SCREENING OF SOME BIOACTIVE CONSTITUENTS FROM THE BARK OF *Cinchona succirubra* PAV. (KWI-NEING)

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Abstract

Cinchona succirubra Pav. (Kwi-neing in Myanmar) has been used in traditional medicine due to the presence of alkaloids such as quinine, quinidine, cinchonine and cinchonidine. The alkaloids contained in cinchona bark are powerful drugs and so *C. succirubra* has remarkable biological activities. The present study focuses on screening of some bioactive constituents from the bark of *C. succirubra* Pav. (Kwi-Neing). Preliminary phytochemical investigation was carried out by the reported chemical methods. The extractable matters of the bark of *C. succirubra* were prepared by extracting powdered sample with polar solvents such as ethanol and water using WHO standard method. Some organic constituents, compound 1 (terpenoid), compound 2 (phenolic compound) and compound 3 (alkaloid) were isolated from EtOH crude extract by column chromatographic separation technique. The isolated compounds were identified by treating with specified reagent on TLC and by UV and FT IR spectroscopic methods. The antimicrobial activity of crude extracts and isolated compounds 2 and 3 was determined by agar well diffusion method against six species of microorganisms namely *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albicans* and *E.coli*. Both extracts showed the pronounced antimicrobial activity against *Bacillus subtilis* (inhibition zone diameter = 30 mm). In addition, compound 3 showed the strong antimicrobial activity against *E.coli* (inhibition zone diameter = 33 mm). Antioxidant activity of crude extracts and compounds 2 and 3 was also screened by Dot-Blot and DPPH staining method. It was found that the violet colour of DPPH disappeared from 400 μg up to minimum concentration of 100 μg for EtOH extract and 200 μg for H_2O extract. In addition, compound 2 showed potent antioxidant activity with sample concentration 25 μg (31.25 μg/mL). The mineral contents of bark of *C. succirubra* were quantitatively determined by AAS method. Calcium (Ca) was found to be the principal element.

Keywords: *Cinchona succirubra* Pav., antimicrobial activity, antioxidant activity, AAS

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Introduction

In the developing countries, large numbers of the world's population are unable to afford pharmaceutical drugs and they continue to use their own systems of indigenous medicine that are mainly plant based. There is an increasing awareness of the potential of natural products, which may lead to the development of much-needed new drugs. *Cinchona succirubra* Pav. is known to be native to south America and was long used and its alkaloids, quinine, quinidine, cinchonine and cinchonidine, as herbal medicine (Pelletier and Caventou, 2015). Quinine has remarkable biological activities such as anti-inflammatory, anti-aging, anti-tumor, anti-bacterial and astringent activity (Skogman, 2012). At the start of world war II, cinchona production became a military objective. The development of synthetic drugs replaced the widespread use of quinine to treat malaria, and as a result its plantations decreased (Achan, 2011). In Myanmar, cinchona plant is cultivated in Thantaung Gyi and Laketho township, Kayin state. The photographs of *C. succirubra* Pav. is described in Figure 1.The present research has focused on screening of some bioactive constituents from the bark of *C. succirubra* Pav. (Kwi-neing).

Botanical Aspects of *Cinchona succirubra* Pav. (Kwi-neing)

Family	:	Rubiaceae
Botanical name	:	*Cinchona succirubra* Pav.
English name	:	Quinine
Myanmar name	:	Kwi-neing
Genus	:	*Cinchona*
Species	:	*succirubra*
Past used	:	Bark

Leaves Flowers Barks

Figure 1: Photographs of *Cinchona succirubra* Pav.

Materials and Methods

In this research work, the bark of *Cinchona succirubra* Pav. (Kwi-neing) was collected from Thantaung Gyi Township, Kayin State during August, 2015 and then identified at Botany Department, Taungoo University. The sample was made to fine powder and stored in air tight container. All chemicals used in this work were reagent grade (BDH) and the instruments consisted of a UV - visible Spectrophotometer (Shimadzu UV-240), Shimadzu FT IR- 8400 Spectrophotometer and AAS (A.Analyst-300 Spectrophotometer: Perkin Elmer).

Preliminary Phytochemical Investigation on Sample

In order to find out the types of phytochemical constituents present in sample, preliminary phytochemical investigation was carried out by using the reported chemical methods (Harborne, 1984) (Robinson, 1983) (Vogel, 1966).

Determination of Extractable Matter Contents of Sample

Dried powdered sample (100 g) was percolated with ethanol (400 mL) for four days and then filtered. The same procedure was repeated three times. The combined filtrate was concentrated under vacuum rotatory evaporator. The dried filtrate was transferred to a weighed porcelain basin and evaporated to dryness on water-bath to obtain ethanol extract.

For water soluble extractable matter, dried powdered sample (100 g) was boiled with distilled water (300 mL) for 2 h. The extract was then filtered and transferred to a weighed porcelain basin and evaporated to dryness on water-bath. The dried filtrate was placed in oven, maintained till constant weight, at 100 °C. The two extracts were stored in a desiccator containing dry silica gel prior using in each experiment.

Isolation and Identification of some Phytoconstituents from the Ethanol Crude Extract

The ethanol crude extract was subjected to chromatography over a silica gel column. The column was initially eluted with pet-ether:ethyl acetate (9:1 v/v)solvent system and the fractions were collected at the rate of one drop per second. The polarity of eluting solvent was gradiently increased by addition of increasing amount of ethyl acetate in petroleum ether, ethyl acetate, and ethanol in ethyl acetate. A quantity of 20 mL was collected for

each fraction and chromatographic separation was monitored by TLC. Spots on TLC were examined under UV lamp (254 and 365 nm). Fractions that showed similar TLC pattern were combined to provide six main fractions. Fraction F_2 provided compound 1 as colourless crystals, fraction F_3 provided compound 2 as pale yellow powder and fraction F_5 provided compound 3 as colourless crystals.

The isolated compounds were characterized by determining R_f values, by treating with 1 % $FeCl_3$ solution, 5 % H_2SO_4, Vanillin-HCl, I_2 vapour and Dragendorff, and by their spectral data, Shimadzu UV-240 UV–visible Spectrophotometer and Shimadzu FT IR-8400 Fourier Transform Infrared Spectrophotometer.

Investigation of Biological Activities of *Cinchona succirubra* Pav. Bark

Investigation of Antimicrobial Activity by Agar Well Diffusion Method

Agar well diffusion method was employed for determining antimicrobial activity of the ethanol and water extracts, and compounds 2 and 3. Two small holes of 10 mm diameter each were cut out in the inoculated agar to place samples to be tested. The volume of each sample placed in each hole was 0.1 mL. The petri dish was then incubated at 27 ºC for 24 h, and the diameter of clear inhibition zone appeared around the hole were measured.

Screening of Antioxidant Activity by Dot-Blot and DPPH Staining Method

The antioxidant activity of ethanol and water extracts, and compounds 2 and 3 was screened by Dot-Blot and DPPH staining method. 5 mg each of the sample was dissolved in 10 mL of corresponding solvent. Test amounts were 400 μg to 1.5625 μg. The samples were carefully loaded on 1×1 cm TLC plate (Silica gel 60 GF_{254}, Merck) by using microliter syringes and dried for 3 min. Loaded samples gave 1 cm diameter (area 78mm^2). Drop of each sample was loaded in order of decreasing concentration along the row. The staining of the silica plate was based on the procedure of Soler-Rives *et al*., 2000. The sheet bearing the dry spot was placed upside down for 10 s in 60 μM DPPH solution. Then, the excess solution was removed with a dryer blowing cold air. The stained silica gel layer revealed a purple background with white spots at the location of the drops, which showed radical scavenger

capacity. The intensity of the white colour depends upon the amount and nature of radical scavenger present in sample.

Determination of Elements in *Cinchona succirubra* Pav. Bark by AAS

The elemental contents in cinchona bark was determined by Atomic Absorption Spectroscopy (AAS) at the Universities' Research Centre (URC).

Results and Discussion

Preliminary Phytochemical Investigation of *Cinchona succirubra* Pav. Bark

The phytochemical tests showed that alkaloids, α-amino acids, flavonoids, glycosides, organic acids, phenolic compounds, saponins, tannins, steroids and terpenoids were present in sample. However, carbohydrates, cyanogenic glycosides, reducing sugars and starch were not detected.

Extractable Matter Contents of *Cinchona succirubra* Pav. Bark

After performing the preliminary phytochemical tests, it is required to investigate some organic constituents present in sample. Ethanol and water crude extracts were prepared and the percentage of crude extract yield was derived from the weight of dried and ground plant material. According to the results, it was observed that the yield of ethanol extract (15.2 %) was higher than that of water extract (12.3 %).

Identification of the Isolated Compounds

Compound 1

Compound 1 (0.002 %) was isolated as colourless crystals from fraction F_2 of the ethanol extract on silica gel by column chromatography using PE:EtOAc (4:1) solvent system. Its R_f value was 0.56 in this solvent system. It provided pink colour on TLC with 5 % H_2SO_4 and also brown colour with vanillin-HCl. Compound 1 may be terpenoid compound since it gave pink colour with acetic anhydride and H_2SO_4 (conc.).

In the FT IR spectrum of compound 1 (Figure 2), the absorption band appeared at 3419 cm^{-1} was attributed to O-H stretching vibration. A strong absorption bands appeared at 2931 cm^{-1} and 2850 cm^{-1} were attributed to asymmetric and symmetric C-H stretching vibration respectively. The C=O stretching vibration was observed at 1734 cm^{-1}. A band in medium intensity at

1641 cm^{-1} was due to the C=C stretching vibration. The absorption band at 1465 cm^{-1} was attributed to C-H bending vibration. The O-H bending vibration appeared at 1377 cm^{-1} and stretching vibration of C-O group was observed at 1092 cm^{-1} and 1022 cm^{-1} (Silverstein and Webster, 1998).

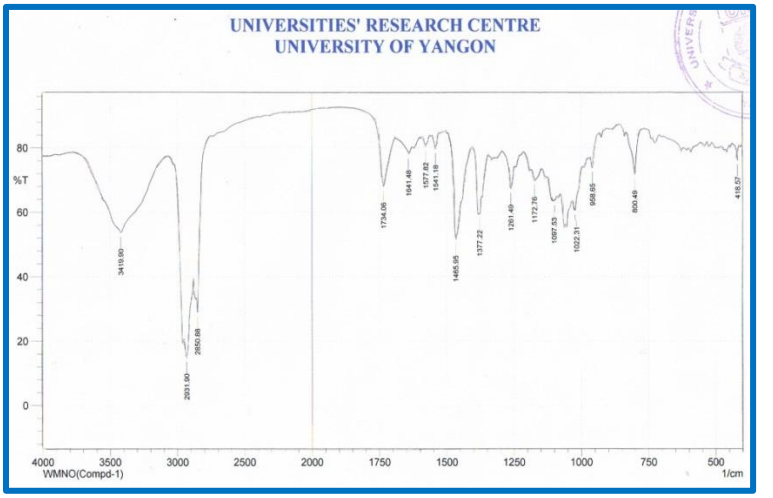


Figure 2: FT IR spectrum of compound 1 (KBr)

Compound 2

Compound 2 (0.0036 %) was isolated as a pale yellow powder from fraction F_3 of the ethanol extract on silica gel by column chromatography using PE:EtOAc (1:4) solvent system. Its R_f value was 0.45 in this solvent system. Compound 2 provided pink colour on TLC with 5 % H_2SO_4 and also with vanillin-HCl. It provided deep blue colour on TLC when treated with 1 % $FeCl_3$ solution suggested that compound 2 may be phenolic compound.

The ultraviolet spectra of the isolated compound 2 in methanol solvent and in methanolic NaOH solution are illustrated in Figure 3. The wavelengths of maximum absorption were found to be 215 nm (K-band) and 292 nm (R-band). Since the bathochromic shift was observed by adding NaOH, the compound may contain phenolic –OH groups. The shift of R band (292 nm) to longer wavelength (317 nm) by addition of NaOH indicating the presence of phenolic OH group in isolated compound 2.

In FT IR spectrum of compound 2 (Figure 4), the broad absorption band appeared at 3350 cm^{-1} was attributed to O-H stretching of phenolic –OH group. The absorption band at 3143 cm^{-1} was due to the aromatic C-H

stretching and the absorption band at 2948 cm^{-1} was assigned as aliphatic C-H stretching. The stretching vibration of aromatic C=C ring was observed at 1691 cm^{-1}, 1610 cm^{-1}, 1519 cm^{-1} and 1465 cm^{-1}. The O-H bending vibration was observed at 1334 cm^{-1} and C-O stretching vibration was observed at 1234 cm^{-1}, 1139 cm^{-1} and 1031 cm^{-1}. The absorption bands at 825 cm^{-1} and 763cm^{-1} were assigned as aromatic C-H out of plane bending (Silverstein and Webster, 1998).

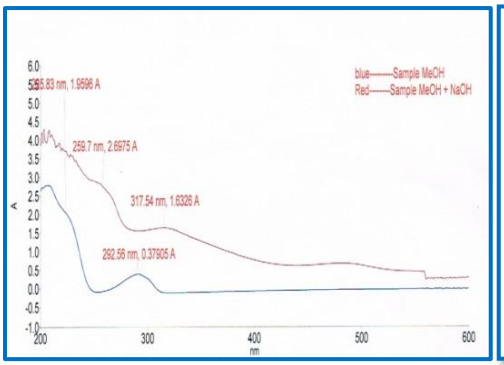


Figure 3:UV spectra of compound 2

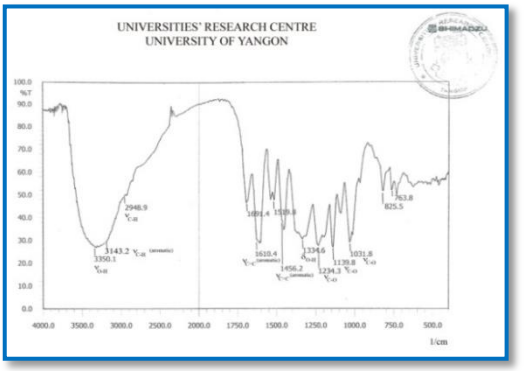


Figure 4: FT IR spectrum of compound 2 (KBr)

Compound 3

Compound 3 (0.061 %) was isolated as colourless crystals form fraction F_5 of the ethanol extract on silica gel column by eluting with EtOAc:EtOH:HOAc (9:1:0.5) solvent system. Its R_f value was 0.31 in this solvent system. Compound 3 may be an alkaloid compound since it gave yellow colour on TLC when treated with iodine vapour and also orange colour with Dragendorff"s reagent.

The ultraviolet spectrum in ethanol of compound 3 (Figure 5) showed wavelengths of maximum absorption (λ_{max}) at 241, 270, and 392 nm due to $\pi \rightarrow \pi^*$ transition.

In FT IR spectrum of compound 3 (Figure 6), a band appeared at 3375 cm^{-1} was interpreted as O-H stretching vibration. The presence of asymmetric and symmetric C-H stretching vibration were also confirmed by the bands at 2932 cm^{-1} and 2867 cm^{-1} respectively. Absorption bands at 1620cm^{-1} was due to the presence of C=C stretching of olefinic group. Aromatic C=C ring stretching appeared at 1486 cm^{-1}, 1450 $cm^{-1}$and 1431cm^{-1}.

Absorption bands at 1239 cm^{-1} and 1227 cm^{-1} were due to the presence of C-O stretching vibration. C-N stretching vibration was observed at 1077 and 1028 cm^{-1}. C-H out of plane bending vibration was appeared at 821 cm^{-1} (Silverstein and Webster, 1998). Photographs of the isolated compounds 1, 2 and 3 are illustrated in Figure 7.

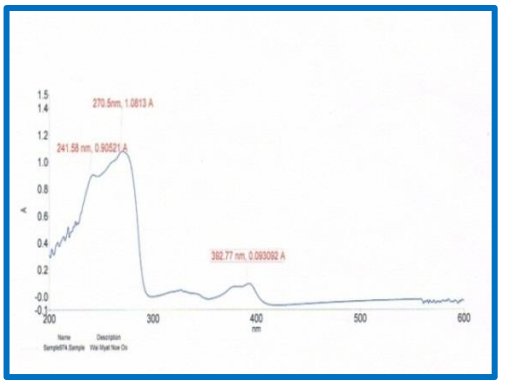


Figure 5: UV spectrum of compound 3 (EtOH)

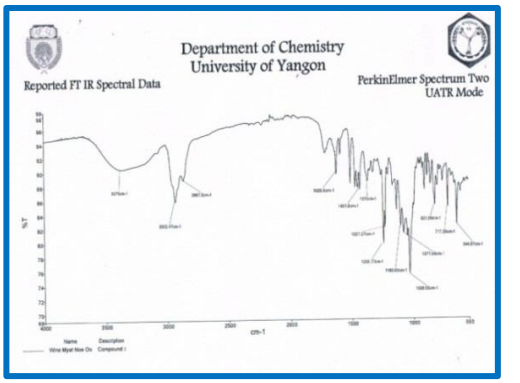


Figure 6: FT IR spectrum of compound 3 (KBr)

Compound 1 Compound 2 Compound 3

Figure 7: Photographs of the isolated compounds 1, 2 and 3

Biological Activities of *Cinchona succirubra* Pav. Bark

Antimicrobial Activity by Agar Well Diffusion Method

The antimicrobial activity of ethanol and water extracts, and compounds 2 and 3 was screened by agar well diffusion method on *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus*, *Candida albicans* and *Escherichia coli* (Figures 8 and 9). Both extracts exhibited antimicrobial activity against all test species with inhibition zone diameters ranged between 20-30 mm. Compound 2 showed antimicrobial

activity with inhibition zone diameter range between 13-20 mm and compound 3 exhibited inhibition zone diameter between 30-33 mm. The results indicated that compound 3 of *C. succirubra* bark showed strong antimicrobial activity against *E.coli* (inhibition zone diameter = 33 mm). Therefore, cinchona bark may be effectively used for the treatment of diseases infected by the microorganisms such as diarrhoea, dysentery, skin infection and wound infections.

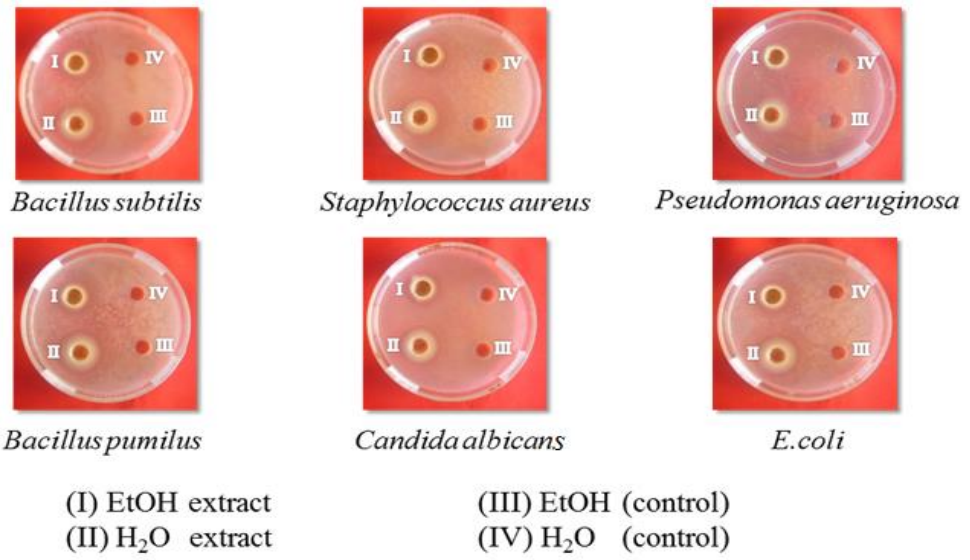
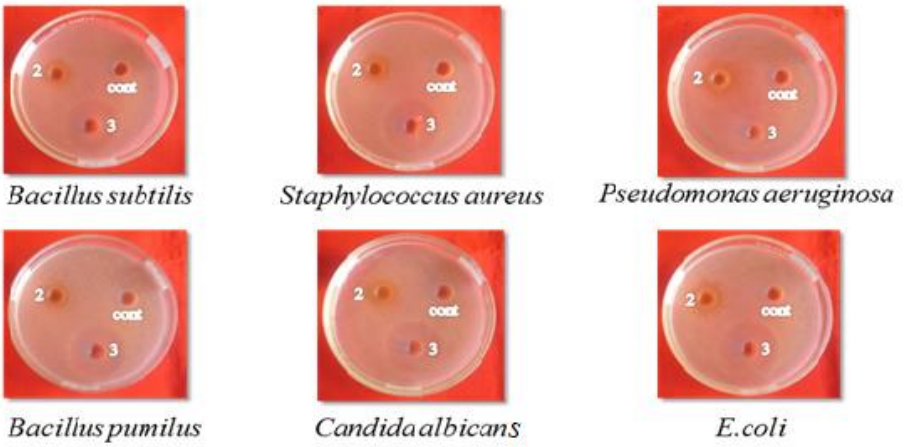


Figure 8: Antimicrobial activity of crude extracts of *Cinchona succirubra* Pav. bark



(2) compound 2
(3) compound 3

Figure 9: Antimicrobial activity of isolated compounds of *Cinchona succirubra* Pav. bark

Antioxidant Activity by Dot-Blot and DPPH Staining Method

The 2, 2-diphenyl-1-picryl hydrazyl (DPPH) radical was widely used in the model system to investigate the scavenging activity of several natural compounds such as phenolic compounds and anthocyanins or crude mixtures such as the alcohol extracts of plants. DPPH radical is scavenged by antioxidants through the donation of a proton forming the reduced DPPH.

To make a semi-quantitative visualization possible, the antioxidant activity of the EtOH and H_2O extracts, compounds 2 and 3 of cinchona bark sample was detected on the TLC plates by DPPH staining method. The appearance of yellow colour in the spots has a potential value for indirect evaluation of test samples in the dot blot (Solar-Rives *et al*., 2000).

When the antioxidant activity was screened by DPPH staining, white spots with strong intensity appeared from 400 μg to minimum concentration of 100 μg for EtOH extract, 200 μg for H_2O extract and 25 μg for compound 2. Antioxidant activity of compound 3 was not detected in concentration range between 200 μg-1.5625 μg. The results indicated that isolated compound 2 contains phenolic – OH group and it is responsible for antioxidant property by

free radical scavenging. Therefore, the bark of *C. succirubra* may be used for the cure of oxidative stress related diseases in Myanmar traditional medicine.

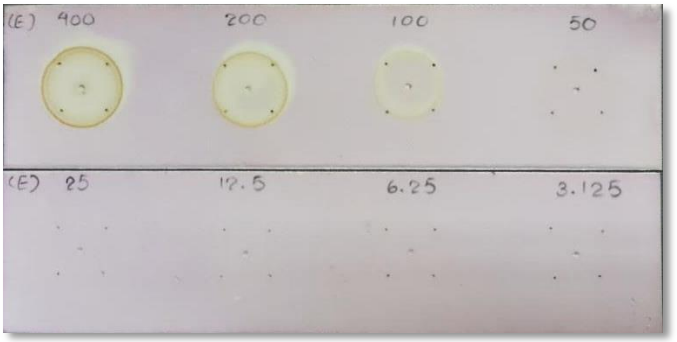


Figure 10: Antioxidant activity of EtOH extract by Dot-Blot and DPPH staining method

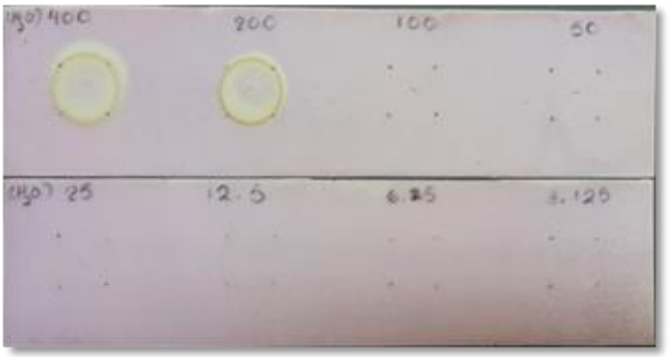


Figure 11: Antioxidant activity of H_2O extract by Dot-Blot and DPPH staining method

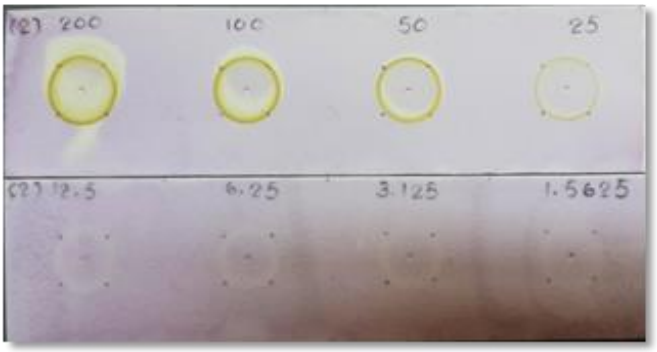


Figure 12: Antioxidant activity of compound 2 by Dot-Blot and DPPH staining method

Quantitative Analysis of Some Elements in *Cinchona succirubra* Pav. Bark by AAS

Quantitative analysis of 8 elements (Fe, Mn, Cu, Cd, Mg, Pb, Zn and Ca) in cinchona bark was carried by AAS and the data are described in Table 1. AAS method has high sensitivity and several elements are easily analyzed and measured in the range between ppm and ppb. Among these elements, calcium content in cinchona bark is significant. In the human body, Ca ranks fifth after oxygen, carbon, hydrogen and nitrogen and make up 1.9 % of the body by weight. Ca is an essential nutrient that plays a vital role in neuromuscular function, many enzyme mediated processes in blood clotting and providing rigidity to the skeleton by virtue of its phosphate salts. Therefore, cinchona bark may be used in Myanmar traditional medicine.

Table 1: Elemental contents in C*inchona succirubra* Pav. Bark by AAS

No.	Elements	Content (ppm)
1	Fe	3.978
2	Mn	1.551
3	Cu	0.233
4	Cd	0.06
5	Mg	8.802
6	Pb	0.492
7	Zn	0.266
8	Ca	115.6

Conclusion

The preliminary phytochemical tests on bark of *C. succirubra* revealed the presence of alkaloids, α-amino acids, flavonoids, glycosides, organic acids, phenolic compounds, saponins, tannins, steroids and terpenoids in it. In extractable matter contents, ethanol extract (15.2 %) was found to be higher than water extract (12.3 %). On silica gel column chromatographic separation, compound 1 (terpenoid, 0.002 %), compound 2 (phenolic compound, 0.0036 %) and compound 3 (alkaloid, 0.061 %) were isolated from ethanol crude extract and then identified by UV and FTIR spectroscopic methods. Antimicrobial activity of ethanol and water extracts, compounds 2 and 3 was screened by agar well diffusion method. Among the tested samples, compound 3 showed the pronounced antimicrobial activity with inhibition

zone diameter range between 30-33 mm. In addition antioxidant activity was also screened by Dot-Blot and DPPH staining method. Among the tested samples, compound 2 exhibited the potent antioxidant activity. In the elemental analysis of plant sample by AAS, Ca is more predominant than other elements such as Fe, Mn, Cu, Cd, Mg, Pb and Zn. Therefore, the bark of *C. succirubra* may be effectively used for the treatment of diseases infected by the microorganisms such as diarrhoea, dysentery, skin infection and wound infection and also as antioxidant in the cure of oxidative stress related diseases.

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