AAB BIOFLUX

Advances in Agriculture & Botanics-
International Journal of the Bioflux Society

Radical Scavenging Activities and TLC-Guided Bioautography of the SC-CO_2 extract of *Ganoderma applanatum*

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Abstract. SC-CO_2 extracts from the fruiting bodies of *Ganoderma applanatum* were characterized by reverse phase High Performance Liquid Chromatography (HPLC) and Fourier Transform Infrared Spectroscopy (FTIR); and its antioxidant compounds were determined through DPPH Radical Scavenging Assay and TLC-guided Bioautography. The extracts were found to be essential oils in 10 MPa and secondary metabolites in 20 & 30 MPa. DPPH Assay revealed that the SC-CO_2 extracts have an antioxidant activity through the determination of its radical scavenging activity along with ascorbic acid which can be summarized in the order: 10 MPa < 30 MPa < 20 MPa < ascorbic acid. Results of TLC-bioautography using 2.54 mM DPPH showed that the compounds have antioxidant property through the change in color of the extracts in the TLC plate, which confirms the DPPH Radical Scavenging Assay. These results suggest that the extracts serve as a source of compounds with radical scavenging activity that can be used as antioxidants aside from its traditional uses.

Key Words: *Ganoderma applanatum*, SC-CO_2 extracts, reverse-phase HPLC, FTIR, DPPH Radical Scavenging Assay, TLC-guided Bioautography.

Introduction. *Ganoderma* active compounds are known for their numerous pharmacological uses. Species of *Ganoderma* such as *Ganoderma lucidum* has been used in traditional medicine in the treatment of cancer, hypertension, chronic bronchitis, and as sedative or tonic in China, Japan, and Korea (Yoshikawa et al 2002; Ming et al 2002). Almost all medicinal properties have been attributed *to G. lucidum*, which make it known as 'mushroom of immortality' in China, Japan and Korea (Joseph et al 2009). In the Philippines, little information is available on *Ganoderma*. This mushroom was considered as "neutriceuticals" which has curative effect on various diseases like hypertension, high blood sugar levels, high blood cholesterols, allergic reaction, insomnia, and cancer as reported by the research conducted by Benguet State University since most of the indigenous people in the area were using *G. lucidum* and *G. applanatum* in herbal medicine and health uses (Fernandez 2006).

Presently, the commonly used medicinal *Ganoderma* include *G. lucidum* and *G. applanatum*. The fruiting bodies of *G. lucidum* contain a variety of chemical substances, major components being terpenoids and polysaccharides. Currently 130 triterpenoids, and more than 100 types of polysaccharides are reported from *G. lucidum* with which have known antioxidant activities (Gao et al 2004). However, studies on *G. applanatum* in determination of secondary metabolites with potential antioxidants are not yet established. Antioxidants are deployed to prevent and scavenged the generation of ROS. Potentially harmful reactive oxygen species (ROS) are produced during the normal aerobic metabolism in biological systems (Chatterjee et al 2011). Deficiency of antioxidative defenses may lead to oxidative stress, which might be associated with a variety of disorders such as coronary heart diseases, neural disorders, diabetes, arthritis and cancers (Spiteller 2001). Recent epidemiological studies conducted indicates that

high intake of antioxidants was positively associated with the reduced risk of coronary heart diseases and ageing related diseases (Flora 2007; Valko et al 2007). However, BHA and BHT which are synthetic antioxidants are proven to be potentially toxic and carcinogenic (Thamavit et al 1985; Williams et al 1984). Accordingly, there is an increase of interest in the search for antioxidants naturally found in plants, fruits, and functional herbs. Because of the complicated compositions of natural products, it is very challenging to screen potential components of it with antioxidant activities (Gu et al 2009).

Nowadays, a number of screening assays were developed and established to search for potential antioxidants. Some of these assays are DPPH radical scavenging capacity (RDSC) assay (Cheng et al 2006), HO radical scavenging capacity (HOSC) assay (Moore et al 2006), and thin layer chromatography (TLC) bioautography assay (Cimpoiu 2006; Jayasinghe et al 2004). From the assays mentioned, TLC Bioautography method was reported to have a quick detection and separation of the active components in a very complicated plant extract and has additional advantages such as convenience, being simple to run, and requiring no specialized equipment (Gu et al 2009). Herein, an activity-detection and determination of natural antioxidants from the supercritical fluid (SC-CO_2) extract of *G. applanatum* were established using TLC Bioautography method and the conventional 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical Assay.

In continuous search for medicinal uses of *G. applanatum*, only the crude extracts and the fruiting bodies of these fungi were used in different studies. However, studies on the use of the supercritical fluid extracts of these *Ganoderma* species were limited. Supercritical fluid extraction (SC-CO_2) on the other hand has recently become an attractive alternative to traditional liquid extraction methods and one of the commonly used extraction techniques in the course of analysis and preparation (Xu et al 2011).

In the present study, the SC-CO_2 extract of *G. applanatum* was used and an activity-guided purification was conducted to isolate the free radical scavenging components from it. In addition, semi-quantification of the extracts was carried out by reverse phase High Performance Liquid Chromatography (HPLC) and Fourier Transform Infrared Spectroscopy (FTIR).

Material and Method

Supercritical carbon dioxide (SC–CO_2) extraction. The pulverized fruit bodies of *G. applanatum* was processed using the AKICO supercritical CO_2 extraction and separation equipment (AKICO Co., Japan) available in the Department of Chemical Engineering Technology, MSU-Iligan Institute of Technology, Iligan City with three (3) pressure levels (10 Mpa, 20 Mpa, and 30 Mpa) wherein carbon dioxide (CO_2) was used as the solvent. The extraction yields of the mushroom samples were determined. The resulting extracts were collected in a 5 ml test tube, sealed with laboratory film, wrapped with foil and stored in the refrigerator until use. The flow rate of CO_2, extraction temperature, and pressure was adjusted and as well as the extraction time was measured. Absolute ethanol (EtOH) acted as the modifier. The supercritical CO_2 flow rate was maintained at 15 g min^{-1} and also, the duration of static extraction will be fixed at 30 minutes. The powdered *G. applanatum* (93.5 g) was placed into the extractor vessel. All obtained extracts were collected with modifier. In order to remove ethanol extracts, it was vacuum evaporated using a rotary evaporator at 40°C. The extract was then placed in the oven at 40°C for 30 minutes. Finally, the obtained extracts under the optimum and controlled supercritical fluid extract conditions were transferred into brown glass bottles.

Reverse phase high performance thin layer chromatography. To determine the composition of the three (3) SC–CO_2 extracts of *G. applanatum*, reverse phase High Performance Thin Layer Chromatography (HPLC) method was used. The extracts were processed and analyzed through the HPLC machine available in the Chemistry Laboratory, Science Building, Ateneo de Cagayan – Xavier University, Cagayan de Oro City. One (1) mL extract in methanol (Scharlab S.L.) was filtered using filter disks to avoid clogging. About two (2) µL of each extracts (1mg mL^{-1}) was injected through HPLC pump injector into the Wakopak® MS-5C18 GT and was analyzed through Perken Elmer

Binary LC Pump 250 & Perkin Elmer UV/VIS Spectrophotometric Detector with the wavelength of 254 nm (USA). HPLC grade methanol (Scharlab S.L.) was used as the solvent for the whole chromatography process. The results were then recorded.

Fourier Transform Infrared Spectroscopy. To further quantify the composition of the three (3) SC–CO_2 extracts of *G. applanatum*, Fourier Transform Infrared Spectroscopy (FTIR) was done. The extracts were analyzed through the Spectrum 100 Optica (Perkin Elmer, USA) FTIR machine available in the Chemistry Laboratory, Science Building, Ateneo de Cagayan – Xavier University, Cagayan de Oro City. About one (1) µL of pure SC–CO_2 extract (1 mg mL^{-1}) of different pressures were analyzed. The data was recorded and the resulting spectra were determined.

Conventional DPPH Assay. The conventional spectrophotometric 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging capacity assay was established as described by Gu et al (2009) with minor modification. Briefly, an aliquot of 500 µL of different pressures SC–CO_2 extract (1 mg mL^{-1} in methanol) was added to 500 µL of 0.205 mM DPPH methanol solution. After gentle mixing and 40 min of standing at room temperature, the DPPH level was spectrophotometrically determined at 517 nm. The free radical scavenging capacity was expressed as percent of DPPH scavenged that was calculated as [(A0 - A1/A0)] 100 (where A0 was the absorbance of the reagent blank, and A1 was the absorbance with antioxidants). Ascorbic acid was used as a positive control and DPPH in methanol was used as negative control. Duplicate reactions were carried out for each concentration of each individual sample.

TLC – Guided Bioautography. An aliquot of about 3 µL different pressures SC–CO_2 extract (1 mg mL^{-1} in methanol) was directly deposited (as spots or bands) onto the TLC plates. TLC plates were developed in a pre-saturated solvent chamber with chloroform-hexane (50:50) as developing reagents until the solvent front reached one (1) cm from the top of plates. The developed TLC plates were then removed from the chamber, and allowed to air-dry for 30 min, followed by spraying with a 2.54mM DPPH methanol solution for derivatization. Each TLC plate was also monitored under UV light at 254 nm.

Results and Discussion. As established in this study, the extraction method used is SC-CO_2. Unlike the existing studies nowadays, supercritical CO_2 (SC-CO_2) which uses carbon dioxide (critical conditions = 30.9^{o}C and 73.8 bar) as its main solvent, and will make analyte recovery very simple and provides solvent free analytes (Xu et al 2011; Herrero et al 2010). Several researchers studied the extraction of natural compounds from plant matrix by using supercritical carbon dioxide (SC-CO_2) in the past few years (Povh et al 2001; Bimakr et al 2009; Sonsuzer et al 2004). SC-CO_2 extraction is a rapidly developing method to produce bioactive compounds (Stahl et al 1987; King & Bott 1993; Simandi et al 2002).

Table 1 shows the variation of the classification of the compounds obtained from the SC-CO_2 extract of *G. applanatum* in three different pressures. In 10 MPa, the extract is classified as essential oil while secondary metabolites are extracted in 20 MPa and 30 MPa, respectively. The differences of the extracted compounds differ as the temperature and pressure increases (Michielin et al 2005). These secondary metabolites could be identified as triterpenoids such as ganoderic acid which has reported to be present in the fruiting body of *G. applanatum* (Boh et al 2000; Wang et al 2008).

The conventional analytical method applied to characterize the SC-CO_2 extracts involves the use of liquid chromatography such as reverse-phased high-performance liquid chromatography (HPLC) to separate the complex mixtures and identify them based on their absorbance at 254 nm in methanol using ultra-violet (UV) detector. HPLC chromatogram in Figure 1 reveals that the SC-CO_2 extracts of the *Ganoderma applanatum* has two to four distinct peaks in three pressures. A number of secondary metabolites have been purified and identified from *Ganoderma* species using column chromatography and HPLC/GC techniques (Rosecke 2000; Smania et al 1999). These peaks represent functional groups that are the primary components of the extract. These

peaks are further characterized by the spectra observed by the Fourier Transform Infrared Spectroscopy (FTIR).

Table 1

Compounds obtained from the SC-CO_2 extract of *G. applanatum* in three different pressures

Pressure level	*General classification*
10 MPa	Essential oils
20 MPa	Secondary metabolites
30 MPa	Secondary metabolites

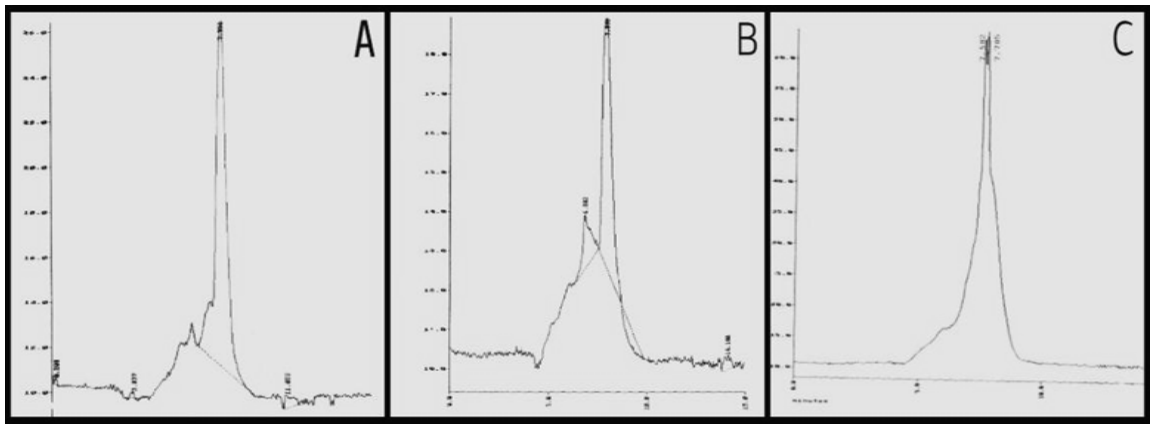

Figure 1. Analytical HPLC chromatogram under UV at 254 nm of the SC-CO_2 extracts of *G. applanatum* in three pressures: (A) 10 MPa; (B) 20 MPa; (C) 30 MPa.

Figure 2 shows the FTIR spectra of the SC-CO_2 extract of *G. applanatum* in three different pressures.

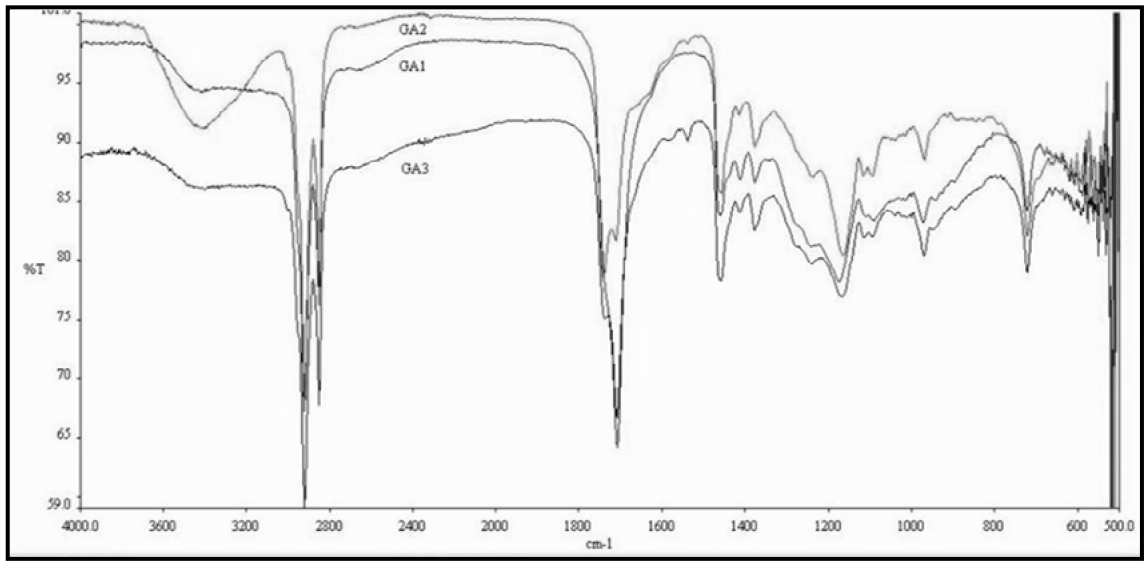

Figure 2. FTIR readings of the SC-CO_2 extracts of *G. applanatum* in three pressures: (GA1) 10 MPa; (GA2) 20 MPa; (GA3) 30 MPa.

The large absorption peak at 3412-3424 cm^{-1} was NH_2 stretch for amines and OH stretch for alcohol while a very strong asymmetric CH_2 stretch was attributed by the absorption peak at 2923-2925 cm^{-1} which was said to be aliphatic. The band at 2853-2854 cm^{-1} was a very strong symmetric aliphatic CH_2 stretch. Absorption peak of saturated aliphatic ester or carboxylic acid anhydrides was found at 1707-1741 cm^{-1} by a C=O stretch. Aliphatic, moderately strong asymmetric CH_3 bending was found at the absorption band of 1457-1460 cm^{-1}. Absorption peaks for aliphatic, strong symmetric CH_3 bending was

observed nearby 1372, 1373, and 1377 cm^{-1}, respectively. Absorption peak for C–O stretch of alcohols or esters was found at 1160-1178 cm^{-1}. The band at 722-724 cm^{-1} was CH_2 rocking vibration for straight chain hydrocarbons of 7 or more carbon atoms. These data is summarized in Table 2.

Table 2

Functional group of the absorption band in the FTIR spectra of the SC-CO_2 extract of *G. applanatum*

Absorption band (cm^{-1})	*Functional group/vibration mode*
3412–3424	NH_2 stretch for amines, OH stretch for alcohol
2923–2925	Aliphatic, very strong asymmetric CH_2 stretch
2853–2854	Aliphatic, very strong symmetric CH_2 stretch
1707–1741	C=O stretch for saturated Aliphatic ester or Carboxylic Acid Anhydrides
1457-1460	Aliphatic, moderately strong asymmetric CH_3 bending
1372, 1373, 1377	Aliphatic, moderately strong symmetric CH_3 bending
1160–1178	C–O stretch of alcohols or esters
722-724	CH_2 rocking vibration for straight chain hydrocarbons of seven (7) or more carbon atoms

The DPPH scavenging activities of all the isolated compounds were estimated using the conventional spectrophotometric DPPH scavenging capacity assay, done in fixed concentration due to limited amount of extracts available. Being a stable free radical, 2,2-diphenyl-1-picrylhydrazyl (DPPH) is usually used to determine radical scavenging activity of natural compounds. In its radical form, DPPH absorbs at 517 nm, its absorption decreases due to the formation of its non-radical form, DPPH–H upon reduction with an antioxidant (Sudha et al 2011). This means that the radical scavenging activity in the presence of a hydrogen donating antioxidant can be monitored as a decrease in absorbance of DPPH solution.

The radical scavenging activity (RSA) using a DPPH generated radical, was tested with a fixed concentration of 1 mg mL^{-1} in different sample extracts due to limited amount, along with ascorbic acid (Table 3).

Table 3

Mean Radical Scavenging Activity of the SC-CO_2 extract of *G. applanatum* in three different pressures and of ascorbic acid with a fixed concentration of 1 mg mL^{-1} and methanolic DPPH

Sample/Extract	*Mean Radical Scavenging Activity (%)*
10 MPa	20.25
20 MPa	62.70
30 MPa	44.05
Ascorbic acid (positive control)	78.81
Methanolic DPPH (negative control)	-

As presented in the Figure 3, the supercritical fluid extracts exhibit high radical scavenging activity. In 20 MPa, the mean radical scavenging activity was 62.70% which was considerably high compared to 10 MPa and 30 MPa which have a mean DPPH activity of 20.25% and 44.05%, respectively. Thus, SC-CO_2 extracts of *G. applanatum* had a potential of DPPH radical scavenging activity similar to those synthetic antioxidants. However, the figure shows that the SC-CO_2 extracts, compared to ascorbic acid, had a low reducing power at the concentration tested (1 mg ml^{-1}). Therefore, such phenomena suggest that the SC-CO_2 extracts of *G. applanatum* may act as free radical scavenger and may react with radicals to convert them to more suitable products and terminate radical chain reactions (Duh & Yen 1997; Jothy 2011). In this manner, the DPPH activity of the extracts can be summarized in the order: 10 MPa < 30 MPa < 20 MPa < ascorbic acid. These results indicate that the SC-CO_2 extracts of *G. applanatum* exhibited the ability to

quench the DPPH radical, which indicated that the extracts were good antioxidant with radical scavenging activity (Sudha et al 2011).

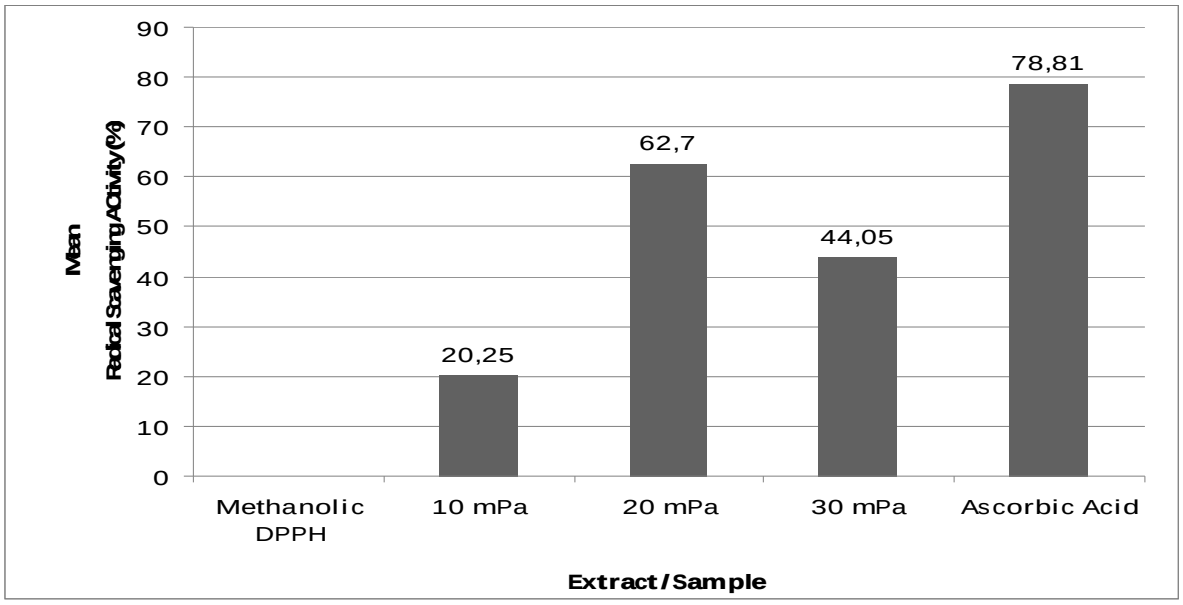

Figure 3. Graphical presentation of the Radical Scavenging Activity of the SC-CO_2 extract of *G. applanatum* in three different pressures and of ascorbic acid with a fixed concentration of 1 mg ml^{-1} and methanolic DPPH.

The TLC bioautography-guided strategy was used to separate the antioxidant compounds from plant extracts (Gu et al 2009). Preliminary identification of active constituents in plants using TLC spray reagents has been performed to detect biologically active compound (Zuhrotun et al 2010). TLC separated the components of SC-CO_2 extracts of *G. applanatum*. The three SC-CO_2 extracts coming from the fruiting body of *G. applanatum* show significant results exhibiting bands in the TLC plates after spraying with 2.54 mM DPPH. Looking at the result of the DPPH reaction of the extracts, it was observed that the three SC-CO_2 extracts had an antioxidant activity, which was confirmed by the appearance of the spots in the TLC plates (Figure 4).

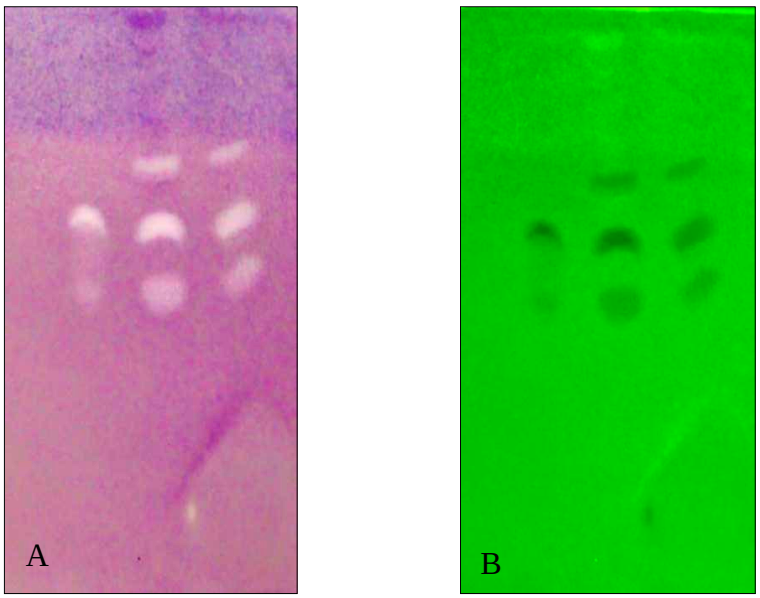

Figure 4. A TLC plate stained with 2.54 mM DPPH solution in methanol visualized under: (A) visible light; (B) UV light in 254 nm.

DPPH is a free radical stable at room temperature, which produces a violet solution in methanol. When the free radical reacts to an antioxidant, its free radical property is lost due to chain breakage and its color changes to light yellow (Badarinath et al 2010). As observed in the Figure 4, there are two (2) visible bands in three different pressures. Thus, two active compounds of the SC-CO_2 extracts of *G. applanatum* were positive with antioxidant property). In the DPPH free radical scavenging capacity assay by TLC, the extracts that produced yellow are white spots in the purple background or were observed as white yellow bands on a purple background exhibit DPPH scavenging activity and thus considered as antioxidants (Gu et al 2009; Badarinath et al 2010).

Conclusions. As established in this study, the extraction method used is SC-CO_2. This study was conducted because studies on *G. applanatum* in determination of secondary metabolites with potential antioxidants are not yet established. Extracts obtained from the fruiting bodies of *G. applanatum* were carried out through SC-CO_2 extraction method and were characterized by Reverse-phase HPLC and FTIR. It was found out that the extract's major components were secondary metabolites, which are aliphatic compounds. SC-CO_2 extracts were found to be essential oils in 10 MPa and secondary metabolites in 20 & 30 MPa. DPPH Assay revealed that the SC-CO_2 extracts have an antioxidant activity through the determination of its radical scavenging activity along with ascorbic acid which can be summarized in the order: 10 MPa < 30 MPa < 20 MPa < ascorbic acid. In addition, the DPPH free radical scavenging capacity assay by TLC with 2.54 mM DPPH, the extracts that produced yellow are white spots in the purple background or were observed as white yellow bands on a purple background exhibit DPPH scavenging activity and thus considered as antioxidants These results suggest that the extracts serve as a source of compounds with radical scavenging activity that can be used as antioxidants aside from its traditional uses.

Acknowledgements. The authors would like to acknowledge the scholarship and research grant given by the Department of Science and Technology – Philippine Council for Industry, Energy, and Emerging Technology Research and Development (DOST-PCIIERD), and Dr. Roberto Malaluan of the School of Engineering Technology, MSU-Iligan Institute of Technology for the support and assistance in the SC-CO_2 extraction.

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Received: 22 January 2013. Accepted: 25 January 2013. Published online: 12 February 2013.
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How to cite this article:
Jose M. A. I., Teves F. G., Madamba M. R. S. B., 2013 Radical Scavenging Activities and TLC-Guided Bioautography of the SC-CO_2 extract of *Ganoderma applanatum*. AAB Bioflux 5(1):20-28.