International Journal of Advances in Science Engineering and Technology, ISSN(p): 2321 –8991, ISSN(e): 2321 –9009
Vol-5, Iss-4, Spl. Issue-2 Dec.-2017, http://iraj.in

ANTICANCER EFFECTS OF DIFFERENT EXTRACTS OF VARIOUS ANTRODIA CINNAMOMEA TYPES ON HUMAN LIVER HEPG2 CELLS AND ANALYSES OF COMPOSITIONS AND BIOACTIVITIES

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Abstract- Antrodia cinnamomea (AC) is a medicinal fungus found locally in Taiwan. Many studies have confirmed that AC is rich in triterpenoids, polysaccharides and other nutrients that may carry functional properties such as detoxification, anti-inflammatory activity, liver function improvement and anti-cancer activity. White AC is thought to be relatively rare and is therefore expensive; it has a greater content of functional compounds than regular forms of AC. Thus, this study was performed in order to develop an ideal culture medium and to compare the contents of bioactive compounds, including triterpenes and polysaccharides, and medicinal functions, such as effects on free-radicals (DPPH) and anti-B liver cancer cells (MTT assay) of the regular form (red AC), the white variant of AC, wild-type red AC in a Petri-dish culture medium, and Basswood AC. The study results showed that the homemade medium enhanced the crude triterpenoid content of mycelia and basidiomatal formation of AC under Petri-dish solid-state fermentation. The polysaccharides contents of regular and white AC were higher in the homemade medium than in the commercial medium. The antioxidant effects (DPPH) of white and red AC in the Petri-dish culture were greater than that of Basswood white AC (culture growth only for 1 month). In addition, it was found that addition of peptone to the culture medium increased the contents of DeEA, DeSA, antcin B and antcin H. Anti-B cancer cell tests showed that approximately 50% inhibition was achieved at 62.5 μg/mL. The wild-type AC fruiting bodies extract concentration was higher than 125 μg/mL, and the anti-B cancer cell effect was over 80%. The results showed that wild-type red AC also exhibited good anti-cancer effects. However, these fungi are not easy to obtain, and 2–3 years are required for the development of fruiting bodies; in addition, other issues must be overcome during culture, such as infection. This study demonstrated the development of a suitable culture medium for solid-state fermentation of AC products that confer protective effects against B liver cancer cells, which has potential applications in the healthcare industry.

Keywords- Antrodia cinnamomea, HPLC; Triterpenoids, Solid-state fermentation, MTT assay.

I. INTRODUCTION

Chronic hepatitis or detoxification leads to severe liver injury. The damaged hepatocytes are initially denatured, and subsequently undergo fibrosis and necrosis, this process eventually leading to hepatoma [1]. Antrodia cinnamomea (AC) is a medicinal fungus locally available in Taiwan. Many studies have confirmed that AC is rich in triterpenoids, polysaccharides and other nutrients that may carry functional properties such as antioxidant activity [2], antiviral effects [3], anti-inflammatory activity[4], liver function improvement effects, and anti-cancer activity[5]. Fruiting bodies of AC are yellow-orange to red-brown in most cases; however, a white variant also occurs in the natural environment. White AC is thought to be relatively rare and is expensive; it has a greater content of functional compounds than the regular form of AC.

Thus, this study was performed in order to develop the ideal culture medium and to compare the bioactive compounds, including triterpenes and polysaccharides, and medicinal functions, such as effects on free-radicals (DPPH) and anti-B liver cancer cells (MTT assay) of the regular form (red AC), the white variant of AC, wild-type red AC in a Petri-dish culture medium, and Basswood AC.

II. DETAILS EXPERIMENTAL

2.1. Fungal strain and chemicals

A. cinnamomea BCRC 35398 was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). White AC was obtained from Dr. Yen's laboratory (Meiho University). Wild-type AC was collected in Chiayi (from the Alishan mountain, which is of approximately 1500 meters in height). The strains were inoculated and maintained in potato dextrose agar (PDA) at 26°C for approximately 15 days, and stored in a refrigerator at 4°C. The abbreviations used in this paper to represent different types ofAC are asfollows: BWAC, Basswood white AC; HMWAC, homemade medium white AC; CMWAC, commercial medium white AC; HMRAC, homemade medium red AC in a15-cm Petri dish; HMNTAC, homemade medium native-type AC; and NTAC, native-type AC. Acetonitrile (ACN, AE0627), methanol and perchloric acid were obtained from Echo Chemical Co.; fetal bovine serum (FBS, 10437) and Dulbecco's modified eagle medium (DMEM, 12800-017) were obtained from Gibco. Dextrose powder, gallic acid, phenol, sodium bicarbonate and dimethyl sulfoxide (DMSO) were purchased from J.T. Backer; 2,2-diphenyl-1-picrylhydrazyl (DPPH) and vanillin were obtained from ACROS (New Jersey, USA); glacial acetic acid

was obtained from Wako-Chem. Co. (Japan); and potato dextrose agar plus and potato dextrose were obtained from Hui-dongfeng Co. For high-performance liquid chromatography (HPLC), a UV-Vis detector L-2420 was obtained from HITACHI, and a HPLC column (Hypersil GOLD C18, 250 mm × 4.6 mm) and a microinjector (Finnpipette F3) were obtained from Thermo Co. Triterpenoid indicators for 8 standard compounds of AC were obtained from Dr. Cheng's laboratory (National Pingtung University, Taiwan).

2.2. Growth of A. cinnamomea

The PDA medium consisted of the following components (w/v): potato 25 g, glucose 30 g, agar 12 g in 1 L distilled water. The culture medium was autoclaved at 15 psi, 121°C for 20 min. A 0.3 × 0.3 cm2 agar blot of AC was placed on a 90 × 20 mm Petri dish containing 23 mL PDA medium. Strain BCRC 35398, red, white and natural-type AC were grown in an incubator at 28°C.

2.3. Sample preparation and extraction

Samples of AC were harvested at 1, 2 and 3 months of age. After growth, AC samples were placed in a drying oven at 50°C for 2–3 days. White and natural-type AC samples were freeze-dried before extraction and analysis.

2.4. Triterpenoids standard compounds of A. cinnamomea

Triterpenoids indicators for 8 standard compounds [antcin A, antcin B, antcin C, antcin H, antcin K, dehydrosulphurenic acid (DeSA), dehydroeburicoic acid (DeEA) and 1,4-dimethoxy-2,3-methylenedioxy-5-methylbenzene (DMMB)] were used for comparative analysis of samples of mycelium or fruiting bodies of AC. These benzenoids were of a purity of >98%.

2.5. Determination of total polysaccharides content

For all samples, 100 mg of AC powder were suspended in 1 mL distilled water. The standard curve of linear regression was obtained from the absorbance of different concentrations of glucose solution. The total polysaccharides content in the culture medium was determined using a phenol–sulfuric acid assay according to Hsieh et al. [6].

2.6. Assay of crude triterpenoids content

The crude triterpenoids content assay was modified from that described by Yanget al. [7].Samples of dried mycelia or fruiting bodies of AC were extracted with 10 mL of 95% ethanol for 72 h. After removal of the precipitate by centrifugation at 3000 ×g for 5 min, the supernatants were dried at 43°C under a vacuum, then mixed with 400 μL 5% (w/v) vanillin–acetic acid solution; 1 mL of perchloric acid was also added, following which the solution was mixed and incubated at 60°C for 15 min. Then, the mixed solution was cooled and diluted to 5 mL with acetic acid. The absorbance was detected at 548 nm in a spectrophotometer. The content of crude triterpenoids was calculated on the basis of a standard curve prepared using ursolic acid.

2.7. DPPH free-radical scavenging activity assay

Thefree-radical scavenging activity assay was performed according to a method modified from Wang et al. [8]. Dried samples of mycelia or fruiting bodies of AC were extracted with 10 mL 95% ethanol for 24 h, and the extraction was assisted by successive sonication for 30 min using a Delta DC400H sonication cleaner. Samples were centrifuged at 3000 ×g for 5 min, and the resulting supernatants were freeze-dried and re-dissolved in 1 mL 95% ethanol. In the first series, each AC sample was mixed with 1 mL freshly-prepared ethanol solution of 0.2 mM DPPH. The reaction mixture was vortexed vigorously for 1 min and kept in the dark at 25°C for 10 min. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm against the blank. Ascorbic acid dissolved in ethanol was used as the positive control. The capability to scavenge DPPH free-radicals was calculated using the following equation:

Inhibition ratio (DPPH scavenging effect) (%) = $100-[(A_{sample}/A_{control}) \times 100]$

where in $A_{control}$ is the initial concentration of stable DPPH radicals without AC and A_{sample} is the absorbance of the remaining concentration of DPPH free-radicals in the presence of samples of AC. All results presented were the average of triplicate analyses.

2.8. HPLC analysis of A. cinnamomea

Samplesof dried mycelia or fruiting bodies of AC were extracted with 10 mL 99.95% methanol for 48 h, and the extraction was assisted by successive sonication for 30 min using a Delta DC400H sonication cleaner. Samples were centrifuged at 13,000 ×g for 2 min, and the supernatants were filtered using a 0.2-μm filter. The method of triterpenoid analysis was modified from Yang et al. [9]and Wu et al. [10]. HPLC was performed on a Hitachi L2420 series with a reverse-phase column (Hypersil GOLD C18, 250 mm × 4.6 mm, Thermo) under 254 nm UV detection. The mobile phase was eluted at a flow rate of 1 mLmin-1 with a linear solvent gradient elution of A (H2O) and B (acetonitrite), with the following gradient program: 0–10 min, 30–30% B; 10–40 min, 30–50% B; 40–50 min, 50–60% B; 50–53 min, 60–90% B; 53–63 min, 90–90% B; 63–90 min, 90–100% B. HPLS was performed at room temperature, with an injection volume of 20 μL.

2.9. Cell line and culture

Human cancer cell line HepG2, a human liver carcinoma, was obtained from Dr. Shi's laboratory

(National Pingtung University, Taiwan). The cancer cell line was maintained in DMEM medium containing 10% FBS, 2 mM glutamine and 1% penicillin. The cells were cultured at 37°C in a 5% CO_2 incubator for one week.

2.10. Cytotoxicity analysis by MTT assay

HepG2 cells were plated in 96-well plates (1×10^4/well) and incubated for one day.The viability of HepG2 cells was examined following treatment with 100 µL of differing concentrations (0, 7.8, 15.6, 31.25, 62.5, 125 and 250 µg/mL) of AC sample extracts for 72 h. The medium was removed and cells were washed once with PBS buffer then incubated in 200 µL MTT solution (0.5 mg/mL) without FBS in DMEM for 2 h at 37°C. Finally, the MTT solution was removed and 100 µL DMSO (100%) were added to each well. The absorbance was measured at 570 nm using a microtitre plate reader [12].

2.11. Statistical analysis

Variations between experiments were estimated from standard deviations, and the statistical significances of changes in physiological parameters were estimated by analysis of variance (ANOVA) using Tukey's multiple comparison test. On all graphs presented, means in each row followed by the same letter are not significantly different ($P<0.05$). All data were obtained from three replications.

III. RESULTS AND DISCUSSION

3.1. Determination of total polysaccharide and triterpenoids contents

The study results showed that the homemade medium enhanced the crude triterpenoids content of mycelia and basidiome formation of AC under Petri-dish solid-state fermentation. The polysaccharides contents of regular and white AC in homemade medium were higher than those in commercial medium (Fig. 1A). The total triterpenoids content of HMRAC (15-cm Petri dish) was the highest of all samples at 67.03 mg/g, followed by HMWAC+P (peptone) with a content of 51.22 mg/g and CMWAC with a content of 49.47 mg/g. BWAC and BWAC (in 4□ for 1 day) had the lowest triterpenoids contents, at 25.15 and 24.13 mg/g, respectively (Fig. 1B).

3.2. HPLC analysis of A. cinnamomea

HPLC analyses were performed on basidiomes and mycelia samples extracted from Petri-dish-grown homemade and commercial medium cultured and Basswood white AC (Fig. 2A–D). Our results showed that the samples cultured in homemade medium contained more antcin K than those in commercial medium; the dehydroeburicoic acid (DeEA) content in samples cultured in homemade medium was approximately185 mAU, which was nearly 3.7 times higher than the content in samples cultured in commercial medium (approximately 50 mAU). The antcin A,Band H,and DeSA and DeEA contents were significantly higher in samples cultured in homemade medium with added peptone, which demonstrated that the addition of peptone could effectively accelerate the formation of basidiomes and enhance the production of bioactive compounds fromAC.

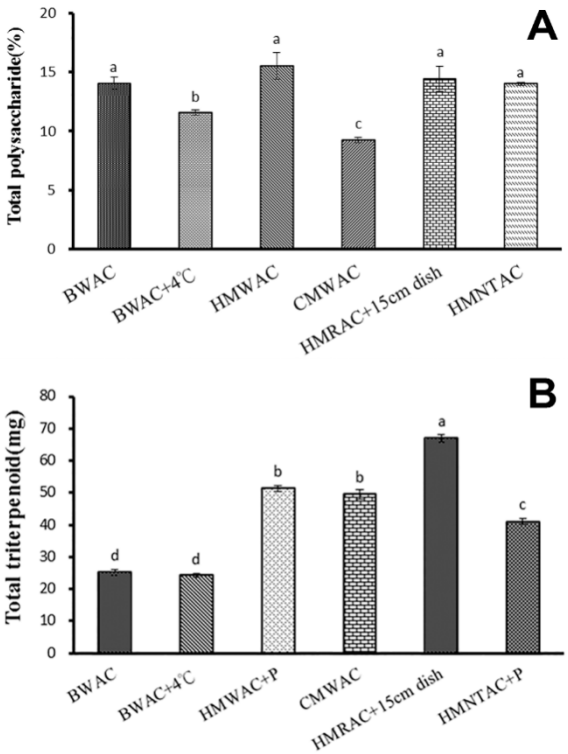

Fig.1. Comparisons of the total (A) polysaccharides and (B) triterpenoids contents of different samples extracted from A. cinnamomea.

Table1: Comparisons of eight triterpenoid indicators (%) in 1 g of extracted samples from different types of A. cinnamomea by HPLC analysis.

Triterpenoid & indicator /samples	A	B	C	D	E	F	G
Antcin A	0.03	nd	0.08	0.06	0.01	1.46	0.17
Antcin B	nd	0.03	0.30	0.60	0.04	9.90	0.58
Antcin C	0.05	0.03	0.05	0.09	nd	3.28	0.36
Antcin H	nd	0.04	0.34	0.29	nd	4.47	0.73
Antcin K	nd	0.08	0.02	0.06	0.03	2.99	nd
DMMB	nd	0.14	0.16	0.63	0.18	1.95	0.57
DeSA	1.02	0.79	0.97	1.62	0.93	3.67	1.40
DeEA	0.74	0.77	1.08	1.57	0.88	0.92	0.39
Total	1.83	1.87	2.98	4.93	2.07	28.65	4.21

A: BWAC (Basswood white AC); B: HMWAC (homemade medium white AC); C: HMWAC (homemade medium white AC); D: HMRAC (homemade medium red AC on a 15-cm Petri dish); E: HMNTAC (homemade medium native-type AC); F: NTAC (native-type AC); G: CMWAC (commercial medium white AC); nd: not detectable.

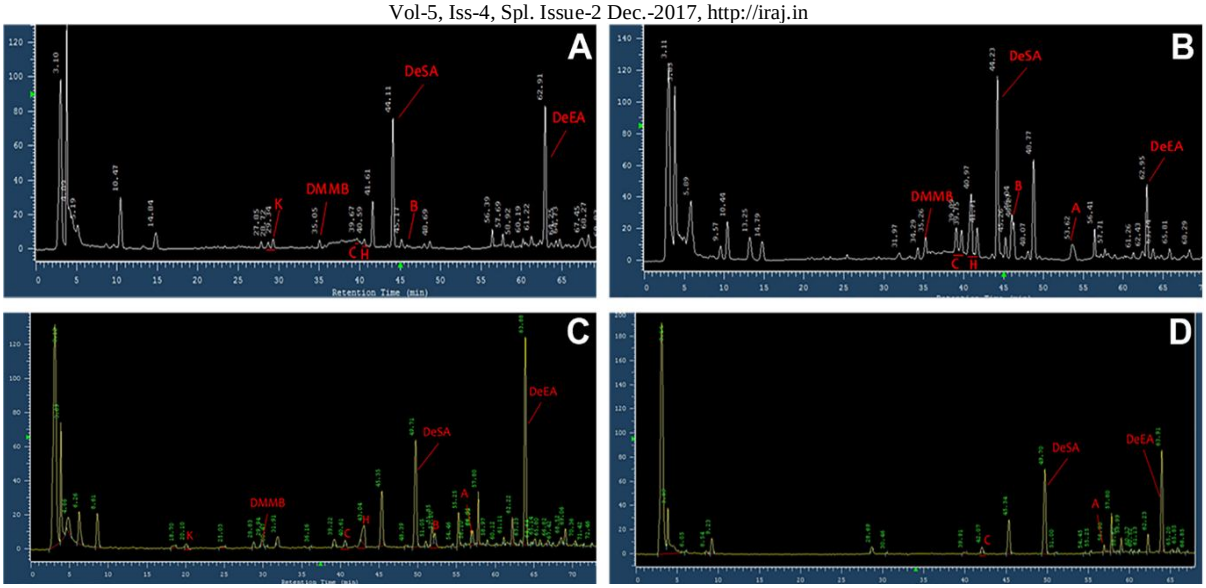

Fig.2. Triterpenoid indicator contents of 8 standard compounds from (A) HMWAC, (B) CMWAC, (C) HMWAC+P, and (D) BWAC grown for 1-2 months.

3.3. DPPH free-radical scavenging activity assay

The antioxidant activity in terms of 0.2 mg DPPH free-radical scavenging ability was determined. Figure 3 shows the results for BWAC, BWAC+4°C, HMWAC, CMWAC, and HMRAC+15-cm Petri dish, and vitamin 10 at 25 ppm. The DPPH free-radical scavenging activity was of the following order: vitamin C 25 ppm, HMWAC (72.9%), CMWAC (69.64%), HMRAC+15-cm Petri dish (65.10%), BWAC (25.99%), BWAC+4°C (13.58) (Fig. 3). Our results showed that the DPPH activities of white AC under homemade Petri-dish culture and red AC cultured in commercial medium were greater than the activity of Basswood white AC.

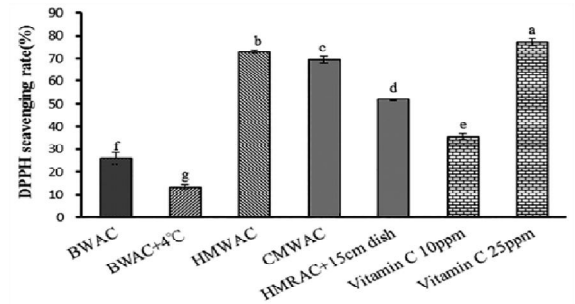

Fig.3. Comparison of antioxidant activities of different extracts of A. cinnamomea.

3.4. MTT-based cell viability assay of different extracts of A. cinnamomea in HepG2 cells

The MTT assay results showed that the inhibitory effect on human liver cell line HepG2 was increased upon treatment with 7.825,15.625,31.25,62.5 and 125 µg/mL of extracts of various types of AC, and all types of AC exhibited similar levels of inhibition at a concentration of 62.5 µg/mL (Fig. 4A). However, the inhibitory effects of CMWAC and HMRAC+15-cm Petri dish decreased upon increased concentration to 125 µg/mL (Figs. 4B & 5). The above-described anti-B cancer cell tests showed that approximately 50% inhibition was achieved at a concentration of 62.5 µg/mL; however, the concentration of wild-type AC fruiting bodies extract was higher than 125 µg/mL, and the anti-B cancer cell effect was over 80%.

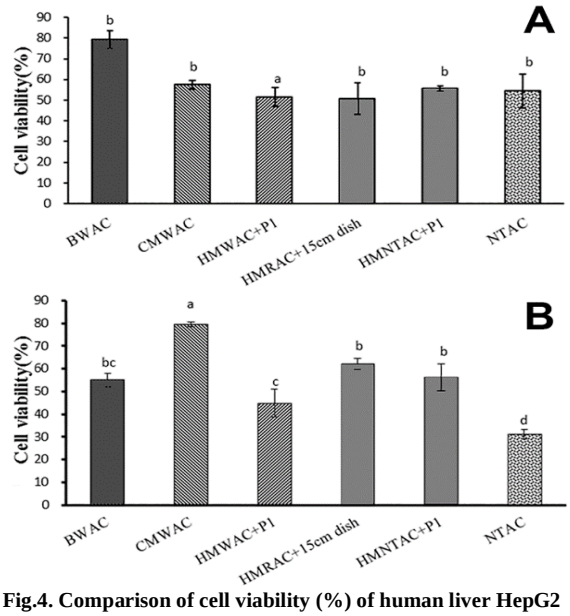

Fig.4. Comparison of cell viability (%) of human liver HepG2 cells incubated with (A) 62.5 µg/mL and (B) 125 µg/mL of different extracts of A. cinnamomea for three days.

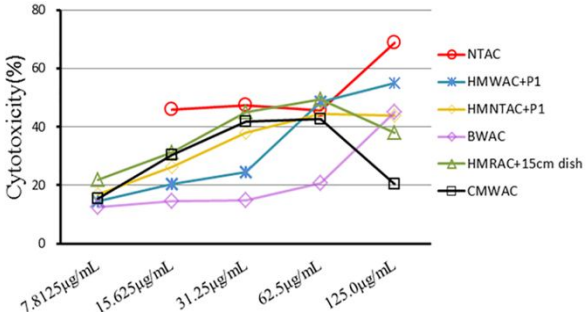

Fig.5. Inhibition effects of different concentrations of extracts of various types of A. cinnamomea against HepG2 cells.

CONCLUSIONS

This study evaluated 8 triterpenoid indicators in different AC extracts in artificial culture medium (homemade medium) modified from commercial medium by HPLC and compared the compositions and bioactivities of regular and white AC. AC has attracted a great deal of attention in Taiwan in recent years owing to its valuable medicinal properties. At present, one of the chief problems is AC cultured on wood Cinnamomumkanehirai Hay) that it takes 2–3 years or more for fruiting bodies to form. In order to enhance production efficiency, control of environmental conditions and modification of the medium composition are required. This study demonstrated the development of a culture medium that resulted in higher contents of triterpenoids, polysaccharides and other bioactive components of AC that may be useful in various health-related products, and hence has considerable potential for application in the healthcare industry.

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