seen

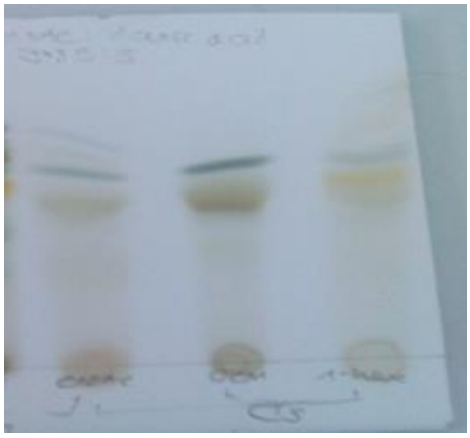

Figure 1. Thin layer chromatography of *C. Simensis*

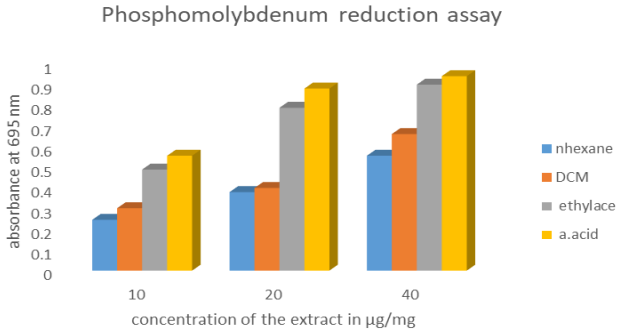

Figure 2. Total antioxidant capacity of different extracts of *C. simensis* according to Phosphomolybdate assay, expressed as µg/ml of ascorbic acid equivalents (AAE) when n= 3

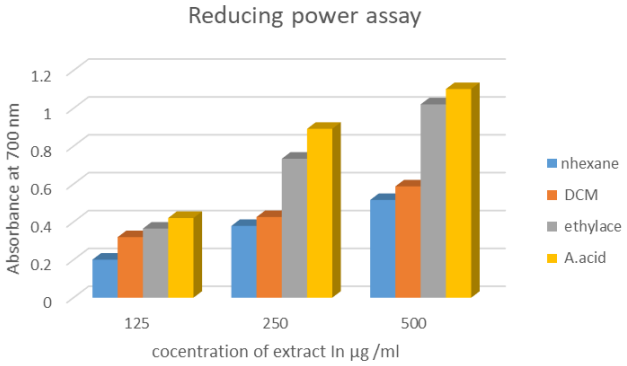

Figure 3. Reducing power assay of different extracts of *C.simensis* expressed as absorbance at 700nm (n=3)

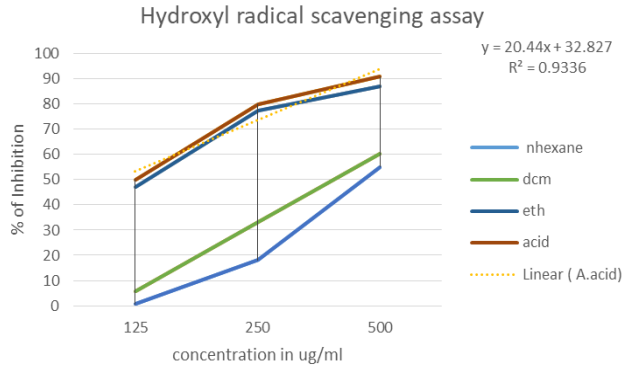

Figure 4. Percentages of free radical scavenging activities of N-hexane, DCM and ethyl acetate extracts of *C. Simensis* as per hydroxyl radical assay when=3

DISCUSSIONS

The present study was done to evaluate the antimicrobial activities of crude extract and antioxidant properties of solvent fractionation. To extract phytochemicals, present in the plant, solvent used for extraction purpose should be easy to remove, inert, nontoxic, and not easily flammable (Mukherjee, 2002). Hydro-alcohol co-solvents have been used in this study. It is hypothesized that alcoholic solvents efficiently penetrate cell membranes, permitting the extraction of high amounts of endocellular components in contrast to lower polarity solvents, which are limited to extracting mostly extra-cellular material. Due to these problems, hydro-alcoholic co-solvents such as 80% methanol seem to possess the optimum solubility characteristics for initial extraction (Messele, 2004). In the present study, 80% methanol was chosen based on the above-mentioned points. Phytochemical screening is a useful tool that gives an idea on some constituents of the plant extract that may be responsible for the pharmacological activities of the plant and serve as a stepping-stone for further isolation studies (Mukherjee, 2002).

In this study, preliminary phytochemical screening was carried out for assessing its antimicrobial and

antioxidant activities. The results of the preliminary screening (Table 1) indicated the presence of some active constituents such as flavonoids, Terpenoids, tannin and phenols. Accordingly, these results disagree with the study conducted in Jimma which reported that 80% of methanol leaf extracts of *C. simensis* was negative for terpinoides (Teshome and Teshale, 2013). The difference existed may be due to geographical difference of the area and chemicals used in screening test. Flavonoids are one of the most widely distributed natural products in plants. Studies done on this group of compounds have reported a variety of biological activities including anti-inflammatory, antibacterial and anti-carcinogenic activities (Jasmine et al., 2007). The presence of these compounds in the study plant contributes their own share for the observed antibacterial activities. The possible mechanism of action is associated with disruption of cell membrane and inhibition of nucleic acid synthesis (Dzoyem et al., 2013). Polyphenols are strong antioxidants and free radical scavengers as well as toxic to microorganisms is due to the site (s) and number of hydroxyl groups present in the phenolic compound (Nostro et al., 2000). Tannins is another class of compounds which are believed to have an astringent property, play a great role in healing of microbial associated inflamed surface of mouth and throat. They result antimicrobial action may be associated with membrane damaging effects and inhibition of metabolic pathways of bacteria (Funatogawa et al., 2004). Terpenoids is another class of compounds present in this plant which shows effective antimicrobial activities against *S.aureus* and *P.aeruginosa* through disruption of cytoplasmic cell membrane (Brehm-Stecher and Johnson, 2003).

Secondary metabolites mention above could be responsible for the observed antimicrobial activity although the exact mode of action is not clearly understood. In this study, the active antimicrobial principles of the crude extract of *C.simensis* were due to the presence of flavonoids, Terpenoids, tannin and phenols. The antimicrobial activity of these plants is in general agreement with the study conducted in Jimma which reported that 80% of methanol leaf extracts of *C. simensis* was active against *S. aureus* and *P. aerugenosa* (Teshome and Teshale, 2013) but differs from the study conducted in Rwanda (Cos et al., 2002). The activity difference observed could be attributed to the slight difference in the chemical constituents of the plants. In a study done on the same genus Clematis the antimicrobial activity of the gradient extracts of *Clematis longicauda staud* and *Clematis burgensis* leaves indigenous to Ethiopia, methanol extract impregnated disk have a mean zone of inhibition is 3.99mm and 7.25mm respectively against *S.aureus* (Hawaze et al., 2012) while in the current study the inhibition zone is 12.00mm. This may indicate that agar diffusion is more effective than disc diffusion method in addition to slight difference in chemical constituents of the plant. Although these activities were not impressive especially as compared to the positive control used. Generally, in this study gram positive bacteria *S. aureus* was more susceptible (highest inhibitory zone was observed) as compared to gram negative bacteria used. This is due to the presence of outer pepitidoglaycane layer which is not an effective permeability barrier (Nostro et al., 2000). In contrast other gram negative bacteria such as *K. Pneumoniae* was less susceptible (lowest inhibition zone was seen) because these bacteria possess an outer phospho-lipidic membrane caring structural lipopolysaccharides components. This makes the cell wall impermeable to the penetration of bioactive phytochemicals (Lambert, 2002). In a study done in Jimma, on the same genus *Clematis longicauda staud* and *Clematis burgensis* leaves the MIC value for both of them is 3.125mg/ml against *S.aureus* (Hawaze et al., 2012), which is almost close to the average value of the current study (4.45mg/ml). In South Africa a study carried out by Suliman, 2010 in the same genus, *Clematis oweniae* (leaves extract) the MIC value against *S.aureus* and *K.pneumoniae* was 2mg/ml by using solvent acetone together with methanol, however, in the current study the MIC value is 4.485mg/ml and 35.507mg/ml against *S.aureus* and *K.pneumoniae* respectively. The observed difference may exist due to types of solvent used, concentration of plant, geographical difference and slight variation in chemical contents of the plant. The antimicrobial activities of the crude extract in terms of inhibition zone against the tested bacteria strain was inversely proportional to their values of MIC and MBC, that is the more susceptible the bacteria (the highest inhibition zone), the less is its cross ponding MIC and MBC values and the less susceptible the bacteria (the lowest inhibition zone), the more is its cross ponding MIC and MBC values. In addition, in this study the MIC value was equals to MBC value against all tested bacteria strain in micro broth dilution test. This indicates the sensitivity of the method in detecting minimum bacterial turbidity than that of visual inspection methods (Ncube et al., 2008). The phytochemical constituents of plants depend on seasonal changes, biotic (genetic) and abiotic (climatic stress, infection and soil fertility) factors (Moure et al., 2001). TLC analyses help as a means of comparing phytochemical composition of different plant extracts developed side by side. Solutions A; n-hexane: ethyl acetate: acetic acid

(30:15:5) was the best mobile phase obtainable for preparing TLC fingerprint of the ethyl acetate fractions, n-hexane fractions and dichloromethane fractions respectively in this work. In this study, identification of compounds can be done using two different mobile phases on the same stationary phase to develop the fingerprint of the extracts and visualization of chromatogram under UV light at 366nm. Yellow bands and blue-black bands were observed. This is in general agreement with, (Wettasinghe et al., 2001) in which it is confirmed that phenolic compounds stain blue black spot with the sulphuric acid spray reagent while flavonoids were stain yellow spot (Rijke et al., 2006) with acetic acid spray reagent (CH3COOH). In the current study, *C.simensis* leaves demonstrated significant in vitro antioxidant potential and free radical scavenging activity. The total antioxidant activity of the leaves extracts of *C.simensis* increased and correlated well with the increasing concentration. The Phosphomolybdenum reduction assay was based on the reduction of green phosphate (VI) to green phosphate (V) in presence of antioxidant compound and subsequent formation of a green phosphate (V) complex at acidic pH and at higher temperature. However, the activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging (Yildirim et al., 2000). According to Tanaka et al. (2002) that have confirmed a direct correlation between antioxidant activities and reducing power of certain plant extracts. This is in general agreement with the current study plant which shows good antioxidant activities with increasing concentration of the plant extracts. Hydroxyl radical is the most reactive oxygen centered species and causes severe damage to adjacent biomolecule. Hydroxyl radical scavenging activity was estimated by generating the hydroxyl radicals. The hydroxyl radicals were formed by the oxidation reaction with the DMSO to yield formaldehyde, which provides a convenient method to detect hydroxyl radicals by treatment with Nash reagent (Kumar et al., 2007). The current study plants have strong radical scavenging activity this may be due to the presence of flavonoid and phenols in the solvent fractionation extract.

CONCLUSION

This study concluded that the antibacterial activity of 80% methanol extracts partly supports the use of this plant for the treatments of various ailments, though it exhibits weak activity as compared to positive control. For all tested bacterial strains, the inhibition zone was in agreement with its MIC and MBC value i.e. the more susceptible the bacterium the lowest plant concentration (highest dilution) required. All the antimicrobial activities observed were due to the presence of phytochemical constituents detected in screening tests like flavonoids, phenolic compounds, Terpenoids and tannin. This study plant has good antioxidant activity so it serves as a potential source of natural antioxidant that could have great importance as therapeutic agents in preventing or slowing the progress of ageing and age associated oxidative stress related degenerative diseases by eliminating ROS. Based on the above conclusions the following points are forwarded;

- ❖ In the present study, the antimicrobial activity test was conducted only by the agar well diffusion method and broth dilution methods against few tested bacteria. However, it is recommended to carry out similar studies using other methods, other strains of the same species and using different bacterial species.
- ❖ Application of TLC bio-autography techniques should be done to identify the active constituent(s) for the demonstrated pharmacological activities.
- ❖ Further research is also required to confirm the traditional claims of *C. simensis*, and for clarification and elucidation of the chemical components within the plant responsible for biological activities.
- ❖ Isolation and characterization of the antioxidant constituents of the plant should also be included in future similar studies.

DECLARATIONS

Authors' contributions

MB conceived the review, coordinated the overall activity, and reviewed the manuscript. HD and AK supervised in all activities

Conflict of Interest

The authors declare that they have no conflict of interest.

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