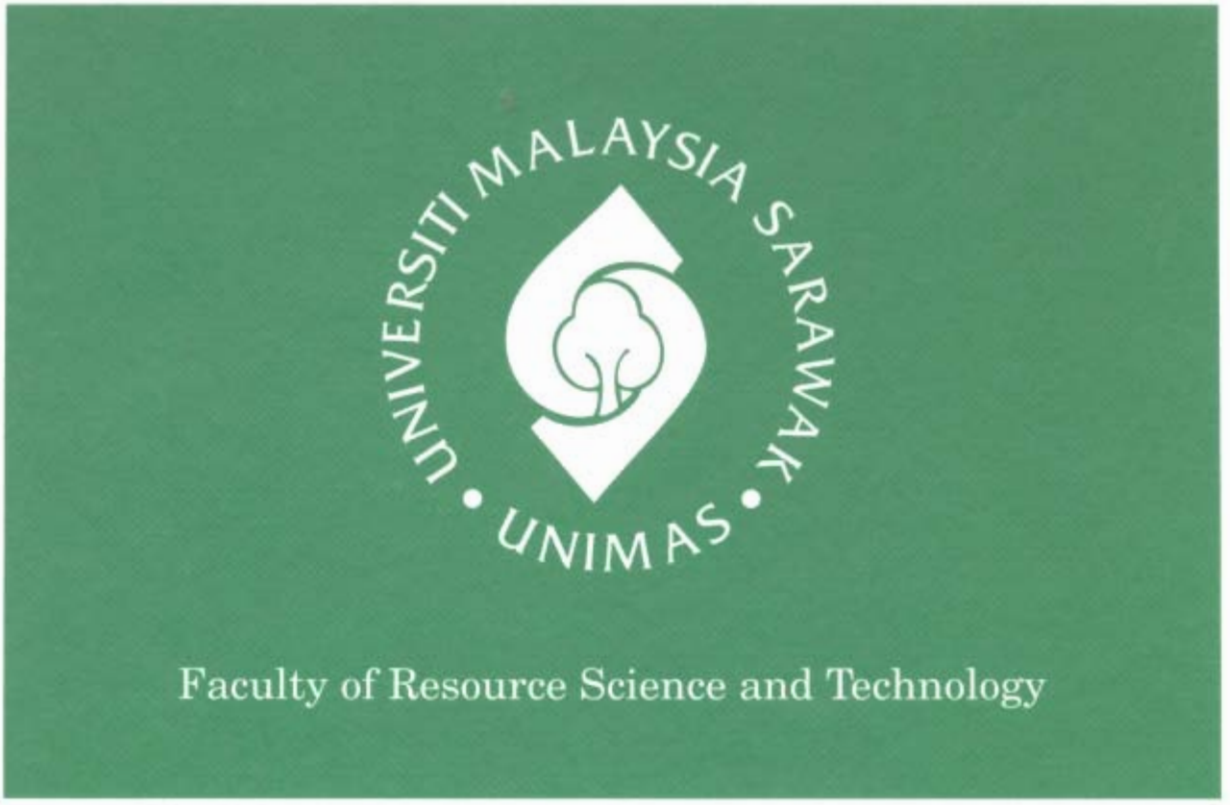


# THE ISOLATION AND CHARACTERIZATION OF TRITERPENOID ACIDS FROM *Ganoderma boninense*

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Bachelor of Science with Honours
(Resource Chemistry)
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This project is submitted in partial fulfillment of the requirement for the degree of

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## DECLARATION

No portion of the work referred to in dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

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## DEDICATION

Firstly, to Allah, for His Humanity and Power to His Creatures to let me finished this final year project. This thesis is dedicated to my beloved mom, Madam Saleha Abdul Rahman, Madam Ramlah binti Selamat, my uncle, Mr Abdul Rashid Abdul Rahman, sisters and family. Thanks for your love, bless and supports for my life.

## ACKNOWLEDGEMENTS

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**Isolation and Characterization of Triterpenoid Acids from *Ganoderma boninense* (SOP2K7 and SOP1K4).**

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**ABSTRACT**-*Ganoderma boninense*, (SOP1K4 and SOP2K7) were isolated and cultivated in 1 L malt extract broth (MEB). 16.6 mg extracellular triterpenoids acids were obtained from *G. boninense* SOP1K4 and about 18.6 mg extracellular triterpenoids acids were obtained from *G. boninense* SOP2K7. Identification of isolated triterpenoid acids was carried out using UV-Vis and FT-IR. Toxicity studies of the isolated triterpenoid acids were conducted against *Artemia salina* indicates that both triterpenoid acids extracts and showed low toxicity against *Artemia salina.*

**Keywords**: *Ganoderma boninense*, triterpenoids acids

***ABSTRAK*** *-Ganoderma boninense (SOP1K4 dan SOP2K7) telah dikultivasikan di dalam 1L MEB. Pengekstrakan asid triterpenoid telah di jalankan di mana sebanyak 16.6 mg ekstrak kasar telah diperolehi daripada G. boninense SOP2K7 dan sebanyak 18.6 mg ekstrak kasar diperolehi daripada G. boninense SOP1K4. Asid tritepenoid dikenal pasti dengan lebih lanjut dengan menggunakan FT-IR dan UV-Vis. Ujian ketoksikan ekstrak asid triterpenoid di jalankan di mana kedua-dua ekstrak menunjukkan ketoksikan yang rendah terhadap Artemia salina.*

***Kata kunci****: Ganoderma boninense, asid triterpenoid*

# CHAPTER 1

# INTRODUCTION

## 1.1 *Ganoderma* spp.

*Ganoderma* is known as wood degrader fungus which usually grows on log or tree stumps. There are approximately 2,500 species of *Ganoderma* in the world (Walting *et al.*, 1999). In ancient China, *Ganoderma* was so rare and highly prized and has been claimed as the primary shen tonic in Chinese herbalism. Herbalist considered *Ganoderma* as an adaptogen, or natural regulator, which suppressing the immune system if it is overactive and boosting it if it is underactive. These claims are based on the presence of high molecular weight polysaccharides and antioxidants in *Ganoderma* extracts.

The tropical climate in Malaysia results to the condusively growth of *Ganoderma* especially in oil palm area. This fungus has caused basal stem rot which is considered as serious disease of oil palm trees in Southeast Asia countries (Elliot and Uchida, 2003). However, this genus is responsible for most of the wood decays which is important economically for its ability to attack and destroy lumber (Alexopoulos and Mims, 1979).

Generally, *Ganoderma* takes the nutrients from the dried trunk or trees that they grow on for their survival. It has been reported that *Ganoderma* attacks a variety of perennial crops including rubber, tea, pineapple and palm trees. For example, *Ganoderma boninense* has been recognized to cause basal stem rot of oil palm trees which contributes to the high crop losses in Malaysia and other regional countries. However, this species appears to be only pathogenic to oil

palm, as it also occurs as saprophyte to a dead palm, such as coconut (Arora, 2004). In addition, *Ganoderma zonatum* plays the same role of causing basal stem rot in the region of United States (Elliot and Uchida, 2003).

The symptoms of *Ganoderma* basal stem rot include a mild to severe wilt, reduced growth and overall off color foliage (paler green color than normal). This wood degrading agent is also capable in causing white rot which involves the degradation of all wood components includes lignin. Degradation of lignin enables them to gain cellulose and hemicelluloses, which serve as their carbon and energy source (Arora, 2004). As the result, the wood turns to beached appearance. This fungus is profoundly involved in the ecosystem process, either directly or indirectly. For example, this fungus serves as food source for many soils organism including bacteria, insects, earthworms and mammals. Indirectly, *Ganoderma* can degrade the dead trunks into simpler components which can be used by other organisms where at the same time, the soil structure can be improved with the presence of organic matters (Carrol and Wicklow, 1994).

In the field of remedial, *Ganoderma* has been used in East Asia for over 4000 years to cure several human diseases such as hypertension, hyperglycemia, bronchitis, nephritis, diabetic mellitus, and others (Habijanic *et al.*, 2001). This genus also has been recognized to promote metabolisms regulation, anti-tumor activity, and immunomodulatory effects (Habijanic *et al.*, 2001, Zhang *et al.*, 2003). Nevertheless, the bioactive compounds isolated from *Ganoderma* have been shown to be effective in psychology therapy as well as in physical healing.

During the past two decades, there are more than 130 triterpenoids have been isolated from the fruiting bodies, spores as well as the mycelia of *Ganoderma*. The activities of triterpenoid acids from *Ganoderma* include as anti-HIV (Ganoderic α, A, B, H, C1) (Hiue and De, 2004, Wang *et al.*, 2006), anti histamine (Wang *et al.*, 2006), antinociceptive (Ganoderic acids A,B,G, and H) (Hiue and De, 2004, Wang *et al.*, 2006), anti cholesterol (Ganoderic acids B, C2) and as inhibitor of angiotensin converting enzyme (Ganoderic acids K, F, S) (Wang *et al.*, 2006). Due to their apparent pharmaceutical activities, triterpenoids have been given serious attention by medical practitioners.

## 1.2 OBJECTIVES

1) To isolate the extracellular and intracellular triterpenoid acids from cultivated *Ganoderma boninense*.
2) To identify and characterize the triterpenoid acids by using UV-Visible and FT-IR spectra.
3) To perform cytotoxicity test against *Artemia salina*.

# CHAPTER 2

# LITERATURE REVIEW

*Ganoderma* is a genus of *Bacidiomycetes* that belongs to *Polyparaceae* of *Aphylpphorales* (Liu *et al.*, 2002) which is easily found in Southeast Asia countries and tropical region (Sudirman and Mujiyati, 2001). There are about 120 species of *Ganoderma* have been described from over than 2,500 species of *Ganoderma* exist in the world (Huie and De, 2004, Walting *et al.*, 1999). Generally, this fungus has an inner brown layer which covered with spines that pierce on outer layer. The fruit bodies of *Ganoderma* is basically thick bracket, soft cork and solitary or in small groups (Ellis and Ellis, 1990). The brackets are dikaryotic, and the basidiospores produced by meiosis (Arora, 2004). The cap color ranges from yellow to black and the bracket size varies from 10-25 cm in diameter (Ellis and Ellis, 1990). *Ganoderma* is usually becoming woody and hardening when old.

*Ganoderma* is considered as one of the most important herbs in Asian traditional healing. It has been reported that there are about 200 elements have been isolated from *Ganoderma.* However, only several elements are being given attention for its medicinal values which includes polysaccharides, triterpenoids, organic germanium, adenosine and ganoderic essence (Wasser and Weiss, 1999). According to Habijanic *et al.* (2001), protein, alkaloid, nucleotides, lactone and fatty acid are the other pharmaceutical bioactive compounds found in *Ganoderma* spp.

Based on literature precedent, *Ganoderma* spp. extract is reported to have the ability to increase the plasma insulin and induce the hepatic key enzymes which are beneficial in diabetic treatment. This is based on a survey made upon the carbohydrate metabolic of a normal mice and an alloxan induced diabetic mice. Alloxan is the diabetic potentiate agent that damages the function of insulin to produce the β cells (Zhang *et al*., 2003). Previous research on *Ganoderma* spp. has shown that this fungus also posses anti viral activity against viral diseases such as Hepatitis B, influenza, cold and canker sores. According to Lu *et al*. (2004), *Ganoderma* spp. is effective in reducing the blood pressure which is due to the stimulation of angiotensin 1- converting enzymes by glucuronic acid.

*Ganoderma lucidum* has been cultivated for its medicinal value. The fruit bodies of this species is commonly called Linzhi in Chinese traditional medicine, and known to the Japanese as Reishi. *Ganoderma lucidum* and its isolates have been used as traditional medicine for several treatments such as chronic bronchitis, bronchial asthma, hepatitis, platelet aggregation, and other (Boh *et al*., 2000). Reishi is responsible in the treatment of cardiovascular weakness, altitude sickness, and as anti viral agent (HIV) (Lu *et al*., 2004). This species has been demonstrated to have anti bacterial activity, against staphylococci, streptococci and bacillus pneumonia. Another species of *Ganoderma* is *Ganoderma oregonense* which holds a crystalline substance that carries a high degree of activity against acid fast and gram negative bacteria (Sudirman and Mujiyati, 2001).

Triterpenes or triterpenoids are known as one of the most benefecial bioactive compounds that have been isolated from the mycelium as well as fruit bodies of *Ganoderma* spp. for recent years. There are over 100 triterpenoids have been isolated from *Ganoderma* spp., which includes ganoderic (highly oxygenated C30 lanostane-type triterpenoids), lucidenic, ganodermic, ganoderenic, ganolucidic, and applanoxidic acids, lucidones, ganoderals and ganoderols (Boh *et al*., 2000). In general, triterpenes have been reported to posses significant bioactivities, such as anti oxidation, hepatoprotection, anti-hypertension, anti allergy and cholesterol reduction. It is also believed that they are able to inhibit the platelet aggregation, due to the inhibition of enzymes such as β-galactosidase, angiotensin converting enzyme, cholesterol synthesis, etc (Huie and Die, 2004).

A number of triterpenes isolated from spores of *Ganoderma* spp. such as ganoderic acid β, luciomol B, ganodermanodiol, ganodermanonriol and ganolucidic acid, show significant activities as anti-HIV-1-protease agent (Huie and Die, 2004). Recent research work has found that the triterpenes fraction which consisted ganoderic acid A, B, C, and D, lucidenic acid B, and ganodermanotriol exhibited highest activities as anti oxidation agent, by testing the ingredient against pyrogallol induced oxidation on erythrocyte membrane and Fe (II)-ascorbic acid induced lipid peroxidative in liver mitochondria (Huie and De, 2004).

Ganoderic acids have received most consideration owing to their well known pharmalogical activities. This is including as anti HIV agent which demonstrated by ganoderic acid α. In addition, ganoderic acids A, B, G, and H

were shown to be active as the antinociceptive components. In the laboratory studies, ganoderic acids were discovered to demonstrate cytotoxicity against mouse sarcoma and mouse lung carcinoma cells (Sliva *et al.*, 2002). Molecular structure of ganoderic acid A is shown in **Figure 1**.

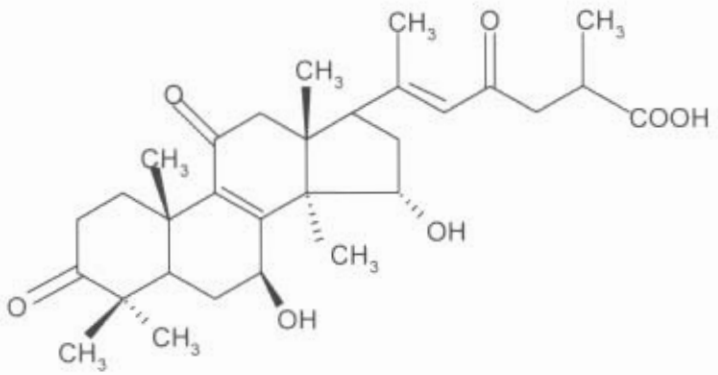


**Figure 1.** Molecular structure of Ganoderic acid A.

Recent investigation by Niu and the co- workers on the bioactive compounds of triterpenoids of *Ganoderma fornicatum* have led to the isolation of two novel triterpenoids, forcinatins A (**Figure 2**) and forcinatins B (**Figure 3**), which represents a novel carbon of 3, 4-seco-25, 26, 27-trinorlanostane (2004). Furthermore, these compounds have been evaluated for their in vitro inhibitory activity against platelet aggregation (Niu *et al.*, 2004). The bioactive triterpenes/triterpenoids isolated from *Ganoderma* spp. are shown in **Table 1**.

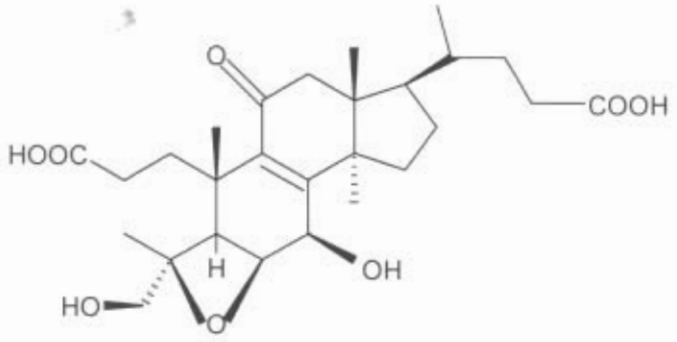


**Figure 2.** Molecular structure of forcinatins A (triterpenoid acid).

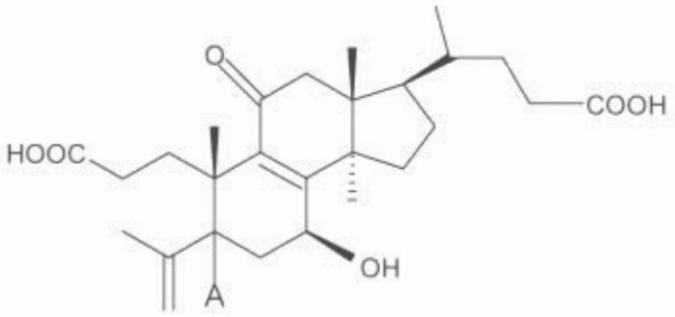


**Figure 3.** Molecular structure of forcinatins B (triterpenoid acid).

**Table 1**: Bioactive triterpenes or triterpenoids isolated from *Ganoderma* spp.

| Triterpenes/Triterpenoids | Usages | References |
|---|---|---|
| Ganoderic acids A, B, H,C1,α<br>Ganodermanondiol, luciomol B,<br>Ganolucidic acids | Anti HIV | Wang *et al.*, 2006<br>Liu *et al.*, 2002<br>Hiue and Di, 2004 |
| Ganoderic acids C2, D | Anti histamine | Wang *et al.*, 2006 |
| Ganoderic acid A, B, C, D<br>Lucidenic acid B,<br>Ganodermanontriol | Anti oxidant | Hiue and Di, 2004 |
| Ganoderic acids A, B, G, H | Antinociceptive | Liu *et al.*, 2002<br>Wang *et al.*, 2006 |
| Ganodermic acids | Inhibitory effects on platelet responses to various aggregating agonist | Liu *et al.*, 2002 |
| Ganoderic acids K, F, S | Inhibitory activity of angiotensin converting enzyme | Wang *et al.*, 2006 |
| Ganodestrone,<br>Ganoderic acids R, S, T<br>Ganoderenic acid A | Liver function stimulant | Wang *et al.*, 2006 |

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Preparation of *Ganoderma* spp. culture

*Ganoderma boninense* was obtained from Plant Pathology Laboratory of University Malaysia Sarawak. The culture was inoculated in petri dish containing malt extract broth (MEB) and incubated at 25° C for 7 days. The experimental media was prepared by dissolving 17 gram broth powder into 1 L of distilled water in a culture bottle. The media was autoclaved to sterilize all microorganisms which may cause contamination to the culture .The mycelia agar was obtained from the Malt Extract Agar (MEA) plate by cutting it in square shape (0.5 cm x 0.5 cm) using surgical blade and was inoculated in broth media at constant temperature 25 ° C for 30 days. Then, the culture was ready to be used at the next stages.

## 3.2 Extraction of extracellular triterpenoid acids

The extraction methodology was adapted from Boh *et al*., 2000.

The broth (250 ml) was added with 250 ml of methanol. The solution was extracted with chloroform (5 x 100 ml). The combined chloroform was concentrated under reduced pressure, 100 mbar at 30° C to 50 ml volume and extracted with saturated aqueous sodium hydrogen carbonate, 5% (3 x 70ml). The combined aqueous layer was acidified with hydrochloric acid 6.0 M to pH 2-3 under ice cooling. After acidified, the solution was extracted with chloroform (3 x 150 ml). The combined chloroform was concentrated and evaporated until dryness.

### 3.3 Extraction of intracellular triterpenoid acids

The mycelium was ground to release the extract using stomacher. That mycelium was extracted with methanol and suspended with purified water. The combined solution was extracted with chloroform (5 x 100ml). The combined chloroform was concentrated under reduced pressure, 100 mbar at 30° C to 50 ml volume and extracted with saturated aqueous sodium hydrogen carbonate, 5% (3 x 70ml). The combined aqueous layer was acidified with hydrochloric acid 6.0 M to pH 2-3 under ice cooling. After acidified, the solution was extracted with chloroform (3 x 150 ml). The combined chloroform was concentrated and evaporated until dryness.

### 3.4 Components separation

Qualitative determination was carried out by using thin layer chromatography (TLC). Silica gel plate (0.25 mm) was used for TLC. Samples were spotted onto TLC plate using capillary tube. The plates were developed using suitable solvent with different polarity. This procedure was used in order to find solvent that gave the best separation of components on TLC plate. The plate was visualized under UV light and the retention factor was determined.

### 3.5 Identification

Triterpenoid acids were identified using UV-Vis and FT-IR.

### 3.6 Bioassay

#### 3.6.1 Brine shrimp cytotoxicity test

Brine shrimp toxicity test was carried out to determine the toxicity of the crude extract against *Artemia salina* larvae (Mclaughin, 1991). About 2 gram of *Artemia salina* larvae was hatched in the saline water under suitable condition. Air was provided continuously and left for 48 hours. 2 mg of samples (SOP1K4 and SOP2K7) were dissolved in 2ml of methanol. From this solution, 500 µL, 50 µL, 5µL were pipetted by using micropipette into vials/test tubes in triplicates. The solvent was removed by using rotovapour. This was followed by the addition of 5 ml of seawater, resulting the final concentration of 100µg/L, 10µg/L, 1µg/L. Exactly 2 ml of samples were transferred into each NUNC multidish. 10 *Artemia salina* were added into each NUNC multidish to perform cytotoxicity test. The observation and the reading were carried out for 24 hours later. After 24 hours contact, amount of survivors was counted and the $LC_{50}$ was determined. Control sample was performed by similar way.

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 Extraction and isolation of triterpenoid acids from *G. boninense* (SOP1K4 and SOP2K7)

Extracellular triterpenoid acids from both samples were successfully obtained. However, attempts to isolate intracellular triterpenoid acids from both mycelia were not successful. We reasoned that both sample (SOP1K4 and SOP2K7) producing very small amount of triterpenoid acids or no triterpenoid acids were formed in the mycelia. The weight of extracellular triterpenoid acids crude extracts was given in the **Table 2**.

**Table 2:** The weight of the tritepenoids acid crude extracts from extracellular SOP1K4 and SOP2K7

| Sample | Weight |
|---|---|
| SOP1K4 | 16.6 mg |
| SOP2K7 | 18.6 mg |

The isolated triterpenoid acids were subjected to Thin Layer Chromatography (TLC) for the quantitative determination of the compounds present in the extract. The best separation for SOP1K4 was given by solvent ethyl acetate: chloroform: methanol in a ratio of 4: 4: 2. The best separation for SOP2K7 was given by solvent system methanol: hexane in a ratio of 2: 3 which showed the presence of two spots on TLC plate. The plates were visualized under UV light and the retention factor for each spot was determined. The $R_f$ values of each spot are given in the **Table 3 (a,b)**.

**Table 3a:** $R_f$ values for SOP1K4 with ratio ethyl acetate: chloroform: methanol (4: 4: 2).

| Spot | Retention factor, $R_f$ |
|---|---|
| 1 | 0.20 |
| 2 | 0.76 |
| 3 | 0.95 |

**Table 3b:** $R_f$ values for SOP2K7 with ratio methanol: hexane (2: 3).

| Spot | Retention factor, $R_f$ |
|---|---|
| 1 | 0.58 |
| 2 | 1.0 |

Due to very small amount of both SOP1K4 and SOP2K7 obtained, column chromatography was not performed.

**4.2 FT-IR spectroscopic characterization**

The FT-IR spectrum of SOP1K4's triterpenoid acids exhibit a band of hydroxyl (OH) group at 3264 $cm^{-1}$. The spectrum also supports the presence of carbonyl group as C=O band appears at 1668 $cm^{-1}$. A strong band of the alkenes group was observed at 1652 $cm^{-1}$. Apart of these identical functional groups, the FT-IR measurement revealed the presence of alkanes with anti symmetric and symmetric C-H absorption bands exist between 2850 $cm^{-1}$ - 2960 $cm^{-1}$. Saturated C-C band shows absorption at 1304 $cm^{-1}$.

Similar results were shown by isolated triterpenoid acids of SOP2K7, which indicates the presence of hydroxyl group (OH) at 3271 $cm^{-1}$. The carbonyl group (C=O) band was also identified at 1667 $cm^{-1}$. A strong absorption at 2924 $cm^{-1}$ and 2854 $cm^{-1}$ corresponds to the absorption by C-H single bond stretching motion which indicates the presence of alkanes group. Saturated C-C bond shows a number of absorption in the 800 $cm^{-1}$ – 1300 $cm^{-1}$ range. Normally, these C-H and C-C peaks are clearly visible in the spectra.

FT-IR spectra of both SOP1K4 and SOP2K7 showed the presence of carbonyl and hydroxyl group which is identical to the triterpenoid acids characteristic. The FT-IR spectra for both extracellular triterpenoid acids are given in **Figure 4 (a,b)**.

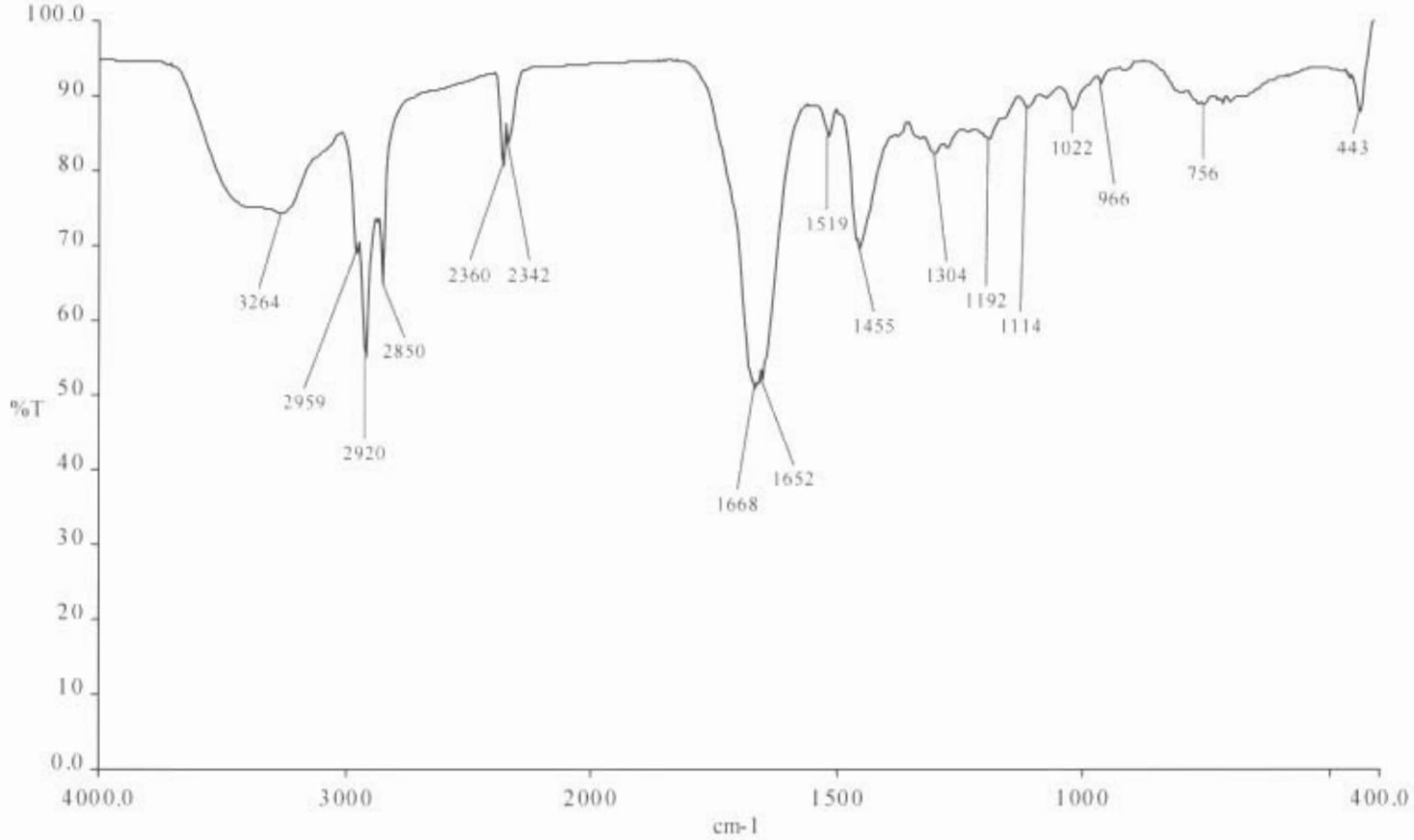


**Figure 4a.** The FT-IR spectrum of extracellular triterpenoid acids from SOP1K4

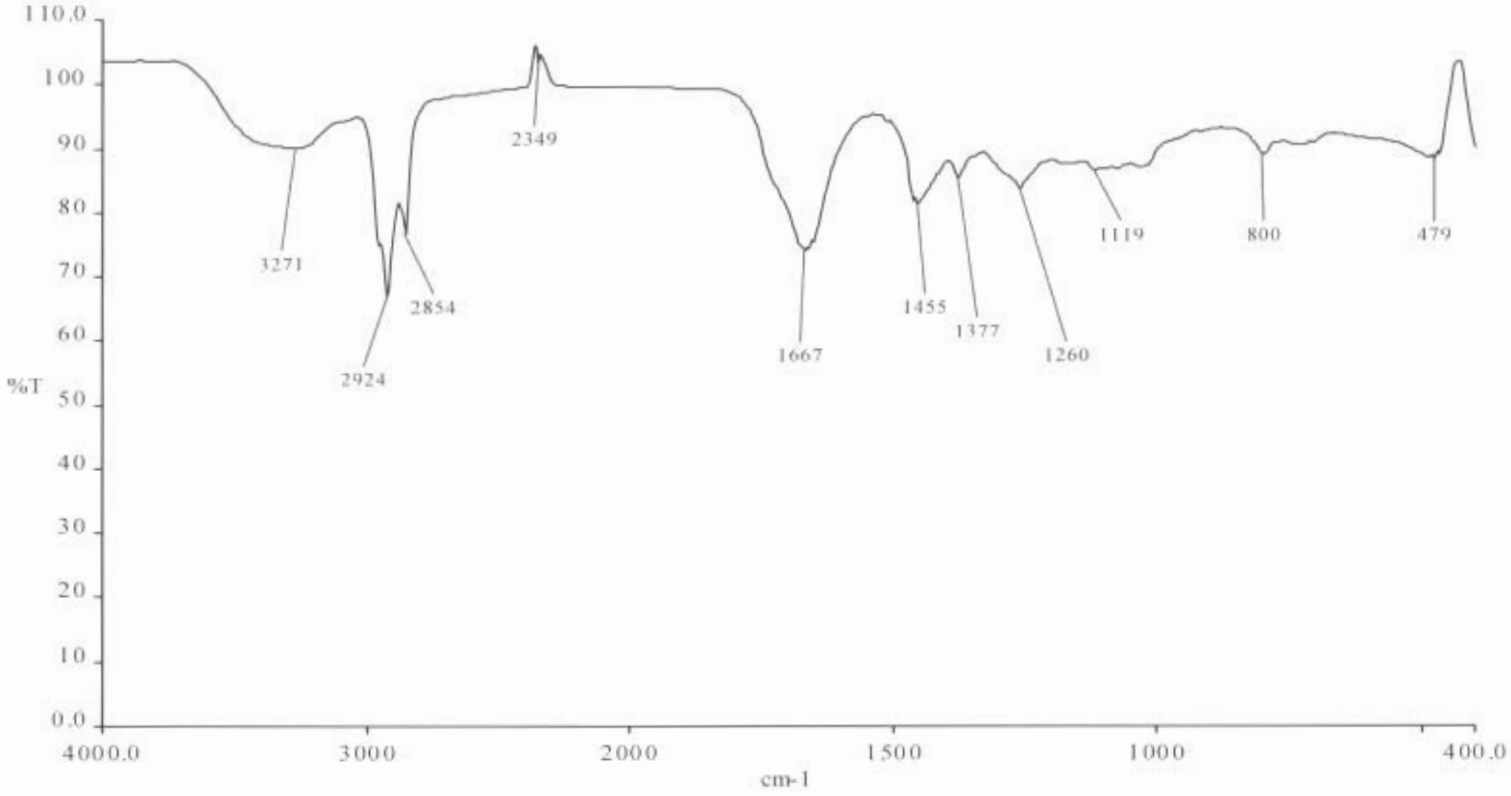


**Figure 4b.** The FT-IR spectrum of extracellular triterpenoid acids from SOP2K7

### 4.3 UV-Visible spectra

UV-Vis spectrum of SOP2K7 extracellular triterpenoid acids shows the absorbance at 243.04 nm which is the characteristic for the unsaturated carbonyl group and is also accordance to the literature data from previous research that found the maximal absorption of ganoderic acid at 240-250 nm (Boh *et al.*, 2000). However, the UV spectrum of SOP1K4 showed absorption at 279.22 nm. The UV absorbance for triterpenoid acids from both samples are given in **Figure 5 (a,b)**.