CHAPTER 4

RESULTS

4.1 Production of antioxidants by *L. squarrosulus* mycelia grown on soya bean, maize and rice supplemented with different concentration of nitrogen sources

The best solid substrate formulation that supported the optimum growth of *L. squarrosulus* mycelia (Plate 4.1) for the production of antioxidants was determined using maize, soya bean and rice supplemented with various nitrogen sources (peptone, malt extract and yeast extract) at different concentrations of 0.04%, 1.0% and 2.0% (Appendix B). The growth of *L. squarrosulus* on each substrate formulation was observed visually.

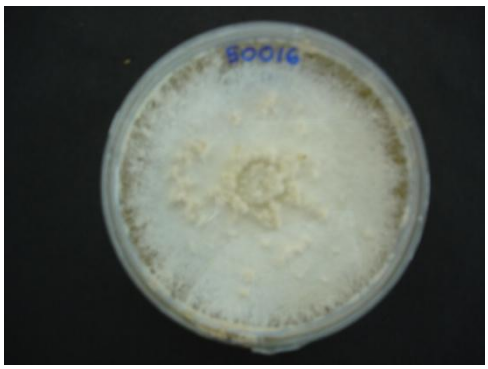


Plate 4.1: *Lentinus squarrosulus* inoculum grown on the Glucose-Yeast-Malt-Peptone (GYMP) agar, after 14 days of incubation at 37°C.

It was observed by visually that the growth of *L. squarrosulus* on soybean was denser compared to rice and maize. This was possibly due to the difference in the water activity of the substrates, whereby soya bean and rice showed the highest percentage moisture content of 42.90% and 40.05% respectively. The growth density observed on maize was the least with moisture content of 37.14%. After 14 days of fermentation, the

mycelia together with the substrates were freeze dried and subsequently soaked in methanol and DCM separately to extract the bioactive compounds and their DPPH radical scavenging activity were then evaluated.

DPPH radical scavenging activity of *L. squarrosulus* extracts from mycelia cultivated on each growth formulation were evaluated using IC_{50} and the values were compared with ascorbic acid ($C_6H_8O_5$, 176.16g/mol), quercetin dihydrate ($C_{15}H_{10}O_7.2H_2O$, 338.26g/mol) and a synthetic antioxidant, BHA ($C_{11}H_{16}O_2$, 180.25g/mol) as positive control. As shown in Appendix C.1 to C.3, ascorbic acid reacted rapidly with the DPPH radical, reaching a steady state in less than 2 minutes, while quercetin dihydrate and BHA approached the steady state in 75 and 60 minutes, respectively. Referring to the IC_{50} values (Figure 4.1), quercetin dihydrate showed the highest DPPH radical scavenging activity compared with ascorbic acid and BHA, with 0.032mg/ml (0.2mM), 0.078mg/ml (0.1mM) and 0.097mg/ml (1.0mM) respectively.

In general, Tables 4.1, 4.2 and 4.3 show that both methanol and dichloromethane (DCM) extracts of *L. squarrosulus* fermented substrates have lower IC_{50} values or better antioxidant activity compared to the unfermented substrates. However, the IC_{50} values of DCM extracts were higher than methanol, indicating that methanol is a better solvent to extract antioxidant compounds. The IC_{50} values of *L. squarrosulus* fermented maize extracted with methanol gave the best DPPH scavenging activity with value ranged from 20.2mg/ml - 22.8mg/ml compared to soya bean (22.2mg/ml - 96.2mg/ml) and rice (20.2mg/ml - 147.1mg/ml) for all the nitrogen source concentrations tested. Considering the methanolic extracts of *L. squarrosulus* fermented maize, the best nitrogen source and concentration for antioxidant production was peptone at concentration 0.04% with IC_{50} value 20.2mg/ml. The best nitrogen source for soya bean

was yeast extract at concentration 2.0% with IC_{50} value of 22.2mg/ml, and rice was 2.0% peptone with IC_{50} value of 20.2mg/ml (Figures 4.1 – 4.3). Maize and rice both supplemented with peptone showed the best antioxidant activity with IC_{50} value of 20.2mg/ml. However, maize only needed 0.04% of peptone concentration compared to rice in order to produce the same IC_{50} value. Thus methanolic fermented maize extract was considered as producing the best antioxidant activity.

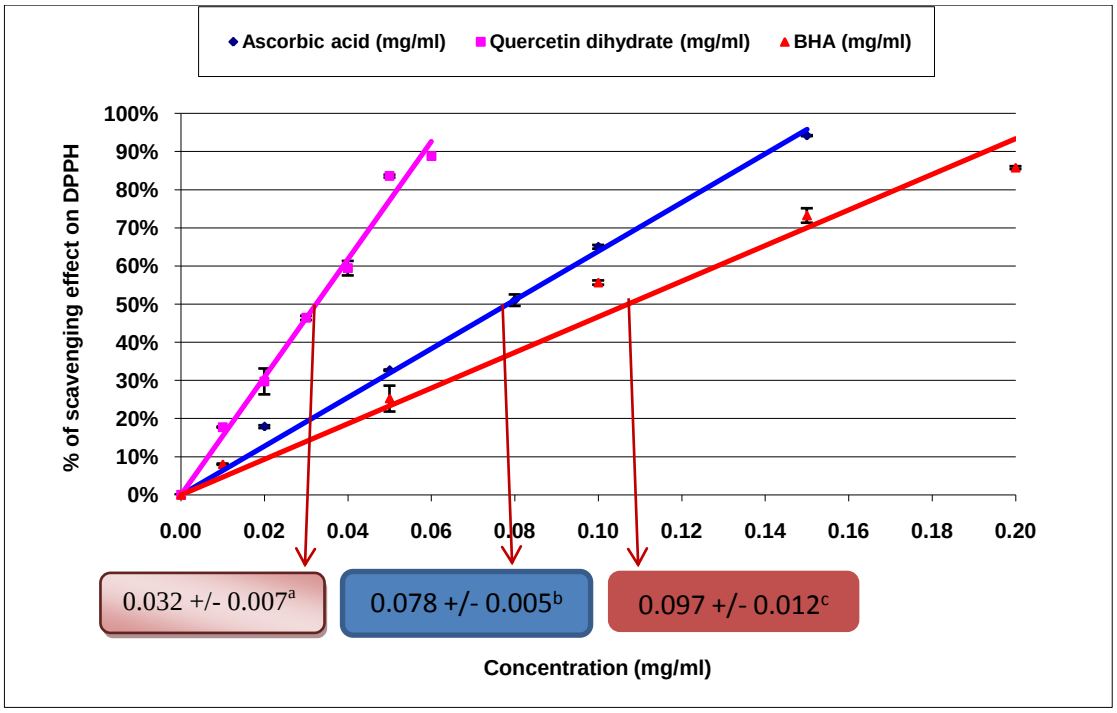


Figure 4.1: DPPH radical scavenging activity of ascorbic acid (at steady state 2 minutes), quercetin dehydrate (at steady state 75 minutes) and BHA (at steady state 60 minutes). *Values are expressed in mean ± standard deviation (n=3). Means with different alphabet indicates significantly different (ANOVA, P<0.05).

Considering the dichloromethane (DCM) extracts of *L. squarrosulus* fermented substrates, for maize the best nitrogen source and concentration for antioxidant production was malt extract at concentration 2.0% with IC_{50} value of 20.4mg/ml. As for soya bean, the best nitrogen source was yeast extract at concentration 2.0% with IC_{50} value of 25.1mg/ml, and rice showed peptone at 2.0% concentration as the best

Table 4.1: IC_{50} values (mg/ml) of methanolic and dichloromethane antioxidant extracts from *Lentinus squarrosulus* grown on soya bean supplemented with various concentrations of nitrogen sources. Values are expressed in mean (n=3 and +/- STD). Raw data for ANOVA and multiple range tests are given in Appendix B.

Sources of nitrogen		Solvent	Concentrations of nitrogen source (%)					
			0.04		1.0		2.0	
			Soya Bean ± STD	Time to reach steady state (min)	Soya Bean ± STD	Time to reach steady state (min)	Soya Bean ± STD	Time to reach steady state (min)
Peptone	Fermented	Methanol	96.2 ± 0.01 [c]	60	50.5 ± 0.01 [b]	60	25.9 ± 0.01 [a]	60
		DCM	69.4 ± 0.01 [d]	60	61.7 ± 0.01 [b]	45	58.8 ± 0.01 [b]	60
	Unfermented	Methanol	116.3 ± 0.01 [e]	60	192.3 ± 0.00 [g]	60	52.6 ± 0.01 [b]	60
		DCM	263.2 ± 0.01 [i]	15	217.4 ± 0.01 [h]	30	131.6 ± 0.01 [f]	60
Malt extract	Fermented	Methanol	69.4 ± 0.00 [c]	60	22.8 ± 0.01 [a]	45	22.3 ± 0.01 [a]	60
		DCM	53.8 ± 0.01 [b]	60	67.6 ± 0.01 [c]	60	28.7 ± 0.01 [a]	60
	Unfermented	Methanol	111.1 ± 0.01 [e]	60	37.6 ± 0.01 [a]	60	66.7 ± 0.01 [c]	60
		DCM	113.6 ± 0.01 [e]	60	142.9 ± 0.01 [f]	60	54.9 ± 0.01 [b]	60
Yeast extract	Fermented	Methanol	67.6 ± 0.01 [c]	60	33.8 ± 0.01 [a]	60	22.2 ± 0.01 [a]	60
		DCM	31.5 ± 0.01 [a]	60	32.3 ± 0.01 [a]	60	25.1 ± 0.01 [a]	60
	Unfermented	Methanol	96.2 ± 0.00 [d]	60	48.5 ± 0.01 [b]	60	96.2 ± 0.01 [d]	60
		DCM	131.6 ± 0.01 [f]	30	98.0 ± 0.01 [d]	60	71.4 ± 0.01 [c]	30

***STD denotes standard deviation value. Means with different alphabet in the tables are significantly different (ANOVA, P<0.05).**

Table 4.2: IC_{50} values (mg/ml) of methanolic and dichloromethane antioxidant extracts from *Lentinus squarrosulus* grown on maize supplemented with various concentrations of nitrogen sources. Values are expressed in mean (n=3 and +/- STD). Raw data for ANOVA and multiple range tests are given in Appendix B. F denotes fermented, U denotes unfermented and STD denotes standard deviation value.

Sources of nitrogen		Solvent	Concentrations of nitrogen source (%)					
			0.04		1.0		2.0	
			Maize ± STD	Time to reach steady state (min)	Maize ± STD	Time to reach steady state (min)	Maize ± STD	Time to reach steady state (min)
Peptone	Fermented	Methanol	20.2 ± 0.01 [a]	30	20.9 ± 0.01 [a]	45	20.7 ± 0.01 [a]	45
		DCM	89.3 ± 0.01 [d]	60	40.3 ± 0.01 [b]	60	46.3 ± 0.01 [b]	60
	Unfermented	Methanol	53.8 ± 0.01 [b]	60	31.1 ± 0.01 [a]	60	29.4 ± 0.01 [a]	45
		DCM	116.3 ± 0.01 [e]	30	86.2 ± 0.01 [d]	60	61.0 ± 0.00 [c]	45
Malt extract	Fermented	Methanol	21.4 ± 0.01 [a]	45	22.8 ± 0.01 [a]	60	22.0 ± 0.01 [a]	60
		DCM	27.8 ± 0.01 [a]	60	20.7 ± 0.01 [a]	60	20.2 ± 0.01 [a]	60
	Unfermented	Methanol	119.1 ± 0.00 [e]	45	60.2 ± 0.01 [c]	60	46.7 ± 0.01 [b]	60
		DCM	200.0 ± 0.01 [g]	60	58.1 ± 0.01 [c]	60	46.3 ± 0.01 [b]	60
Yeast extract	Fermented	Methanol	21.5 ± 0.00 [a]	60	20.8 ± 0.01 [a]	45	21.3 ± 0.01 [a]	60
		DCM	20.8 ± 0.01 [a]	60	20.3 ± 0.01 [a]	60	20.7 ± 0.00 [a]	30
	Unfermented	Methanol	90.9 ± 0.01 [d]	60	72.5 ± 0.01 [c]	60	52.1 ± 0.01 [b]	60
		DCM	156.3 ± 0.01 [f]	60	61.7 ± 0.01 [c]	60	79.4 ± 0.01 [d]	45

***STD denotes standard deviation value. Means with different alphabet in the tables are significantly different (ANOVA, P<0.05).**

Table 4.3: IC_{50} values (mg/ml) of methanolic and dichloromethane antioxidant extracts from *Lentinus squarrosulus* grown on rice supplemented with various concentrations of nitrogen sources. Values are expressed in mean (n=3 and +/- STD). Raw data for ANOVA and multiple range tests are given in Appendix B. F denotes fermented, U denotes unfermented, STD denotes standard deviation value and ND (Non-detected) for the insufficient extract volume for the antioxidant test.

Sources of nitrogen		Solvent	Concentrations of nitrogen source (%)					
			0.04		1.0		2.0	
			Rice ± STD	Time to reach steady state (min)	Rice ± STD	Time to reach steady state (min)	Rice ± STD	Time to reach steady state (min)
Peptone	Fermented	Methanol	37.0 ± 0.00 [b]	60	20.8 ± 0.01 [a]	60	20.2 ± 0.00 [a]	60
		DCM	151.5 ± 0.01 [f]	60	94.3 ± 0.01 [d]	45	69.4 ± 0.01 [c]	60
	Unfermented	Methanol	119.1 ± 0.01 [e]	60	86.2 ± 0.00 [d]	60	62.5 ± 0.01 [c]	60
		DCM	312.5 ± 0.00 [j]	60	156.3 ± 0.01 [f]	30	98.0 ± 0.01 [d]	60
Malt extract	Fermented	Methanol	147.1 ± 0.01 [f]	60	83.3 ± 0.01 [c]	45	62.5 ± 0.01 [c]	60
		DCM	94.3 ± 0.01 [d]	60	96.2 ± 0.01 [c]	60	72.5 ± 0.01 [c]	60
	Unfermented	Methanol	294.1 ± 0.01 [i]	60	227.3 ± 0.01 [h]	60	86.2 ± 0.01 [d]	60
		DCM	200.0 ± 0.01 [g]	60	192.3 ± 0.01 [g]	60	192.3 ± 0.01 [g]	60
Yeast extract	Fermented	Methanol	53.8 ± 0.01 [c]	60	22.5 ± 0.01 [a]	60	21.7 ± 0.01 [a]	45
		DCM	90.9 ± 0.01 [d]	60	96.2 ± 0.01 [d]	60	72.5 ± 0.01 [c]	60
	Unfermented	Methanol	125.0 ± 0.01 [e]	60	106.4 ± 0.01 [d]	60	64.1 ± 0.01 [c]	60
		DCM	ND		ND		ND	

***STD denotes standard deviation value. Means with different alphabet in the tables are significantly different (ANOVA, P<0.05).**

nitrogen source with IC_{50} value of 69.4mg/ml. Comparing the IC_{50} values of DPPH scavenging activity, the best carbon source supporting growth of mycelia biomass with the highest antioxidant activity was maize supplemented with 0.04% peptone.

4.2 Comparison of antioxidant capacities of methanolic extract from *L. squarrosulus* grown in liquid media and maize supplemented with 0.04% peptone using radical scavenging activity of DPPH

Methanolic extract of *L. squarrosulus* mycelia grown on maize supplemented with 0.04% peptone was selected for further antioxidant analysis based on its highest scavenging activity compared to rice and soya bean. The value was compared with *L. squarrosulus* grown in liquid GYMP media supplemented with 0.04% peptone. With regard to IC_{50} values of methanolic extracts, antioxidants produced by fermentation of maize with 0.04% peptone showed better DPPH radical scavenging effect than antioxidants extracted from mycelia obtained by liquid fermentation with values of 23.5mg/ml and 26.9mg/ml respectively (Figure 4.2).

4.3 Inhibition of lipid peroxidation of methanolic extract from *L. squarrosulus* grown in liquid media, maize supplemented with 0.04% peptone and unfermented maize using egg yolk and cooking oil

The selected antioxidant extract was further evaluated for the inhibition of lipid peroxidation by determining whether it can induce a high amount of malondialdehyde (MDA), a secondary product of lipid peroxidation. It was necessary to test the extent of the efficacy of the extracts to inhibit lipid peroxidation present in lipid-rich foods. In egg yolk assay, ferrous ion was used as inducer. Lipid peroxide levels were

progressively suppressed by the addition of increasing amounts of the positive control which is catechin, ascorbic acid, quercetine dihydrate and BHA. Figure 4.3 showed that 50% inhibition of lipid peroxidation by BHA, quercetin dihydrate and ascorbic acid were at 0.085mg/ml, 0.07mg/ml and 0.10mg/ml respectively. The effect of antioxidant concentration on autoxidation rates depends on the antioxidant structure, nature of sample being oxidized and oxidation conditions.

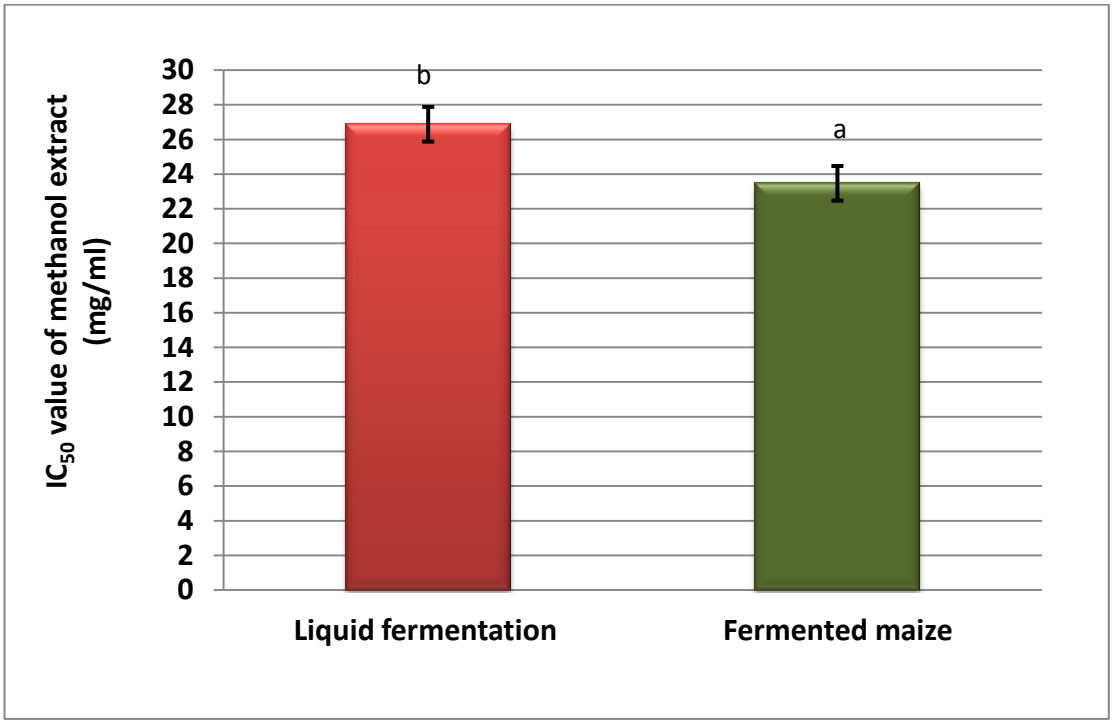


Figure 4.2: IC_{50} values (mg/ml) of methanolic extracts of *Lentinus squarrosulus* grown in liquid GYMP media and maize supplemented with 0.04% peptone using DPPH radical scavenging assay. Means with different alphabet within the bars are significantly different (ANOVA, P<0.05).

In egg yolk assay of inhibition of lipid peroxidation, the *L. squarrosulus* grown in liquid GYMP, unfermented and fermented maize extracts showed a rapid inhibitory activity at low concentrations. The results also showed that the lipid peroxide level was not suppressed by the addition of increasing amounts of extracts and this showed the lipid peroxidation activity was not dose dependent. At maximum inhibition of lipid peroxidation, the *L. squarrosulus* fermented maize extract exhibited the best inhibition

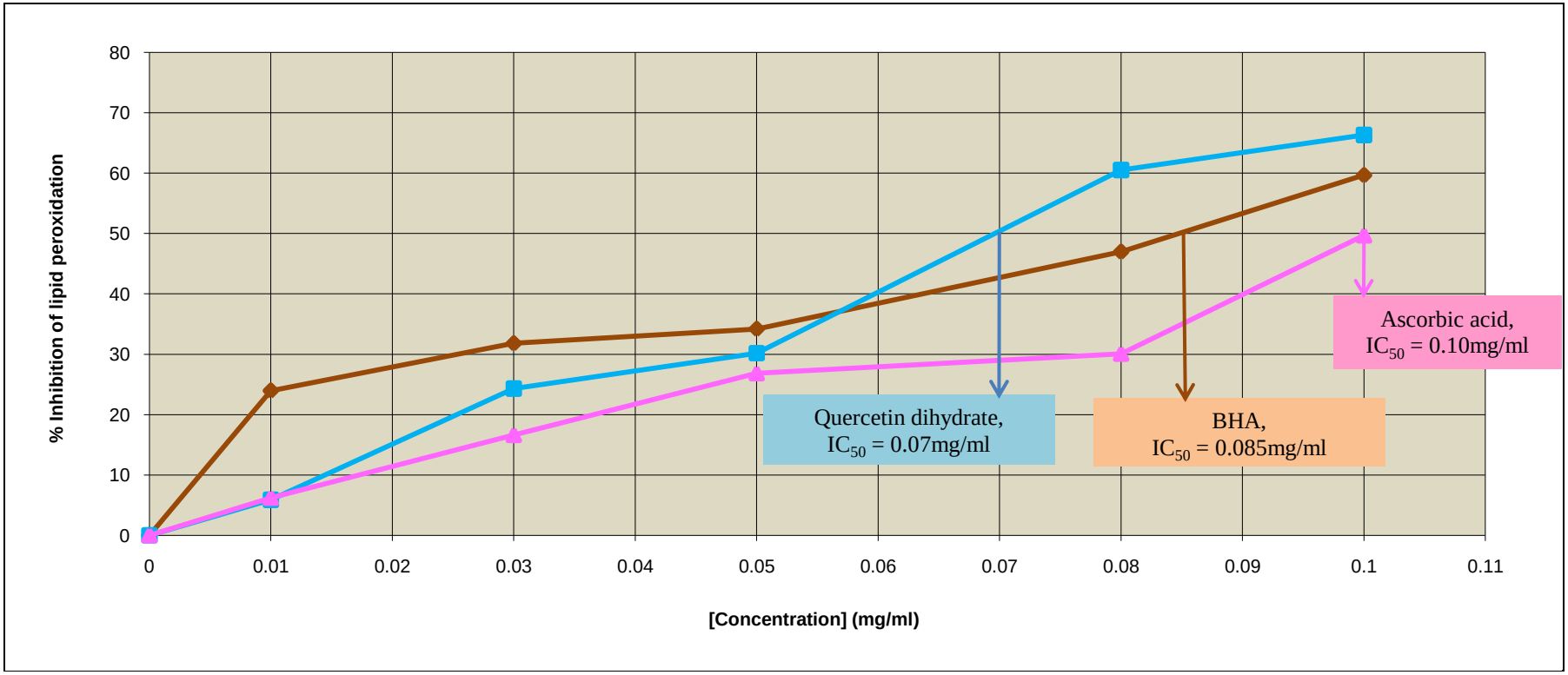


Figure 4.3: Comparison of inhibition of lipid peroxidation by ascorbic acid, quercetin dihydrate and BHA (n=3).

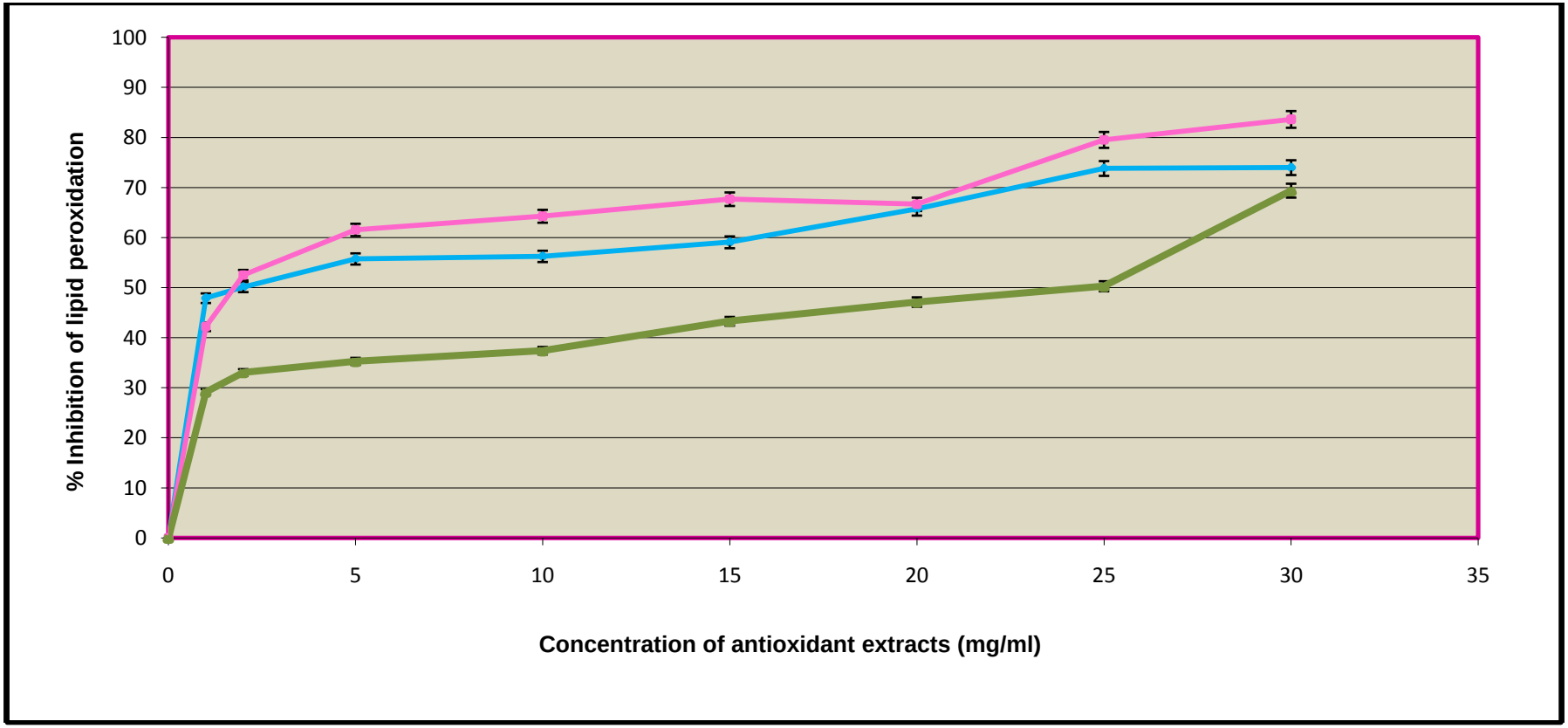
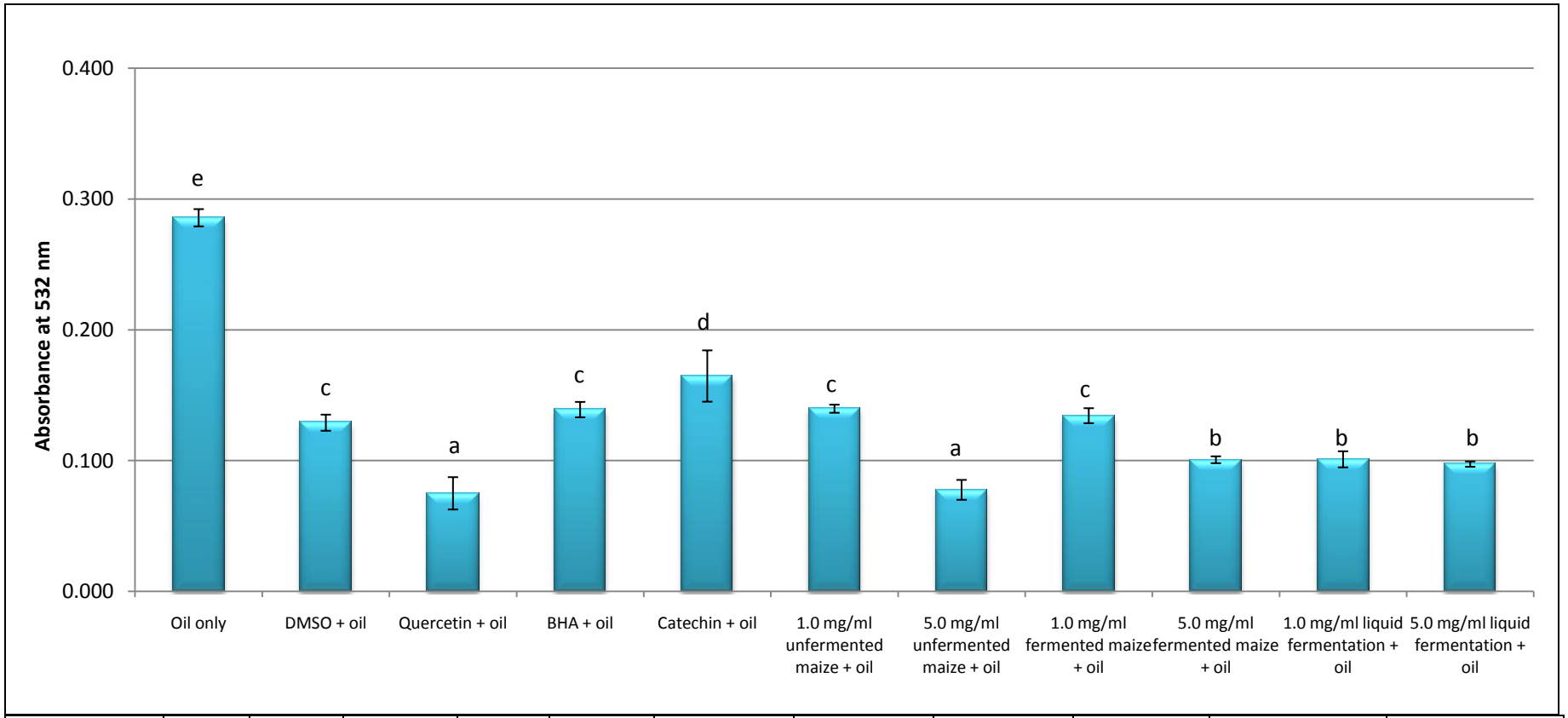


Figure 4.4: Comparison of inhibition of lipid peroxidation activity by methanolic extract from *L. squarrosulus* mycelia grown in liquid GYMP media [ ], *L.squarrosulus* fermented maize supplemented with 0.04% peptone [ ], and unfermented maize [ ] using buffered egg yolk (n=3)

activity at concentration of 0.63mg/ml of 30% inhibition compared to liquid fermentation and unfermented maize extracts with 0.71mg/ml (of 30% inhibition) and 1.03mg/ml of (30% inhibition) respectively (Figure 4.4).

The inhibition of lipid peroxidation was carried out using oil and was compared with quercetin dihydrate, catechin and BHA as positive controls to determine the inhibition of lipid peroxidation in oil. When oil was heated, it induced oxidation to form large amount of polymers such as lipid hydroperoxide, primary products of autoxidation, alcohols, aldehydes, ketones and hydrocarbons. In this study, heated unsupplemented cooking oil showed the highest absorbance value (0.286nm) with the pink supernatant formed in the reaction tube. This was taken as a control for malondialdehyde (MDA) formation in the thiobarbituric acid assay. Based on Figure 4.5, the heated oil supplemented with quercetin dihydrate exihibited the lowest absorbance value compared to catechin and BHA with 0.075nm, 0.139nm and 0.16nm respectively. These indicate that, quercetin dihydrate was very effective in protecting cooking oil from lipid peroxidation activity with 73.8% inhibition than catechin (42.4%) and BHA (51.4%). At concentration 1.0mg/ml, the heated oil supplemented with liquid fermentation of *L. squarrosulus* showed a better activity in inhibiting lipid peroxidation compared to unfermented and fermented maize extract with the percentage 64.7%, 51.2% and 53.0% respectively. However, when the concentration was increased to 5.0 mg/ml the unfermented maize showed the best inhibition activity with 72.8% followed by liquid fermentation (66.0%) and fermented maize (64.8%). The inhibition of lipid peroxidation of unfermented maize was comparable with quercetin dihydrate. On the other hand, at concentration 5.0mg/ml, heated oil supplemented with fermented maize extract, liquid fermentation of *L. squarrosulus* extract and unfermented maize extract



Percentage of Inhibition (%)	Oil only	DMSO + oil	Quercetin + oil	BHA + oil	Catechin + oil	1.0mg/ml unfermented maize + oil	5.0mg/ml unfermented maize + oil	1.0mg/ml fermented maize + oil	5.0mg/ml fermented maize + oil	1.0mg/ml liquid fermentation + oil	5.0mg/ml liquid fermentation + oil
	0.0	54.9	73.8	51.4	42.4	51.2	72.8	53.0	64.8	64.7	66.0

Figure 4.5: Absorbance values for different concentrations of unfermented, fermented maize and liquid fermentation of *L. squarrosulus* antioxidant extracts to demonstrate the inhibition of lipid peroxidation activity in cooking oil. The mean values with the same letter are significant at P<0.05. The statistical analysis was presented in Appendix F.

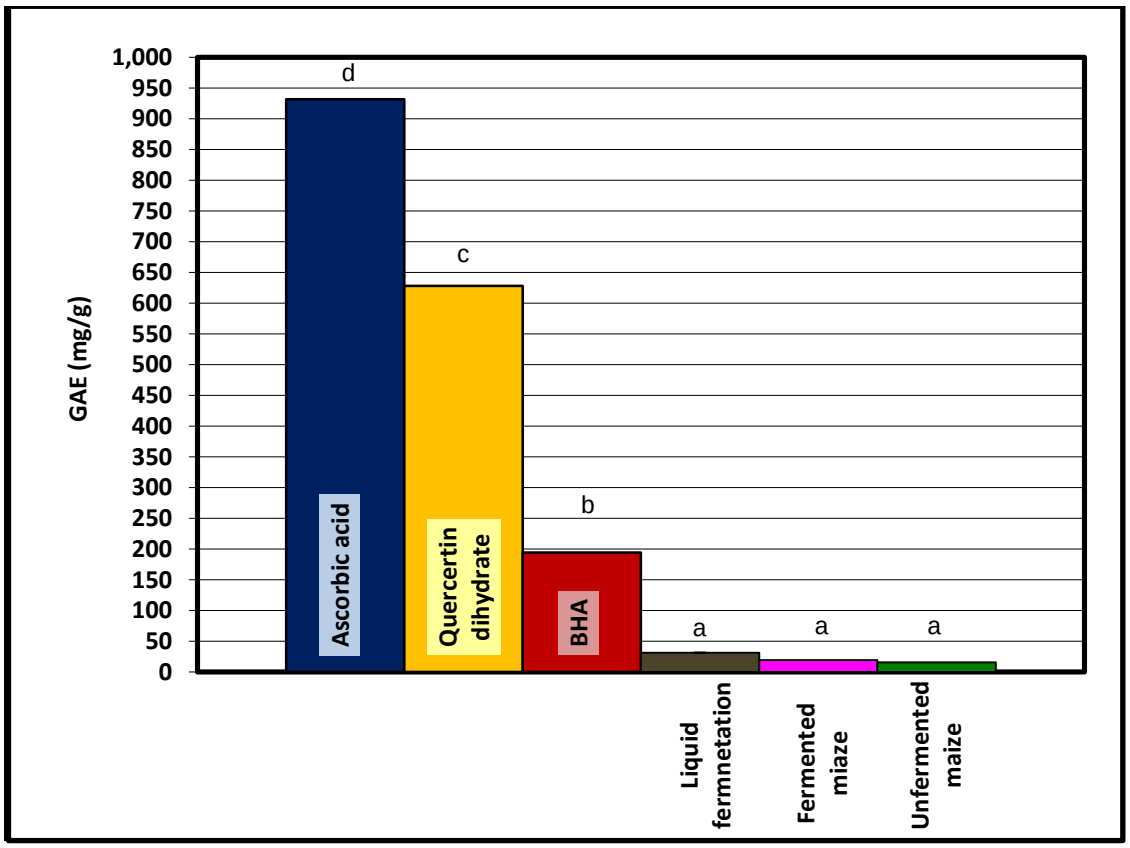


Figure 4.6: Total phenolic content (TPC) values for antioxidant extracts and controls (ascorbic acid, quercetin dihydrate and BHA). Means with different alphabet letters within the bars are significantly different where one way ANOVA showed P<0.05 (Appendix G).

exhibited the best inhibitor of lipid peroxidation than BHA and catechin. As a conclusion, this experiment showed the ability of *L. squarrosulus* antioxidant extract in protecting the oil from lipid peroxidation upon heating.

4.4 Total phenolic content (TPC)

The TPC values of extracts were determined by folin-ciocalteu's method. A standard linear calibration curve of gallic acid which served as a reference was constructed and the equation used was y = 0.0273x (R^2 = 0.997), where y is absorbance at 750 nm and x is concentration of gallic acid in mg/g dried extract (Figure 4.6). Among all the extracts, phenolic compound from *L. squarrosulus* grown on liquid GYMP media had the highest

TPC values with 31.39mgGAE/g extract, while fermented maize extract exhibited 19.34mgGAE/g extract. The unfermented maize showed the lowest phenolic content value of 15.40mgGAE/g extract (Figure 4.6).

Phenolic compounds in food have a significant contribution to the total antioxidant activity and the addition of antioxidants to food is an effective way to prevent the development of various off-flavours and undesirable compounds resulting from lipid oxidation. By referring to Figure 4.6, all the controls showed high phenolic content, the highest exhibited by ascorbic acid (931.86mgGAE/g ascorbic acid) followed by quercetin dihydrate (628.04mgGAE/g quercetin dihydrate) and BHA (194.24mgGAE/g BHA). This corresponds to the high antioxidant activity exhibited.