4.0 RESULTS

4.1 Yield of dried extracts from fresh fruitbodies of *A. auricula-judae*

Fresh fruitbodies of *A. auricula-judae* were extracted using methanol, ethanol and dichloromethane. In addition, polysaccharides and hot aqueous extracts were prepared by using boiling and precipitation by ethanol. The yield was calculated for all five types of extracts as shown in Table 4.1. There was a significance difference in the extraction yield between ethanol, methanol, dichloromethane, hot aqueous and polysaccharides extracts ($p < 0.05$). (Appendix D). The extraction yield varied from 0.057% to 0.968% in the following order: polysaccharides > hot aqueous > dichloromethane > ethanol > methanol.

Table 4.1: Total yield for ethanol, methanol, dichloromethane, polysaccharides and hot aqueous extracts from fresh fruitbodies of *A. auricula-judae* (1000g). Percentages of total yield was calculated based on weight of dry extract (g) / weight of fresh fruitbodies (g)

Types of extract	Weight of dry extract(g)	Total yield (%)
Polysaccharides	9.68	0.968^{e}
Hot aqueous	3.57	0.357^{d}
Dichloromethane	0.98	0.098^{c}
Ethanol	0.71	0.071^{b}
Methanol	0.57	0.057^{a}

Values expressed are means ± S.D of triplicate measurements. Means with different letters in a same column are significantly different ($p<0.005$) (Appendix A)*

4.2 Cytotoxic activity of *A. auricula-judae* against cancer cell lines

In the present study, five extracts of *A. auricula-judae* fresh fruitbodies were evaluated for their cytotoxic activity against six cancer cell lines, namely, human cervical cancer cells (CaSki), human ovarian cancer cells (SKOV), human breast cancer cells (MCF 7), human colon cancer cells (HCT 119), human mouth epidermal cancer cell (KB) and human intestinal colon cancer cells (HT 29). The cells grown in 10 % supplemented medium were incubated with ethanol, methanol, dichloromethane, hot aqueous and polysaccharide extracts from *A. auricula-judae* fresh fruitbodies at 20 µg/ml. This concentration was used to prescreen in order to test the extracts for cytotoxicity. It is known that mushroom or plant extract having ED_{50} less than 20 µg/ml is considered active whereas those exhibiting ED_{50} values greater than 30 µg/ml are considered non-active (Geran *et al.,* 1977). Extracts exhibiting less than 50% cytotoxicity at 20 µg/ml, were not tested at other concentrations to determine ED_{50} value. The incubation was carried out for 72 hours in a 5% carbon dioxide (CO_2) water jacketed incubator at 37˚C. The negative controls consist of the cells without the addition of mushroom extracts (Plate 4.1). These controls exhibited normal proliferation rate and showed no signs of death after 72 hours incubation time.

Generally, all *A. auricula-judae* fresh fruitbodies extracts were weakly in cytotoxic against all six cancer cell lines. The inhibition percentages of the selected cell lines by *A. auricula-judae* extracts are shown in Figures 4.1-4.6 and Table 4.2. ED_{50} value were not determined for all the extracts, as the inhibition percentage were less than 50% at the prescreening concentration of 20 µg/ml. Among the cancer cell lines, the human ovary cancer cells (SKOV) showed higher sensitivity towards all extracts of *A. auricula-judae* fresh fruitbodies. The inhibition percentages value of the SKOV cells proliferation which were in the range of 18.6 - 36.7% (Table 4.2) (Figure 4.1). Methanol extract derived from

A. auricula-judae showed high range of inhibition compared with the rest extracts, which is 36.7 %. This range is followed by hot aqueous, dichloromethane, ethanol with inhibition percentages of 30.2, 29.3, and 27.1 respectively. Polysaccharides possess the least inhibition of SKOV cells with a very low inhibition rate, 18.6%.

Based on the Table 4.2 and Figure 4.3, dichloromethane from *A. auricula-judae* shows high range of inhibition towards MCF 7 compared with the other extracts, which is 36.1%. This range is followed by polysaccharides, hot aqueous, methanol with inhibition percentages of 29.7, 21.9 and 6.1 respectively. Crude ethanol extracts seems to have a very low inhibition percentage towards MCF 7 which is 1.7. The results revealed that hot aqueous extract from *A. auricula-judae* possess high cytotoxic effect against KB cell with 28.5 inhibition percentage. This range is followed by crude ethanol, dichloromethane, polysaccharides extracts with inhibition percentages of 20.5, 16.1, and 8.4 respectively. Methanol, derived from *A. auricula-judae* acts as a cytotoxically weaker extract, which possess only 7.9% of KB cells inhibition.

From the results (Figure 4.6 and Table 4.2), it was shown that polysaccharides extract exhibited high antiproliferative activities on HT 29 cells with the inhibition percentage of 22. Followed by ethanol, methanol and dichloromethane with inhibition percentages 11.7, 10.5 and 8.4 respectively. Hot aqueous extracts seems to have a very low HT 29 inhibition percentage, of 4.5. Based on the Figure 4.5, polysaccharides which was derived from *A. auricula-judae* shows high range of inhibition against HCT 119 compared with the rest extracts, by 24.4%. This range is followed by methanol, ethanol and dichloromethane with inhibition percentages of 22.8, 13.9 and 13.7 respectively. Hot aqueous extracts seems to have a very low against HCT 119 with inhibition percentage of 6.6. The dichloromethane possess high inhibition percentage of CaSki cell compared with the rest extracts with 31.8%. This range is followed by ethanol, methanol and hot aqueous

with inhibition percentages of 25.4, 8.8 and 6.1 respectively. Polysaccharides acts as a weak extract which possess only 1.7% of CaSki cells inhibition.

Although none of the extracts are considered active cytotoxically against all cell lines tested, comparison among extracts showed that the extract which produce the highest inhibition percentage was methanol extract (36.7%) against SKOV cell line, followed by dichloromethane extract (36.1%) against MCF 7 cell line (Table 4.2) (Figure 4.1 and Figure 4.3). The rest cancer cells, KB and HCT 119 were achieved (20-30)% inhibition percentage from five types of extracts randomly (Table 4.2) (Figure 4.4 and Figure 4.5).

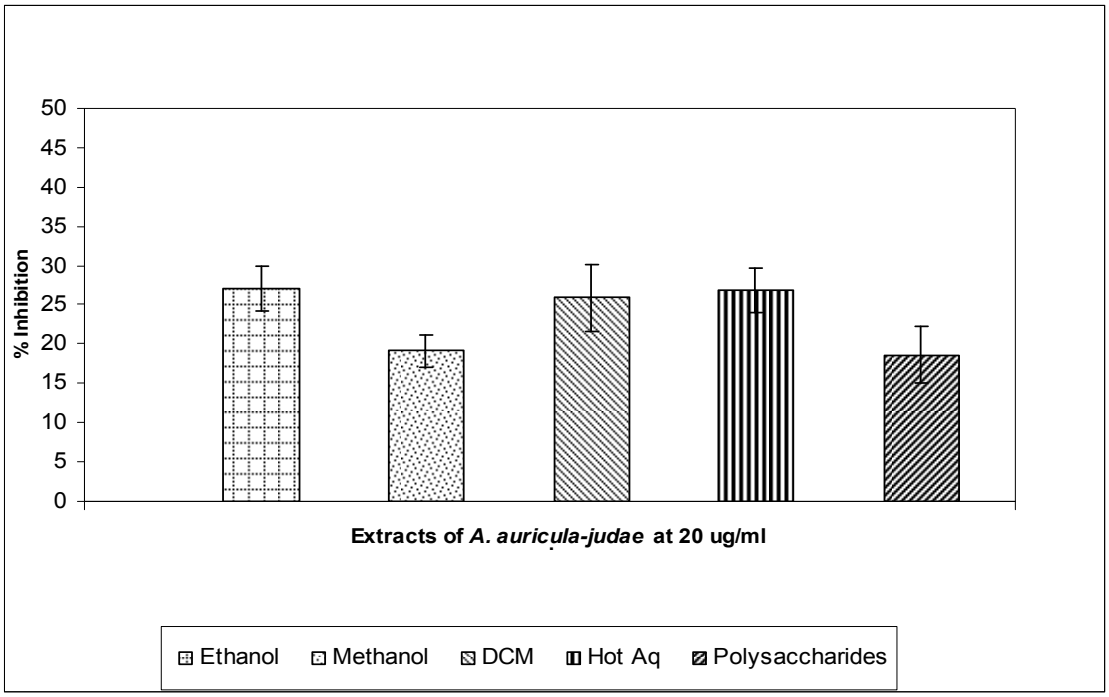
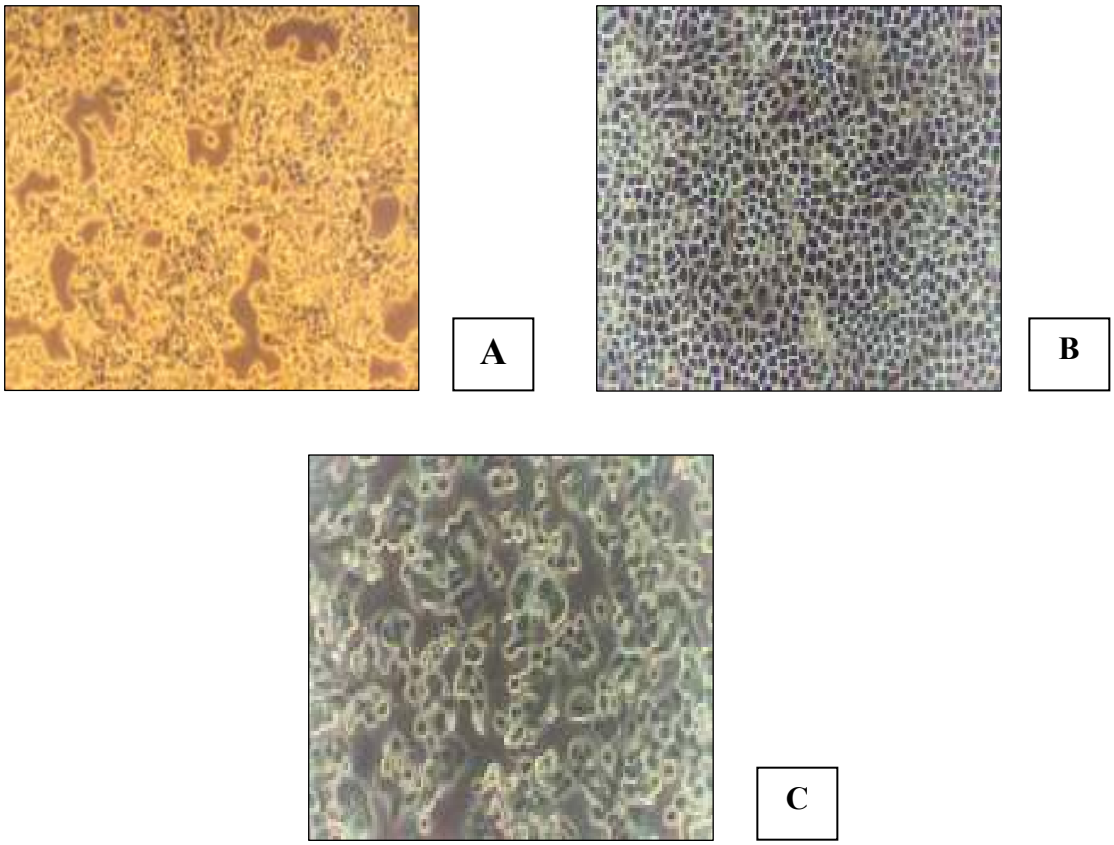


Figure 4.1: *In vitro* growth inhibition by 20 µg/ml ethanol, methanol, dichloromethane, hot aqueous and polysaccharides extracts of *A. auricula-judae* fresh fruitbodies against SKOV cell line.

Plate 4.1: Photomicrograph of: (A): KB cells in Medium 199 (B): CaSki cells incubated in RPMI 1640 medium (C): HT29 cells in RPMI 1640 medium. (Magnification 100x).



4.2.1 Cytotoxic activity of *A. auricula-judae* against normal cell line (MRC 5)

Crude ethanol, methanol, dichloromethane, hot aqueous and polysaccharides extracts of *A. auricula-judae* were evaluated for cytotoxic activity against normal epithelium lung cell line (MRC 5).

Generally, all fresh fruitbodies extracts from *A. auricula-judae* mushroom did not show any active cytotoxic activity against normal epithelial cell line (MRC 5). Percentage of inhibition of MRC 5 cell line by ethanol, methanol, dichloromethane, hot aqueous and polysaccharide were 2.1, 1.6, 1.5, 3.1 and 0.7 respectively.

Table 4.2: Cytotoxicity of *A. auricula-judae* fresh fruitbodies extracts at 20 µg/ml concentration against selected cell lines.

	Types Of Extract				
Cancer cell line	**Ethanol**	**Methanol**	**Dichloromethane**	**Hot Aqueous**	**Polysaccharides**
Skov	27.1 ± 2.56	36.7 ± 2.14	29.3 ± 4.27	30.2 ± 2.88	18.6 ± 3.54
CaSki	25.4 ± 2.22	8.8 ± 2.78	31.8 ± 5.19	6.1 ± 3.57	1.7 ± 0.61
MCF 7	1.7 ± 1.05	6.1 ± 0.79	36.1 ± 3.08	21.9 ± 3.52	29.7 ± 2.19
KB	20.5 ± 4.38	7.9 ± 1.21	16.1 ± 2.96	28.5 ± 3.44	8.4 ± 3.39
HCT 119	13.9 ± 4.03	22.8 ± 3.34	13.7 ± 0.74	6.6 ± 1.96	24.4 ± 2.63
HT 29	11.7 ± 5.36	10.5 ± 5.43	8.4 ± 3.05	4.5 ± 1.48	22.0 ± 0.92
Normal cell (MRC 5)	2.1 ± 2.98	1.6 ± 0.47	1.5 ± 2.11	3.1 ± 1.60	0.7 ± 0.46

The inhibition percentages were expressed in mean value of three replicates together with standard deviations

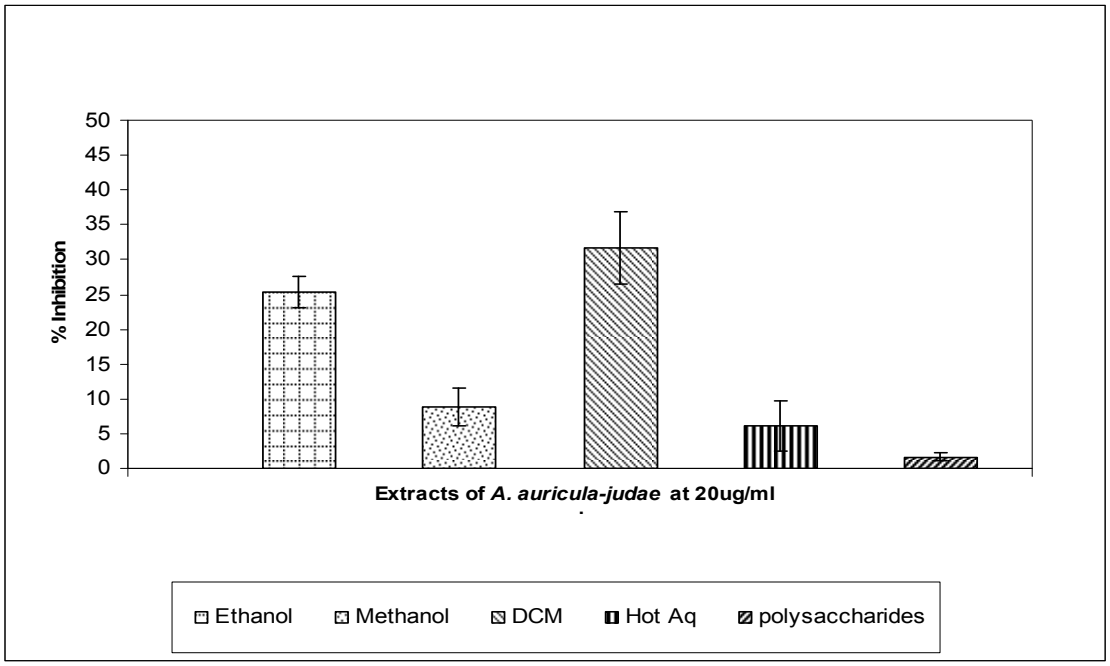


Figure 4.2: *In vitro* growth inhibition by 20 µg/ml ethanol, methanol, dichloromethane, hot aqueous and polysaccharides extracts of *A. auricula-judae* fresh fruitbodies against CaSki cell line.

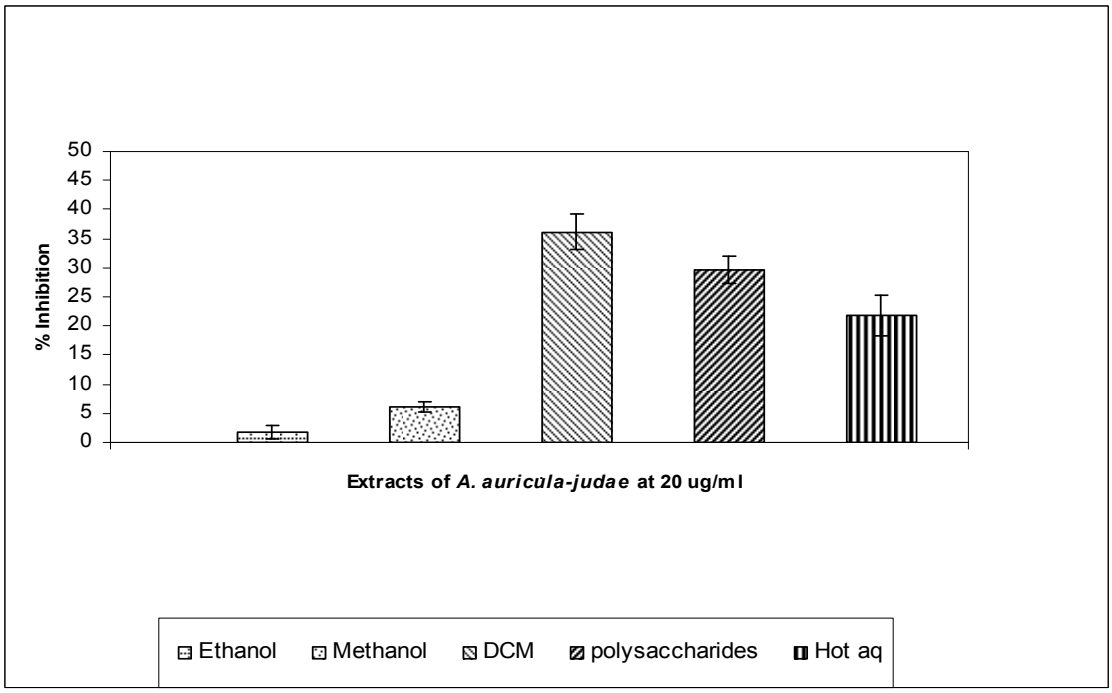


Figure 4.3: *In vitro* growth inhibition by 20 µg/ml ethanol, methanol, dichloromethane, hot aqueous and polysaccharides extracts of *A. auricula-judae* fresh fruitbodies against MCF 7 cell line.

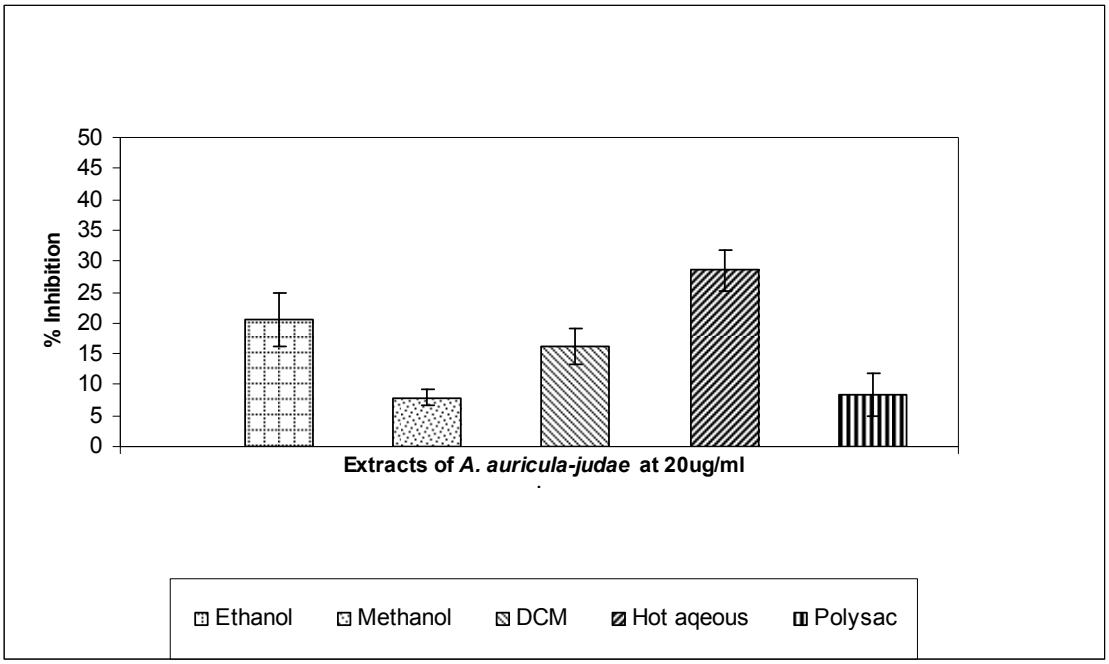


Figure 4.4: *In vitro* growth inhibition by 20 µg/ml ethanol, methanol, dichloromethane, hot aqueous and polysaccharides extracts of *A. auricula-judae* fresh fruitbodies against KB cell line.

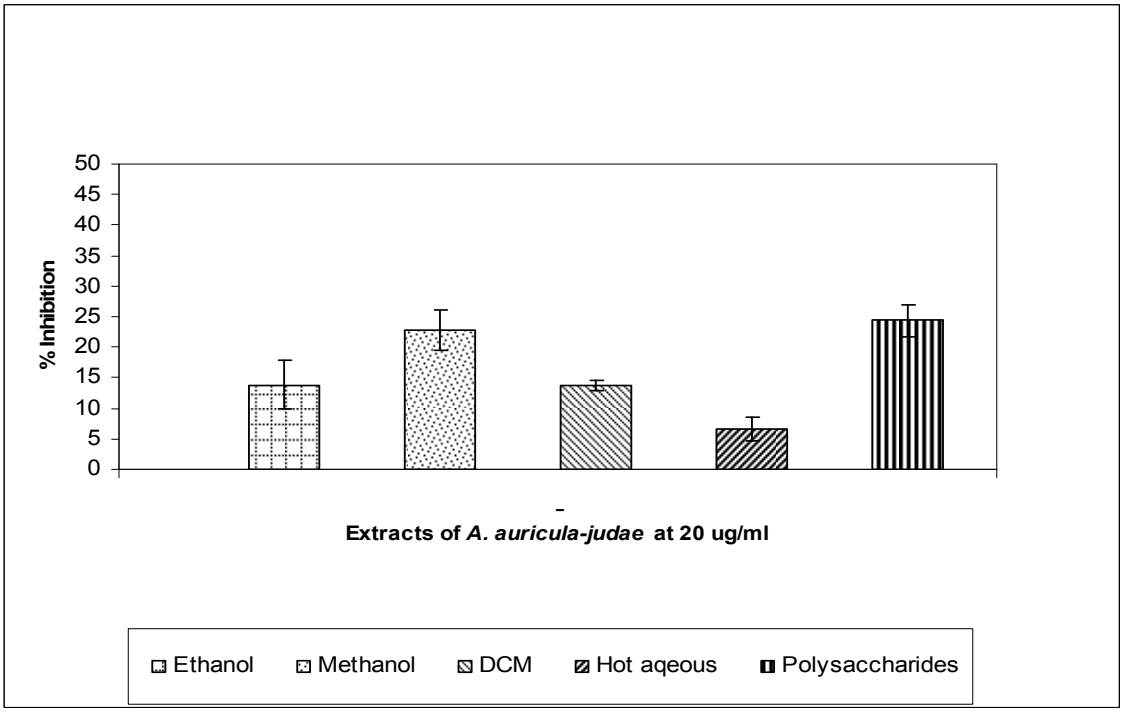


Figure 4.5: *In vitro* growth inhibition by 20 µg/ml ethanol, methanol, dichloromethane, hot aqueous and polysaccharides extracts of *A. auricula-judae* fresh fruitbodies against HCT 119 cell line.

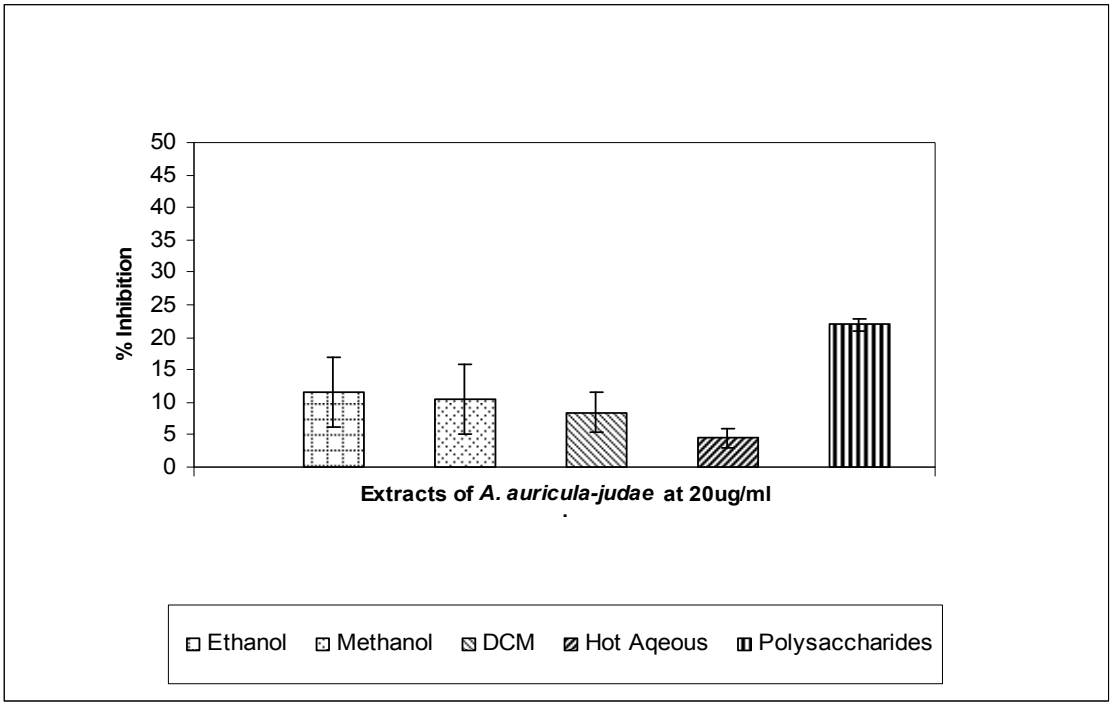


Figure 4.6: *In vitro* growth inhibition by 20 µg/ml ethanol, methanol, dichloromethane, hot aqueous and polysaccharides extracts of *A. auricula-judae* fresh fruitbodies against HT29 cell line.

4.3 Anti HPV-16E6 Oncoprotein Activity

Crude ethanol, methanol, dichloromethane, hot aqueous and polysaccharide extracts from *A. auricula-judae* were screened for possible anti HPV-16E6 activity. The anti HPV - 16E6 oncoprotein activity was evaluated using HPV16 containing cervical cancer HPV 16 containing derived cell line, CaSki. The CaSki cells were treated with *A. auricula-judae* fresh fruitbodies extracts at two concentrations, 25 µg/ml and 100 µg/ml for 72 hours at 37˚ C. In this study, the two step indirect HRP – DAB immunoperoxidase method using anti HPV-16E6 monoclonal antibody was successfully applied to analyze the expression of E6 oncoprotein in treated and untreated CaSki cells.

The presence of E6 oncoprotein was noted based on the appearance of reddish brown stain either in the nuclear or cytoplasmic regions of CaSki cells. The staining intensity was categorized as: no stain (-), weak (+1), moderate (+2), strong (+3) and very strong (+4) in Figure 4.8; Appendix B. Simultaneously, the suppressing effect of the testing extract is considered as very weak for (+4), weak for (+3), moderate for (+2) and high for (+1).

This study utilized two types of negative controls; the CaSki cells not treated with the extract but incubated with anti HPV-16E6 monoclonal antibody and CaSki cells not treated with the extract, and not incubated with anti HPV-16E6 monoclonal antibody. The staining results for untreated CaSki cells with and without anti HPV-16E6 monoclonal antibody is shown in Figure 4.7 (a) and (b). The negative controls incubated with monoclonal antibody were morphologically intact and demonstrated very strong reddish brown stain (+4). Such staining indicates high expression of HPV-16E6 protein detectable with anti HPV-16E6 monoclonal antibody. However, no stain (-) was observed for untreated CaSki cells without incubation with anti HPV-16E6 monoclonal antibody [Figure 4.7 (b)].

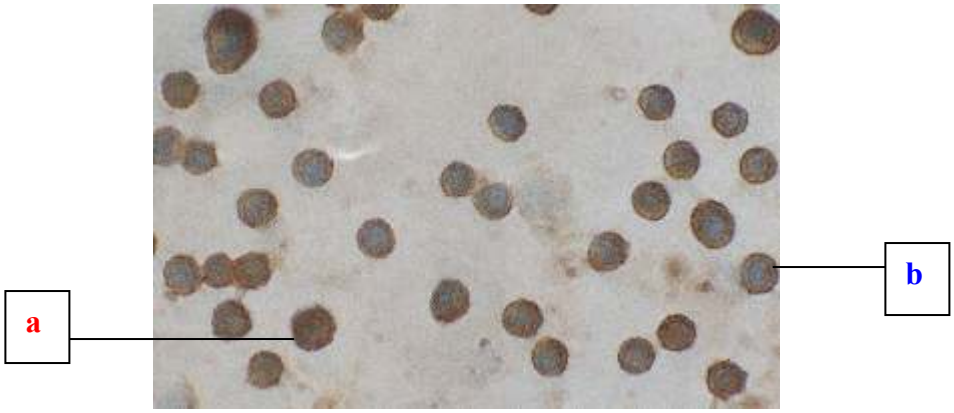


Figure 4.7 (a): CaSki cells not treated with *A. auricula-judae* extracts but incubated with anti HPV -16E6 monoclonal antibody. Cells showed very strong staining (+4) in the cytoplasmic region. (Magnification 400x). a shows staining in the nuclear region; b shows staining in the cytoplasmic region.

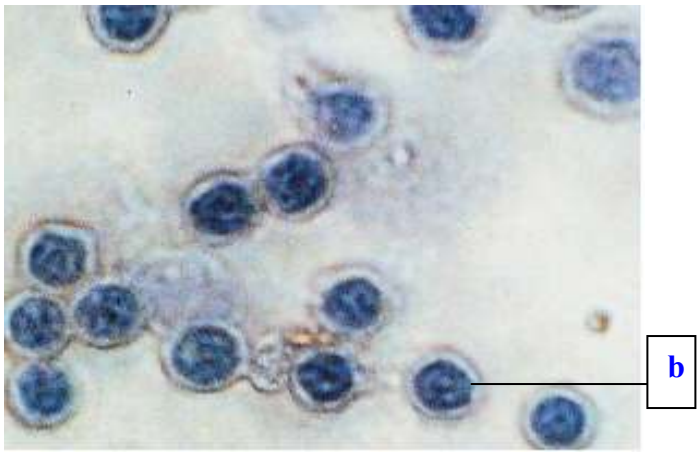
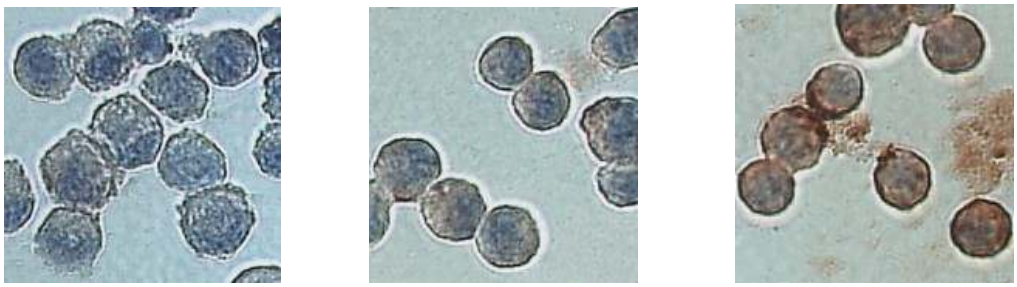
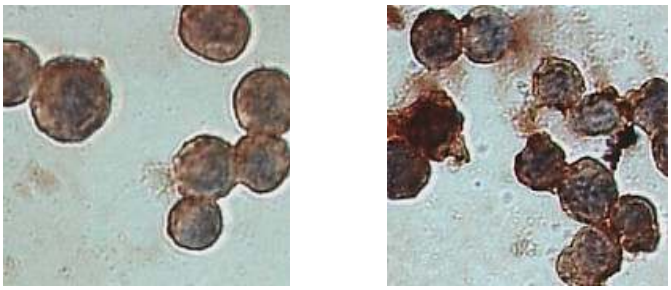


Figure 4.7 (b): CaSki cells not treated with *A. auricula-judae* extracts and not incubated with anti HPV -16E6 monoclonal antibody. Cells showed no staining (-) in the cytoplasmic region (b). (Magnification 400x)



(a) No Stain (-) (b) Weak (+1) (c) Moderate (+2)



(d) Strong (+3) (e) Very Strong (+4)

Figure 4.8: The classifications of reddish-brown stain intensity that indicates the presence of E6 oncoprotein in CaSki cells after ICC. (Magnification 400x). (Chan, 2008)

The staining intensities reflect the expression of E6 oncoprotein HPV16 of the CaSki cells treated with different extracts of *A. auricula-judae* at two concentrations were compared with each other and with the negative controls [Figure 4.7 (c)]. The morphology and the expression of HPV-16E6 oncoprotein in CaSki cells treated with extracts were displayed and summarized in Appendix B.


25 µg/ml


100 µg/ml

Figure 4.7 (c): Examples of *A. auricula-judae* hot aqueous extract treated CaSki cells incubated with anti HPV-16E6 monoclonal antibody for 25 µg/ml and 100 µg/ml concentrations. (Magnification 400x)

4.3.1 Anti HPV-16E6 Oncoprotein Activity in *A. auricula-judae*

As shown, the intensity of reddish brown stains in CaSki cells treated with extracts derived from *A. auricula-judae* fresh fruitbodies decreased with the increment in concentrations of the mushroom extracts. All extracts especially hot aqueous were successful in inhibiting the expression of HPV-16E6 oncoprotein in a dose dependent manner, where higher extract concentration (100µg/ml) exhibited greater suppressing effect of E6 oncoprotein (Figure 4.9).

Among the five types of extracts, three of them (ethanol, methanol, dichloromethane) showed very strong (+4) reddish brown staining intensity and very weak suppression of HPV-16E6 oncoprotein at lower concentration. However, at higher concentration, 100 µg/ml, the E6 oncoprotein were highly suppressed. The hot aqueous extract showed higher suppression activity against HPV-16E6 oncoprotein compared with

other extracts in lower concentration, 25 µg/ml. Staining intensity of hot aqueous extract in CaSki cell indicates low amount of HPV-16E6 oncoprotein being expressed at 25 µg/ml of hot aqueous extract. This suggests strong suppression of HPV-16E6 oncoprotein's expression by hot aqueous extract at this concentration. However at the higher concentration of the ethanol extract (100 µg/ml) the reddish brown stain were not observed. This indicates total suppression of the anti HPV-16E6 oncoprotein by ethanol extracts happened at this concentration. Slight reddish brown stain was observed at hot aqueous, methanol, dichloromethane and polysaccharides extracts (100 µg/ml) showing that, high suppression of E6 oncoprotein occurred. Furthermore, for all extracts of *A. auricula-judae* the staining of reddish brown colour was clearly detected and seen at the cytoplasmic and nucleus region of treated CaSki cells.

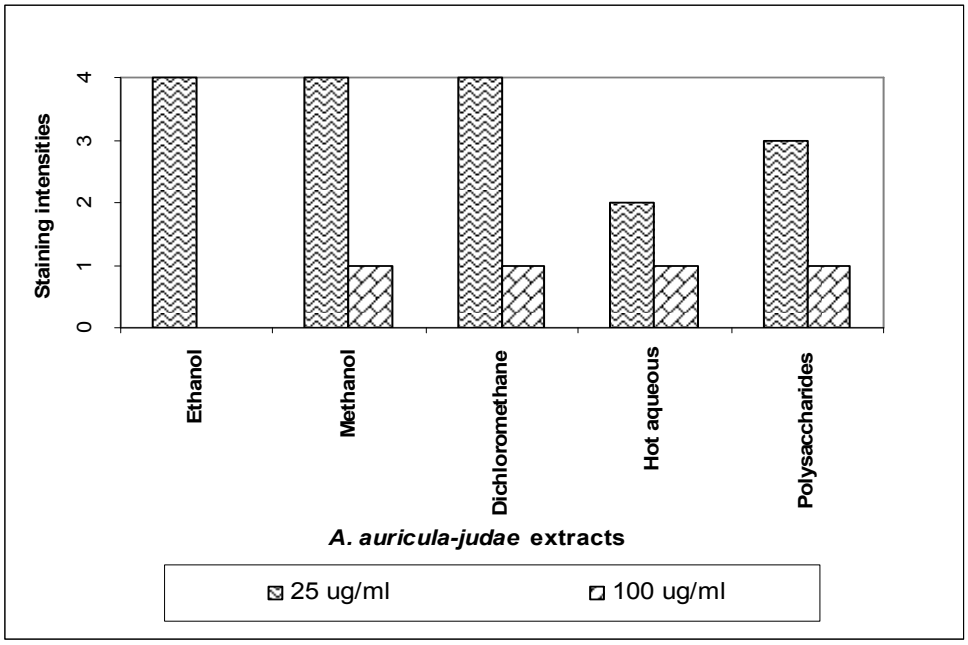


Figure 4.9: Reddish brown staining intensities of CaSki cells containing HPV-16E6 onkoprotein, treated with *A. auricula-judae* ethanol, methanol, dichloromethane, hot aqueous and polysaccharides extracts. Staining intensities measured at two concentrations, 25 and 100 µg/ml.

4.4 Scavenging activity by extracts of *A. auricula-judae* on DPPH radicals

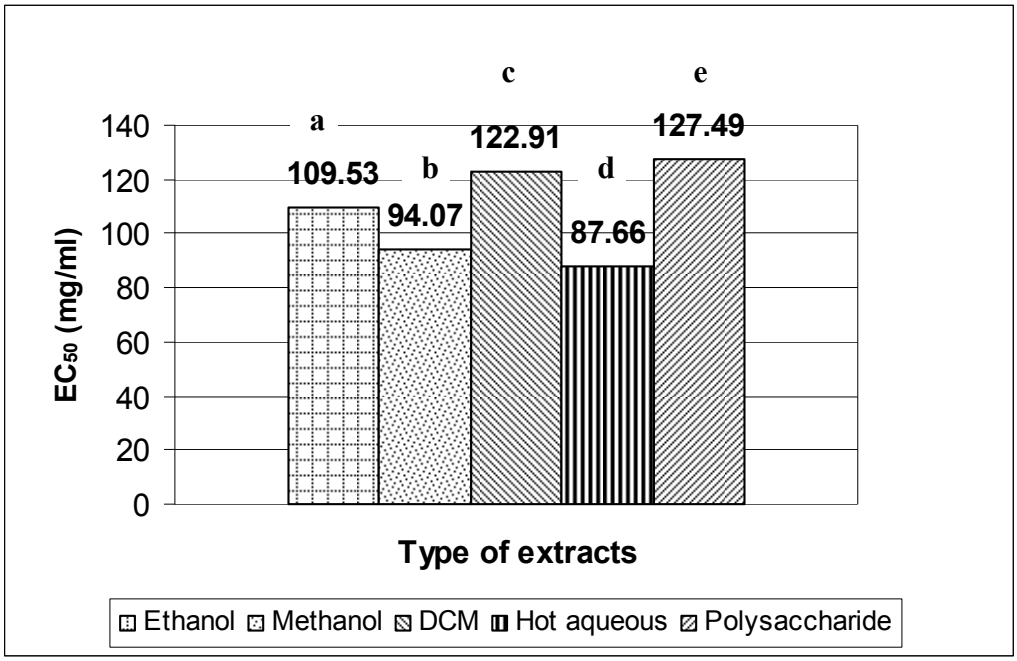
The free radical scavenging activity was evaluated using DPPH radical scavenging method. According to this assay, the antioxidant activity was expressed by using EC_{50} value. The EC_{50} value (50% effective concentration) which is defined as the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50%, was determined from the plotted graph of scavenging activity against the concentration of extracts after 60 minutes. In this study, the scavenging activity of fresh fruitbodies extracts on DPPH radical increased with increasing concentration and reached steady state after 45 minutes of reaction time. This implied a dose dependent DPPH radical scavenging by the extracts.

The EC_{50} value of extracts in DPPH radical scavenging of hot aqueous, methanol, ethanol, dichloromethane and polysaccharides were 87.66 mg/ml, 94.07 mg/ml, 109.53 mg/ml, 122.91 mg/ml and 127.49 mg/ml respectively (Fig 4.10). Based on this DPPH radicals scavenging method, the antioxidant activity of extracts from *A. auricula-judae* decreased in descending order: hot aqueous > methanol > ethanol > dichloromethane > polysaccharides. Meanwhile, Butylated hydroxyanisole (BHA) a synthetic antioxidant and ascorbic acid were used as a positive control, with EC_{50} values at 0.12 mg/ml (Appendix A), 0.021 mg/ml respectively (Appendix A).

4.5 Ferric reducing antioxidant power (FRAP) of *A. auricula-judae* extracts

The ferric reducing antioxidant power (FRAP) of *A. auricula-judae* fresh fruitbodies extracts were determined by measuring the absorbances at 700 nm. In this study, all *A. auricula-judae* fresh fruitbodies extracts showed very promising reducing

power activity. There was a strong significant difference between FRAP values at different concentrations for each extract (Appendix B).



EC_{50} values of positive controls (mg/ml), BHA = 0.021 mg/ml; Ascorbic acid = 0.12 mg/ml

Figure 4.10: EC_{50} values (mg/ml) of *A. auricula-judae* fresh fruitbodies extracts in DPPH radical scavenging assay. The BHA and Ascorbic acid were used as positive controls for the assay. Different letters denotes significance, P> 0.05, (Appendix D)

BHA and ascorbic acid were used as the positive controls, showed increasing FRAP absorbance (2.682 nm and 1.995 nm respectively) at 10 mg/ml concentration. At the concentration of 10 mg/ml to 20 mg/ml, both BHA and ascorbic acid reducing power entered the steady state and no further increase was observed in the absorbance. Among the extracts, the hot aqueous extract showed highest reducing power which indicated by a significantly highest absorbance (Appendix A). This is due to the FRAP absorbance increased as concentration increased from 0 to 5 mg/ml reaching 1.658 nm. However, increasing concentration up to 20 mg/ml did not significantly increase the absorbance (Figure 4.11). Further increase in concentration of BHA and ascorbic acid showed similar

dose dependent increase up to a concentration of 5 mg/ml and achieved absorbance of 2.539 nm and 1.932 nm respectively.

Methanol, ethanol and dichloromethane showed a gradual increase in absorbances until 20 mg/ml concentration (Figure 4.11). Methanol and ethanol extracts showed similar FRAP capacity with methanol exhibiting higher absorbance of 1.463 nm compared to ethanol (1.208 nm) at the concentration of 20 mg/ml. Generally, all *A. auricula-judae* fresh fruitbodies extracts showed good reducing power activity, except for polysaccharides, which showed very low reducing power (0.4397 nm) at 20 mg/ml. The hierarchy of ferric reducing ability for each extract and positive controls were:- BHA > ascorbic acid > hot aqueous > methanol > ethanol > dichloromethane > polysaccharides.

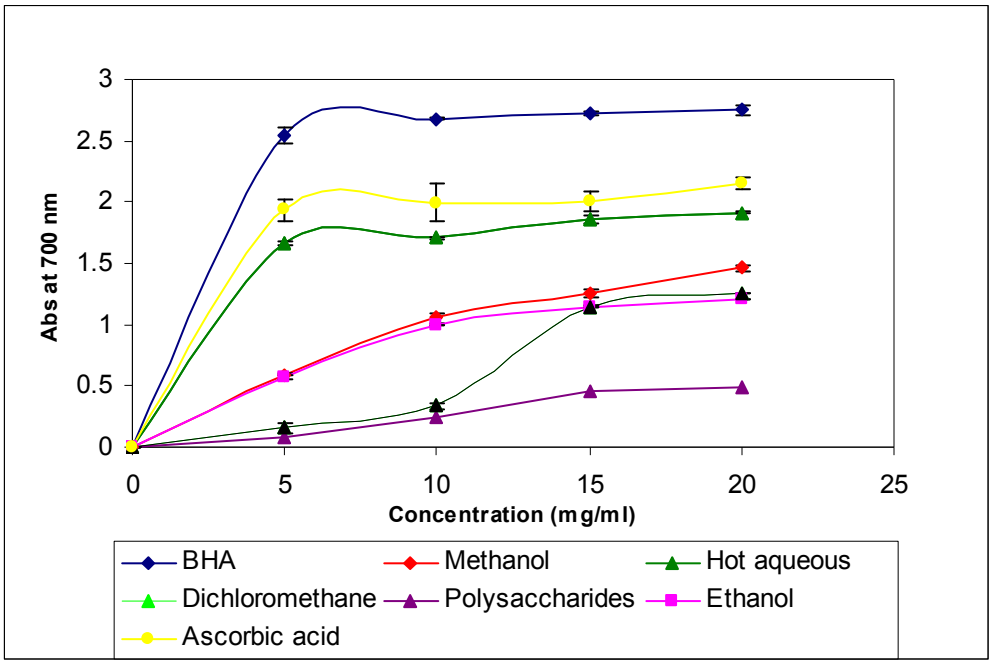


Figure 4.11: Reducing power values of ethanol, methanol, dichloromethane, hot aqueous and polysaccharides from *A. auricula-judae* fresh fruitbodies. The one way ANOVA for each extracts absorbances at each concentration was shown at (Appendix D), $p < 0.05$ shows significant value for absorbances in different concentration.

4.6 Determination of total phenolic contents of *A. auricula-judae* extracts

The Folin-Ciocalteau method was used to measure the total phenolic content in the ethanol, methanol, dichloromethane, hot aqueous and polysaccharides extracted from *A. auricula-judae* fresh fruitbodies. In this study, gallic acid was used as the reference standard, while BHA and ascorbic acid were used as positive controls.

The plot of different concentrations of gallic acid versus absorbances yielded a straight line passing through the origin, as depicted in (Appendix A). The total phenolic content expressed as gallic acid equivalent (GAE) per gram dried extracts was shown in Table 4.3. Based on the Table 4.3, the hot aqueous extract from *A. auricula-judae* fresh fruitbodies contained of significantly high amount of phenolics than other extracts (56.89 mg GAE/g extract). Subsequently, the methanol, ethanol, and dichloromethane extracts possessed total phenolic content within the range of (1.38-1.93 mg GAE/g extract), whereas the total phenolic content of polysaccharides extract, showed low value with only 0.923 mg GAE/g extract. In the meantime, the BHA and ascorbic acid as positive controls possessed 1627.31 mg GAE/ g BHA and 932.90 mg GAE/g ascorbic acid. Thus, it was proven that the positive controls have more pronounced total phenolic content than the *A. auricula-judae* extracts.

Overall, the total phenolic content of extracts and positive controls shown in a decreasing order: BHA > ascorbic acid > hot aqueous > methanol > ethanol > dichloromethane > polysaccharides. There was a moderate correlation (R^2= 0.6243) between scavenging ability on DPPH radicals and the total phenolic content of fresh fruitbodies extracts of *A. auricula-judae*.as shown in Figure 4.12 (a). There was a moderate correlation (R^2= 0.5074) also between reducing power activity FRAP absorbances with the

total phenolic content in all the fresh fruitbodies extracts of *A. auricula-judae* [Figure 4.12(b)].

Table 4.3: Total phenolic content values of *A. auricula-judae* fresh fruitbodies extracts;expressed as gallic acid equivalents (GAE) per gram extracts of fresh fruitbodies. Ascorbic acid and BHA were used as standard for the assay.

***Auricularia auricula-judae* (Extract types)**	**Total phenolics content mg GAE/ g extract**
Ethanol	1.590^{c} ± 0.000
Methanol	1.930^{d} ± 0.001
Dichloromethane	1.380^{b} ± 0.006
Hot aqueous	56.890^{e} ± 0.001
Polysaccharides	0.923^{a} ± 0.004
BHA	1627.310^{g} ± 0.003
Ascorbic acid	932.900^{f} ± 0.002

Values expressed are means ± S.D of triplicate measurements. Means with different letters in a same column are significantly different ($p<0.05$) (Appendix D).

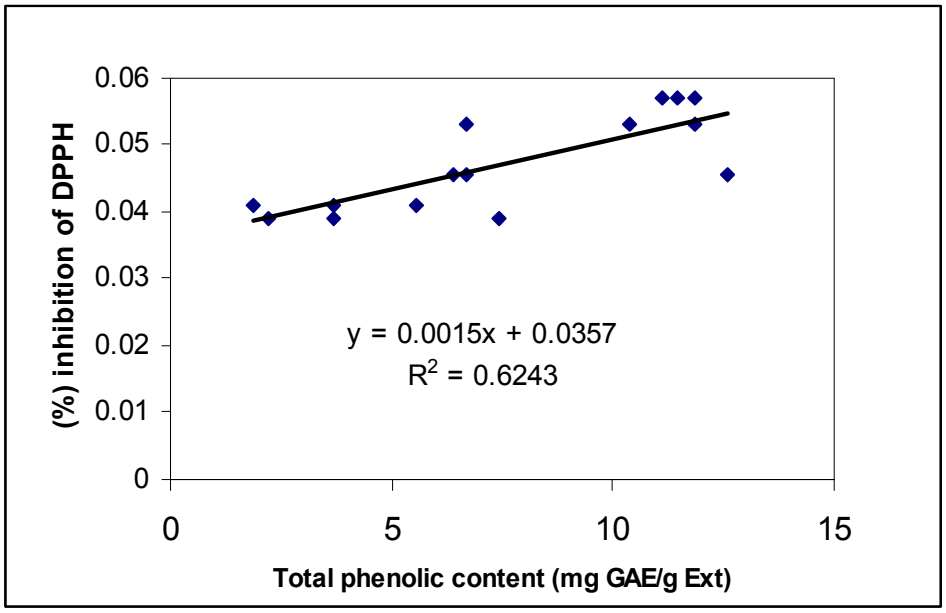


Figure 4.12 (a): Correlation graph of DPPH radicals (%) scavenging activity with total phenolic content in mg GAE/ g extracts of *A. auricula-judae* fresh fruitbodies extracts.

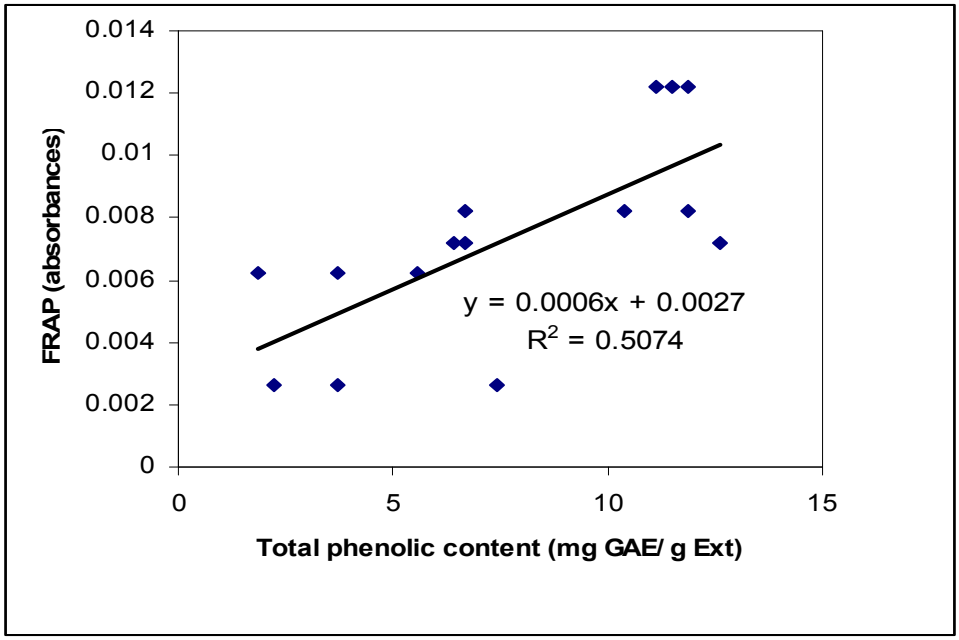


Figure 4.12 (b): Correlation graph of reducing power activity with total phenolic content in mg GAE/ g extracts of *A. auricula-judae* fresh fruitbodies extracts.