Journal of Pharmaceutical Biology

www.jpbjournal.com

e-ISSN - 2249-7560
Print ISSN - 2249-7579

POTENTIAL ANTI-VIRAL ACTIVITIES (%) OF THREE ORGANIC SOLVENT FRACTIONS OF WILD *GANODERMA LUCIDUM* (HIGHER BASIDIOMYCETE) EXTRACT AGAINST INFECTIOUS BURSAL DISEASE (IBD)

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ABSTRACT

The anti-viral activity of different organic fractions of *Ganoderma lucidum* extract against infectious bursal disease (IBD) virus was calculated by relative optical density absorbance method. Fractions of methanol, ethyl acetate and n-butanol of the mushroom were tested against non neuraminidase producing IBD virus as antigen using fetuin as substrate. The antigen was serially diluted to 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512 and 1:1024 (converted to Log_{10}- 3.2, 1.8, 1.3, 1.2, 1.1, 1.0, 1.0,1.0, 1.0, 1.0) and used the optical density absorbance was measured using spectrometer. Calculated viral inhibitory activity of the methanolic soluble fraction of *Ganoderma lucidum* extract was 1.6 %, 3.5 %, 1.8 % and 1.4 % at viral concentrations (log_{10}) of 1.3, 1.0, 1.0 and 1.0 respectively. Ethyl acetate soluble fraction of the extract showed inhibitory activity of 2.9 %, 2.3 %, 16.6 % and 17.6 % at viral concentration (log_{10}) of 1.3, 1.0, 1.0 and 1.0. The n-butanol soluble fraction of the extract showed inhibitory activity against IBD virus antigen of 15.5 %, 8.5 %, 12.7 % and 3.9 % and 11.1 % at viral concentrations (log_{10}) of 3.2, 1.8, 1.0, 1.0 and 1.0 respectively. The finding indicates that n-butanol fraction of *Ganoderma lucidum* extract inhibits infectious bursal disease viral growth compared to methanol and ethyl acetate fractions and can be exploited in the management of infectious bursal disease infection in poultry.

Keywords: *Ganoderma lucidum*, Organic soluble fractions, Virus antigen, Infectious bursal disease.

INTRODUCTION

Infectious bursal disease (IBD, Infectious bursitis, Gumboro or Infectious avian nephrosis) is a contagious, highly pathogenic disease of poultry especially chickens [1]. The disease is caused by double stranded RNA (ds-RNA) birnavirus virus of the family Avibirnaviridae [2]. The virus replicates by infection of lymphoid cells of the bursa of fabricus as the main target organ [3]. Variants of IBDV are highly cytolytic and cause rapid bursal atropy (within 72 h p. i) with minimal inflammatory response [4].

The main emphasis for the control of IBDV is by vaccination of the dam in order to obtain chicken that possess passive immunity (which can be variable and unpredictable) for the first 4-5 weeks of life. Humoral immunity is the primary mechanism of the protective immune response [5]. Due to stability of IBDV in the environment, control through sanitation and isolation is not practical for commercial poultry production [6].

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Research have shown that some mushrooms exhibit antiviral [7,8] anti-bacterial [9] activities. One of such mushrooms is the wild growing *Ganoderma lucidum* (Reishi).

Medicinal properties of extracts from *Ganoderma lucidum* has been investigated against various disease conditions such as anti-tumor effect [10], anti-hypertensive activity [11] related to cholesterol lowering activity [12], anti-bacterial activity [13], hepatoprotective activity [14] anti-human immunodeficiency virus (HIV) activity [15] and anti-inflammatory potentials through regulating histamine-mediating allergies [16].

Recent studies by Shamaki et *al* [17] and Bala *et al* [18] reported anti-viral activity of *Ganoderma lucidum* methanolic extract against avian influenza (H5N2) and Newcastle viruses through neuraminidase inhibition *in vitro*. However, Kilbourne *et al* (1968) suggested that there are several points in the viral life cycle that can be potentially inhibited to inhibit viral replication.

The activity of *Ganoderma lucidum* extracts against infectious bursal disease virus has not been ascertained, hence, the need to evaluate the anti viral property of the inhibitory activity of different organic soluble fractions of *Ganoderma lucidum* extract against non neuraminidase producing IBDV.

MATERIAL AND METHOD

The anti-viral activity of various fractions of *Ganoderma lucidum* was determined *in vitro* using adapted methods described by Aymard-Henry *et al* [19]. Infectious bursal disease virus vaccine was used as viral antigen with fetuin as a substrate.

Preparation of reagents for neuraminidase assay

Preparation of phosphate buffer

Two solutions of (a) 14.33 g of disodium hydrogen orthophosphate dissolved in 500 ml of distilled water to produce 0.4M concentration and (b) 6.24 g of sodium dihydrogen orthophosphate was dissolved in 500 ml of distilled water to produce 0.4M concentration. One hundred milliliter (100 ml) of buffer was prepared from these two solutions by mixing 19 ml of solution (a) and 81 ml of solution (b) to give 0.4M buffer, and the pH was then adjusted to 5.9. To prepare 6 mM of calcium chloride dihydrate solution, 0.088 g of Calcium chloride (Fisons Scientific apparatus, Loughborough, Essex, England) was dissolved in 100 ml of distilled water to give a final concentration of 6 mM [20].

Preparation of fetuin

Twelve gram (12 g) of fetuin powder was dissolved in 250 ml distilled water to give a concentration of 48 g/L. A 1:2 dilution of this fetuin and phosphate buffer containing 6 Mm Ca^{2+} was prepared and kept for use.

Preparation of periodate reagent

This solution was prepared by dissolving 4.28 g of sodium periodate ($NaIO_4$) in 38 ml of distilled water. Sixty two milliliter (62 ml) of orthophosphoric acid was added and the solution (100 ml) was mixed well and stored in a glass-capped brown bottle.

Preparation of arsenite reagent

This was prepared by dissolving 10 g of sodium arsenite ($NaAsO_2$) and 7.1 g of anhydrous sodium sulphate in 100 ml of distilled water, and 0.3 ml of concentrated sulphuric acid was then added and mixed.

Preparation of thiobarbituric acid reagent

Thiobarbituric acid reagent was prepared by dissolving 1.2 g of thiobarbituric acid and 14.2 g of anhydrous sodium sulphate in 200 ml of distilled water by heating in a boiling water bath at 100^0C for 2 minutes.

Preparation of butanol solution

Butanol solution was prepared by adding 5 ml of concentrated hydrochloric acid to 100 ml of butanol.

Antigens used for anti-IBD test

Infectious bursal disease vaccine (IBDV) was obtained from National Veterinary Research Institute Vom, Plateau State Nigeria. The antigen was used as source of neuraminidase in the test against the various extract fractions of *Ganoderma lucidum*.

Preparation of the Extract for anti viral test

Each of the extracts was weighed (0.2 g) and reconstituted with 10 ml of distilled water. This was then used to prepare 200 mg/ml concentration, which was then used for the study.

METHOD

Efficacy studies of fractions of the extract against IBD activity

This was carried out by enzyme assay method as described by Aymard-Henry et al [20]. The test is recommended by WHO influenza laboratory centers worldwide. This assay of anti IBD activity is a control group in the study of neuraminidase activity based on the release of N-acetyl neuraminic acids from the fetuin substrate by the action of neuraminidase (not produced by IBD virus).

Assay of anti-viral activity against IBD antigen

A two-fold serial dilution of the virus antigen in 0.15 ml of normal saline were prepared and placed in seven (7) test tubes, while two test tubes were maintained as blank controls. Virus antigen dilutions of 0.05 ml were placed into the seven test tubes and 0.05 ml of normal saline, and 0.1 ml of reconstituted fetuin was added, these was mixed well and allowed to stand at 37^0C for 18 hours.

The tubes with contents were then cooled to 20^0C. Periodate solution (0.1 ml) was then added to all the test tubes including the blanks. The test tubes were thoroughly mixed using a electric rotatory mixer (IKA-VIBRO-FIX. Germany) and allowed to stand at 20^0C for 20 minutes. Arsenite reagent solution (1.0 ml) was added to each test tube, and a brown colour was observed. The colour disappears after shaking the test tubes. Thiobarbituric acid solution (2.5 ml) and 0.5 ml of the reconstituted extract (200 mg/ml) was then added to each test tube, they were thoroughly mixed and the test tubes were immediately placed in a boiling water bath for 15 minutes. It was then observed for colour change (red colour). The production of red colour indicated neuraminidase activity, while absence of the red colour production indicates inhibition of neuraminidase activity by the extract. The test tubes were then cooled to room temperature in ice water and 4.0 ml of prepared butanol was added to each test tube. It was then mixed using electric mixer and placed in a centrifuge (Universal 32, Hettuch zentrifugen, Germany) for 5 minutes at 1000 G. Using a single channel pipette (Dragon Med, 100- 1000 µl. China), the upper (butanol) phase was pipetted into a corvette and absorbance was read at 549 nm against a fetuin blank using digital spectrophotometer (Acurex Diagnostics, Dba, Fort Meigs Rd. USA).

RESULTS

In vitro anti-viral activity of the three organic solvent fractions of *Ganoderma lucidum* extract against IBD virus antigen is presented in Table 1. The result showed that methanolic extract have inhibitory activity of 1.6 %, 3.5 %1.8 % and 1.4 % while ethyl acetate fraction have inhibitory activity of 2.9 %, 2.3 %, 16.6 % and 17.6 %. No inhibition was observed in the rest of the viral dilutions.

Table 1. *In vitro* anti-viral activity of three organic fraction of wild *Ganoderma sp*. extract against infectious bursal disease virus (IBD)

		Absorbance			
Extract fraction	**Virus dilution (log_{10})**	**Virus+ fetuin**	**Virus+ extract +fetuin**	**Anti-neuraminidase activity (%)**	**Remark**
Methanol	3.2	0.510	- 0.010	-1.9	NI
	1.8	0.804	- 0.075	-9.3	NI
	1.3	0.809	0.013	1.6	I
	1.2	0.854	- 0.058	-6.8	NI
	1.1	0.902	- 0.070	-7.8	NI
	1.0	0.846	- 0.083	-9.8	NI
	1:0	0.840	0.029	3.5	I
	1:0	0.886	-0.090	-10.2	NI
	1:0	0.609	0.011	1.8	I
	1:0	0.650	0.009	1.4	I
Ethylacetate	3.2	0.519	- 0.058	-11.2	NI
	1.8	0.608	- 0.058	-9.5	NI
	1.3	0.804	0.023	2.9	I
	1.2	0.818	- 0.025	-3.1	NI
	1.1	0.848	- 0.035	-4.1	NI
	1:0	0.609	- 0.042	-5.9	NI
	1:0	0.620	0.014	2.3	I
	1:0	0.619	- 0.105	-16.9	NI
	1:0	0.625	0.0104	16.6	I
	1:0	0.623	0.110	17.6	I
n-butanol	3.2	0.490	0.076	15.5	I
	1:8	0.810	0.069	8.5	I
	1:3	0.856	0.075	-8.8	NI
	1:2	0.908	-0.068	-7.0	NI
	1:1	0.902	- 0.072	-8.0	NI
	1:0	0.804	- 0.051	-6.0	NI
	1:0	0.612	- 0.072	-11.8	NI
	1:0	0.620	0.079	12.7	I
	1:0	0.619	0.024	3.9	I
	1:0	0.628	0.070	11.1	I

I= inhibition, NI= no inhibition

DISCUSSION

The three organic soluble fractions of *Ganoderma lucidum* extract under study showed *in vitro* antiviral activity against infectious bursal disease (IBDV). This finding agrees with that of Shamaki *et al* [21] that reported anti-viral activity of methanolic extract fraction of *Ganoderma lucidum* while Hu *et al* [22] also reported that different anti-viral agent and other agents from Chinese Traditional Medicine possess anti influenza property. This antiviral activity could be due to low pH levels of the extracts. Shamaki *et al* agreeing with findings of Stachke *et al* [23] and Russell *et al* [24] who reported that substances of small molecular mass have effect on viral replication. All the three extract fractions of *Ganoderma lucidum* extract tested low pH levels with methanol 5.78, ethylacetate, 3.26 and n-butanol 3.84, the anti-viral activity appears more with the n-butanol soluble fraction then ethylacetate and methanolic fractions. This could be due to the difference in pH agreeing with finding of Benton *et al* [6] who reported that infectious bursal disease virus inactivated at pH 12.0 and unaffected at pH 2.0 at temperatures of 30^0C for 1 h and other organic solvents. The difference in pH may have altered the polarities of the extracts. This difference in pH polarity of the extracts could possibly explain the difference in inhibitory activity of the different fractions of the *Ganoderma lucidum* extracts with the methanol fraction exhibiting less inhibitory activity than the n-butanolic and ethylacetate fractions as shown by reduced optical density absorbance. The anti-viral activity of this mushroom can also be attributed to detectable quantities of flavonoids and terpenoids present. These compounds are reported to possess anti-viral activities [25]. Detectable quantities of flavonoids and terpenoids were found in n-butanol soluble fractions of the *Ganoderma lucidum* extract and a combination of which may have accounted for high percentages of inhibitory activity observed in this fraction compared to that of ethylacetate fraction and methanol. The extracts may have effect on the IBDV structural protein VP4, a non structural polypeptide (Nagey *et al*., 1987) or its precursor VP2a (Fahey *et al*., 1989) which may possibly induce production of virus neutralizing antibodies that may passively protect chicken. All the three extracts have high anti-viral activity with increase in dilution, indicating that the effect of the extract is directly proportional to viral concentration.

CONCLUSION

Variations in the degree of calculated activity observed in infectious bursal disease virus (IBDV) by the three extracts appeared to be mediated by effect of acidic pH of the extracts of *Ganoderma lucidum*, and possibly the presence of detectable quantities of flavonoids and terpenoids that are reported to have antiviral effects.

ACKNOWLEDGEMENT

We express our deep appreciation to the management of the University of Maiduguri, Nigeria for sponsoring this work. We equally appreciate the laboratory assistance and space provided for this work by the staff of Chemical Pathology unit of the University of Maiduguri Teaching Hospital (UMTH).

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