Mysore J. Agric. Sci., 51 (2) : 359-365, 2017

Screening of Wild Mushrooms for Antiviral Property and Their Molecular Characterization

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Abstarct

Five wild mushrooms were collected from Nettana village of Dakshina Kannada district of Karnataka, India. One of the mushroom was identified as *Ganoderma lucidium*based on its phenotypic characters and the other four mushrooms were identified as *Lentinus* sp. *Pleurotus djamour, Pluteus cervinus*and *Micromphale foetidum*by Internal Transcribed Spacer (ITS) sequence homology. Crude extract (1:1 w/v) was made from these mushrooms in sterile distilled water and screened for antiviral property against *Bovine herpes virus-1*(BoHV-1) and *Morbili virus* grown on madin derby bovine kidney cells (MDBK cells) and Vero-cells respectively. The crude extract of the mushrooms except *P. djamour* showed cytotoxic effect on the host cells before dilutions. The diluted extract of *G. lucidium* and *Lentinus* sp. at 10^{-6} and 10^{-4} dilutions respectively inhibited both the BoHV-1 and *Morbilli viruses* without affecting host cells. However, *P. djamour, P. cervinus* and *M. foetidum* did not affect the viruses and found non cytotoxic once after dilutions were made. This study reveals that the *G. lucidum* and *Lentinus* sp. as potential antiviral mushrooms.

Keywords: Mushroom extract, antiviral property, *bovine herpes virus-1*, *morbilivirus*

Many mushrooms were described under traditional medicine to set right the disorders and to cure the diseases. Mushroom extracts were reported to have antibacterial, hematological, antiviral, antitumor, hypotensive, antioxidental, anticancerous and hepatoprotective effects (Barros *et al*., 2007). The isolated active compounds appeared to play a direct role by acting as inhibitors of viral enzymes, synthesis of viral nucleic acids or absorption and uptake of viruses into cells. The direct antiviral effects were exhibited in particular by small molecules and the indirect antiviral effects (Santoyo *et al*., 2012)

Herpes viruses contain DNA as their genetic material and are important pathogens in humans as well as animals. *Bovine herpes virus-1* (BoHV-1) is an important pathogen of cattle causing significant economic losses to the livestock industry worldwide. The virus has been associated with a variety of clinical disease manifestations including rhinotracheitis, vulvovaginitis, balanoposthitis, abortions, conjunctivitis, neurological disorders and generalized systemic infections (Benoit *et al*., 2007). Similarly, the *Morbilli virus* is an RNA virus belonging to the family *Paramyxovirdae* is a causative agent of highly fatal disease called Peste *des* petits ruminant's (PPR) in Sheep and Goats (Harish *et al.,* 2009). Both of these causes major diseases in livestock. Since there were no specific drugs to control the viral diseases directly so far in the modern medicine, exploitation of the plant and fungi resources is very much essential in *bioprospecting*.

Characterization of mushroom species requires basic knowledge on the structure of the fungi. The phenotypic characters used for identification of mushroom species are shape, size, texture, colour and odour of the fruiting body (Arora, 1986). However, in recent years, molecular tools well supported the mushroom taxonomy. Molecular markers, particularly DNA based techniques are quick and reliable to establish identities of wild mushrooms.Identification of mushroom species using ITS (Internal Transcribed Spacers) region sequence not only provides appropriate information to classify the organism up to species level but useful in identification of young fruit bodies. Several researchers have been reported characterization of fungi based on ITS sequence analysis. Oyetayo (2014) identified the *Trametes* species collected from Nigerian forest by using Internal Transcribed Spacer

(ITS) region of the rDNA. In the present study, five mushrooms were screened for antiviral property and characterized.

Material and Methods

Collection of wild mushrooms and preparation of mushroom extract: Mushrooms were collected in a paper cover bags during rainy seasonat forest, Nettana village area of Dakshina kannada district located in Western Ghats (WG) of Karnataka, India. Field characters, such as season, soil type, vegetation, *etc*., were recorded at the time of collection.These mushrooms were brought to the laboratory and designated as WG-1, WG-2, WG-3, WG-4 and WG-5 (Fig.1). One gram of fresh mushroom tissue was ground with one milliliter of sterile water and centrifuged at 10,000 rpm for 10 min. The supernatant was filtered through sterile micron syringe fixed with 0.45 µm membrane filter and stored in -20°C.

Collection of viruses:Two viruses *viz*., *Bovine herpes virus-1* (DNA virus) and *Morbili virus* (RNA virus) were collected from the Department of Virology, Institute of Animal Husbandry and Veterinary Biological, Hebbal, Bengaluru-560024, India. The experiment was conducted in Molecular Microbiology laboratory, Department of Agricultural Microbiology, UAS, GKVK, Bengaluru-560065, India.

Screening of mushroom extract for antiviral property against DNA virus (Bovine herpes virus-1): *Bovine herpes virus-1* adapted to grow in madin derby bovine kidney cells (MDBK) was used to study antiviral property of the mushroom extracts. The antiviral activity was analysed by virus neutralization test using mushroom extracts, in 96 well cell culture plates, as per the procedure described by Rovozzo and Burke (1974). Serial dilution of mushroom extract (1:10) was made in a sterile water. 0.5 ml from each dilution was dispensed in eight cryovials. In to this, 0.5 ml of diluted *Bovine herpes virus-1*was added and incubated for 1 hr at 37°C.After incubation, 200 µl of this neutralized mushroom extract-virus mixture was added to the corresponding well of 24 hrs grown MDBK cells and incubated for one hour at 37°C for viral adsorption. Then these cultures were added with 200 µl of Dulbecco's modified eagle's medium (DMEM) maintenance media at 37°C and five per cent CO_2. The monolayers were observed for production of cytopathic changes at 24 hours intervals till the seventh day post of inoculation and the inhibition of virus was recorded.

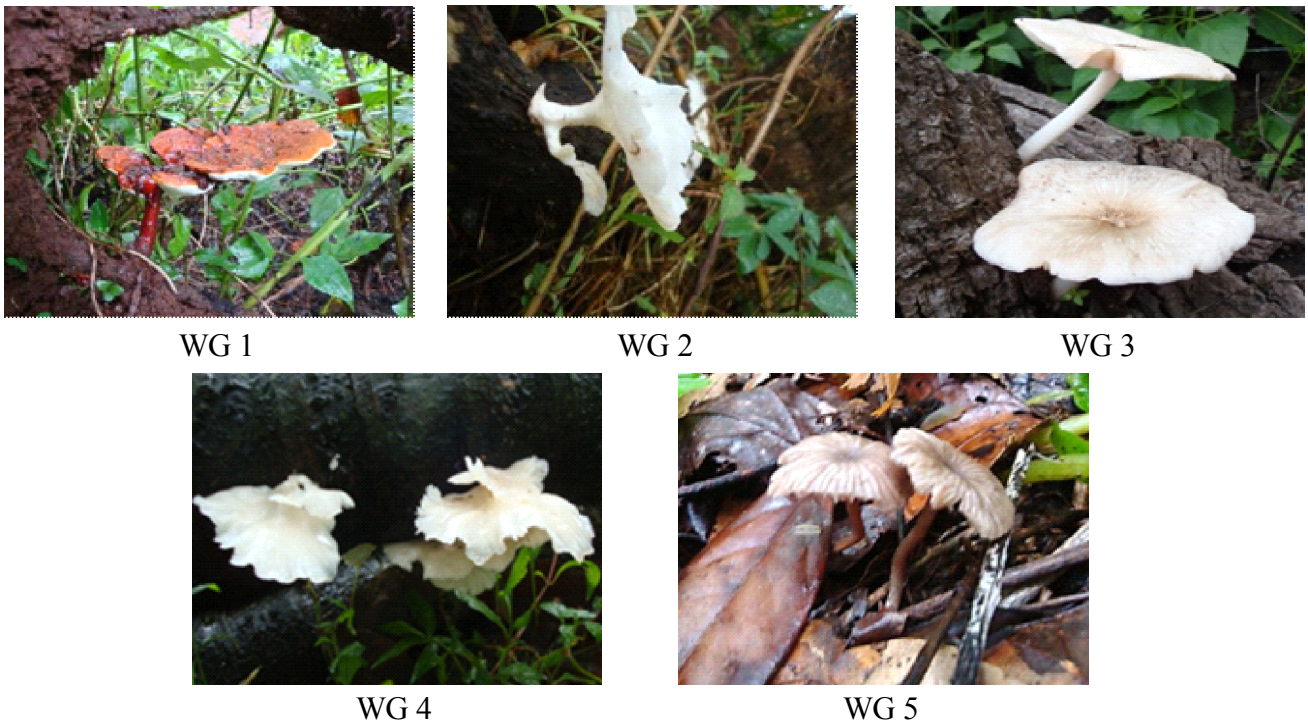


Fig 1: Wild mushrooms collected from nettana village of Dakshina kannada district located in Western Ghats of Karnataka

Screening of mushroom extracts for antiviral property against RNA virus (Peste des petits ruminants virus): The *Morbilli virus* adopted to grow in Vero cell (African green monkey kidney cell) line was used to study antiviral property of the two mushroom extracts.The antiviral property was determined using the same procedure mentioned above for*Bovine herpes virus-1* (Rovozzo and Burke, 1974).

Molecular identification: Total genomic DNA from the cap tissue of the mushroom fungus was extracted using CTAB lysis buffer. Then 0.2 gram of dried mushrooms sample was ground into fine powder using liquid nitrogen and sample was transferred into 2 ml of extraction buffer containing CTAB and incubated at 65°C for 30 minutes. After incubation the tubes were centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred into a fresh centrifuge tube and equal volume of chloroform and Iso-amyl alcohol (24:1 V/V) was added, it was mixed by inverting the tubes and centrifuged at 10,000 rpm for 10 minutes. The above step was repeated till no white interface is seen. Clear supernatant was collected and DNA was precipitated by adding 0.6 volumes of chilled Isopropanol and placed in -20ºC for 2 hours. After incubation the mixture was centrifuged and the pellet was washed with 70 per cent ethanol, further air dried, dissolved in Tris-EDTA (10:1) buffer and stored at -20°C.

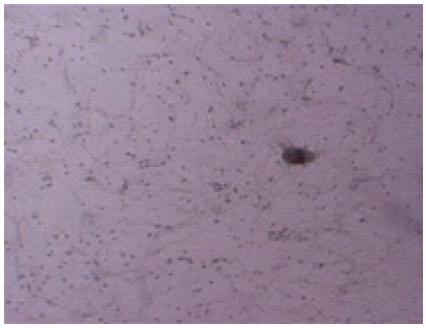
The DNA thus extracted was checked for purity using UV spectrophotometer at 260 / 280 nm wave length. Concentration was measured using nano drop (ependorff). Then 50-100 ng DNA was used for PCR amplification in a 20 µl reaction mixture containing 2.0 µl of 10 x PCR Taq. Buffer, 2.0 µl of 10 mM dNTP's mix, 1.0 µl of ITS primers, 0.3 µl of Taq. DNA polymerase, 1.0 µl of Template DNA, 13.7 µl of Sterile distilled water in Sterile PCR tubes. ITS-1 (5' TCCGTAGGTGAACCTGCGG 3') as a forward primer and ITS-4 (5' TCCTCCGCTTATTGATA TGC 3') as a reverse primer were used as a primer. The reaction was carried out in a Thermal Cycler (Applied biosystems).

The PCR programme was standardized with initial denaturation at 96°C for 4 min, 40 cycles of denaturation of 94°C for 1 min, annealing at 60°C for 30 seconds and extension at 72°C for one min and final extension at 72°C for 10 min. The amplified products were separated by agarose gel electrophoresis. The gel was visualized under UV light and documented using Alpha innotech Gel documentation unit. The visualized band was excised and purified by using the Gene Jet™ Gel Extraction Kit. The DNA thus eluted was sequenced by Sci Genom Labs Private Ltd. Kerala, India using ITS-1 forward and ITS-4 reverse primers. The sequence homology was searchedat NCBI GenBank (*http// www.Ncbi.nlm.nih.gov/BLAST/*).

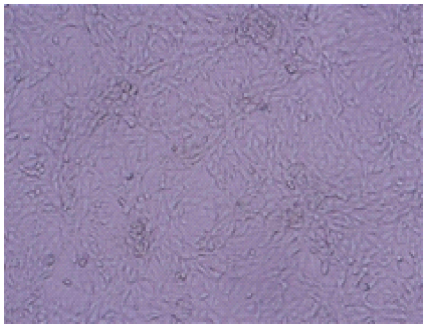
Screening of mushroom extracts for antiviral property against DNA virus (Bovine herpes virus-1) and RNA virus (Peste des petits ruminants virus or Morbili virus): *Bovine herpes virus-1* (BoHV-1) is an important pathogen of cattle causing significant economic losses to the livestock industry worldwide. This virus causes variety of diseases such as neurological disorders and generalized systemic infections in livestock. Similarly, the *Morbilli virus* causes highly fatal disease called Peste *des* petits ruminant's (PPR) in Sheep and Goats. Hence both the viruses contribute to major economic losses in livestock industry. Therefore, present study is focused on the identification of potential antiviral mushroom against both the viruses. Screening of antiviral properties of mushrooms against BoHV-1 (DNA virus) andPeste *des* petits ruminants virus (RNA virus) was done by using extracts of *G. lucidium, Lentinus* sp. *P. djamour, P. cervinus*and *M. foetidum* mushrooms by using 96 well cell culture plates containing MDBK and Vero cells separately. The observations were recorded up to seven days.

Resul'ts and Discussion

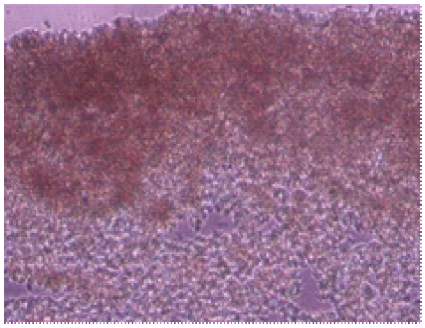
The *Ganoderma lucidium* and *Lentinus* sp.inhibited the viral activity of *Bovine herpes virus-1* (DNA virus) in MDBK cell culture plate at 10^{-6} and 10^{-4} dilution, respectively and *Morbilli virus* (RNA virus) in Vero cell culture plate at 10^{-6} and 10^{-4} dilution, respectively. Both the mushrooms exhibited the potential antiviral properties at 10^{-6} and 10^{-4} dilutions respectively. Mushroom extracts of *G. lucidium* and *Lentinus* sp. up to dilutions of 10^{-5} and 10^{-3} affected the cell growth respectively. But dilutions above 10^{-6} of *G. lucidium* and above 10^{-4} of *Lentinus* sp., did not



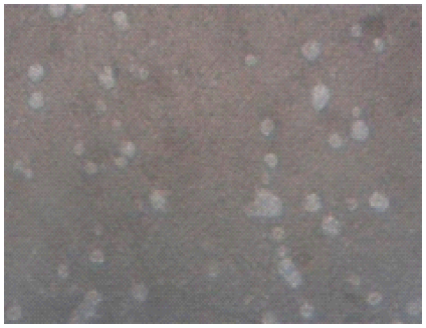
Ganoderma lucidium Mushrooms extract alone on MDBK cells



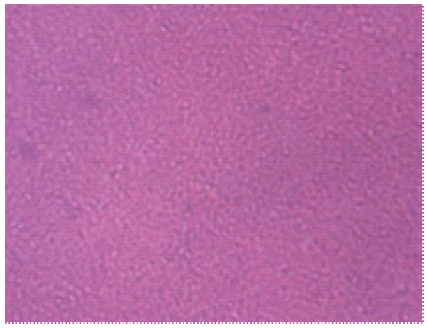
Ganoderma lucidium extract and BoVH-1 on MDBK cells (Inhibition of viral activity at 10^{-6} dilution)



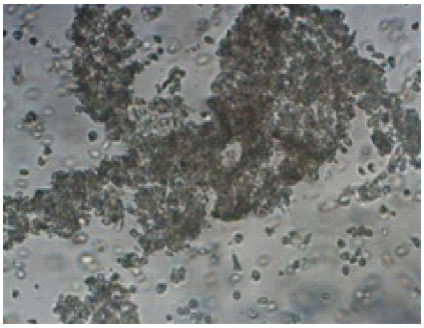
Cytopathic effect of virus control on MDBK cells



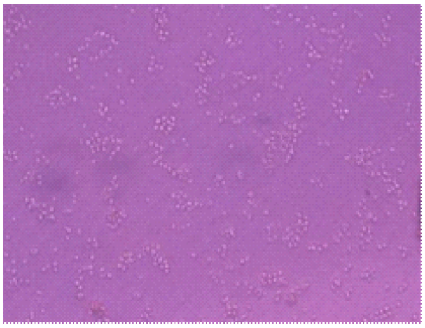
Lentinus sp.mushroom (WG 80) extract alone on MDBK cells



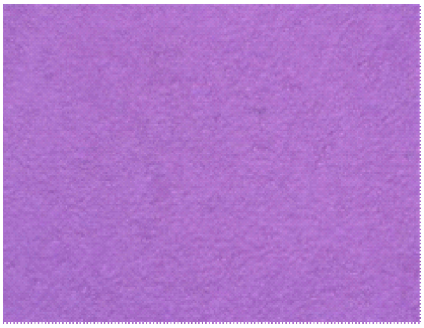
Lentinus sp.+ BoHV-1 on MDBK cells (at 10^{-4} dilution)



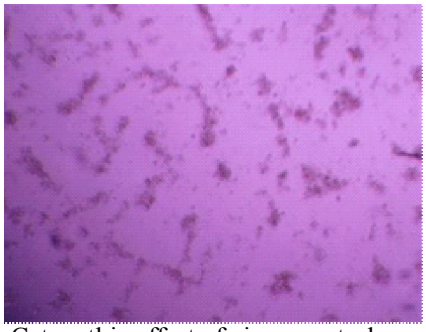
Cytopathic effect of Virus control on MDBK cells



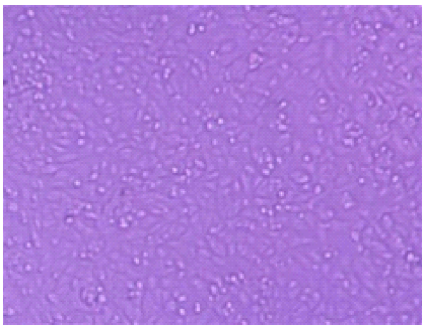
Ganoderma lucidium mushroom extract alone on Vero cells



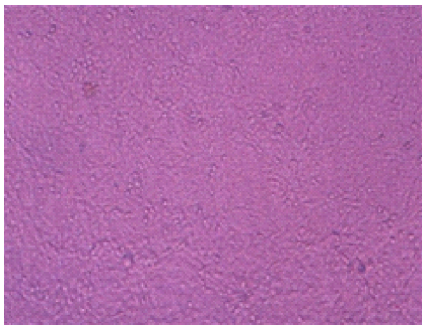
Ganoderma lucidium extract + *Morbili virus* on Vero cells (Inhibition of viral activity at 10^{-6} dilution)



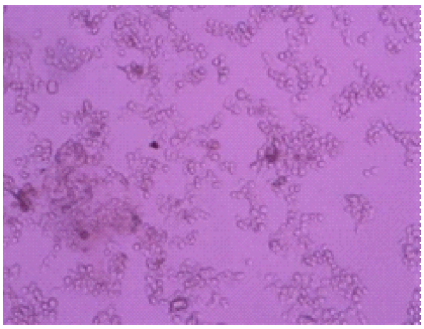
Cytopathic effect of virus control on Vero cells



Lentinus sp.mushrooms (WG 78) extract alone on Vero cells



Sample W 78 + *Morbilli virus* on Vero cells (Inhibition of viral activity10^{-4} dilution)



Cytopathic effect of Virus control on Vero cells

Fig 2: Mushrooms which are showing antiviral properties

CCTCTTCCGTAGGTGAACCTGCGGAAGGATCATTATCGAGTTTTGAAACGGGTTG
TAGCTGGCCTTCCGAGGCATGTGCACGCCCTGCTCATCCACTCTACACCTGTGCA
TTTACTGTGGGTTTCAGGAGCTTCGAAAGCGAGAAAGGGGCCTTCACGGGCTTTT
TCTTGCGTAGTTGTTACTGGGCCTACGTTTCACTACAAACACTTATAAAGTATCA
GAATGTGTATTGCGATGTAACGCATCTATATACACTTTCAGCAACGGATCTCTTG
GCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG
AATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGTCCTTGGTATTCCGAGG
AGCATGCCTGTTTGAGTCATGAAATTCTCAACCTAACGGGTTCTTAACGGGACTT
GCTTAGGCTTGGACTTGGAGGTTCTTGTCGGCTTGCTTCAATGTCAGGTCGGCTC
CTCTTAAATGCATTAGCTTGGTTCCTGTGCGGATCGGCTCACGGTGTGATAATTGT
CTACGCCGCGACCGTTGAAGCGTTTTATAGGCCAGCTTCTAGTCGTCTCTACGAG
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GCATATCAATAGGCGGGGGGAAAA

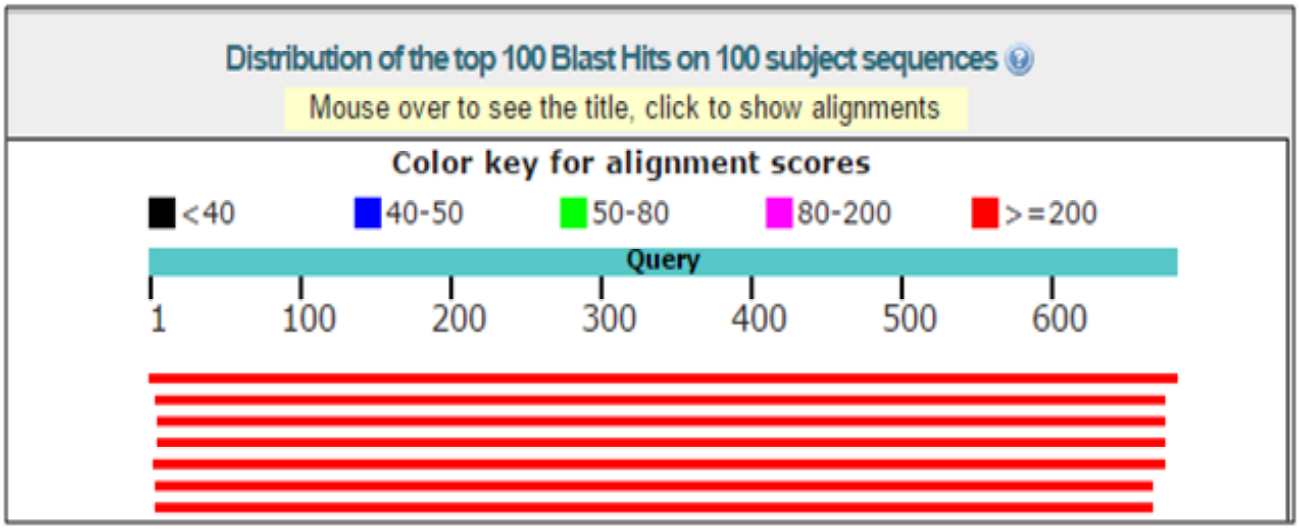

Fig 3: Full length sequence and homology search of Lentinus sp. (WG 2)

TABLE I

Field information and phenotypic character of mushrooms

Designated samples	Habitat	Character of Pileus		Character of Gills	Annuals	Character of Stipe	Name of identified mushrooms	Family
		Colour	Shape					
WG 1	Soil	Reddish brown	Kidney shape	Absent	Absent	Equal	*Ganoderma lucidum*	Ganodermataceae
WG 2	Wood	White	Uplifted	Absent	Absent	Tapering upwards	*Lentinus* sp.	Polyporaceae
WG 3	Wood	Creamy white	Round	Present	Absent	Tapering downwards	*Pluteus cervinus*	Pluteaceae
WG 4	Wood	White	Irregular	Present	Absent	Tapering downwards	*Pleurotus djamour*	Pleurotaceae
WG 5	Soil	Brown	Convex	Present	Absent	Tapering downward	*Micromphalefoetidum*	Marasmimaceae

have lytic effects on the cells and these dilutions when inoculated on the cells along with the virus, yielded effective antiviral effects as evidenced by absence of cytopathic changes by mainly inhibiting intracellular replication of BoHV-1 in MDBK cell culture and *Morbili virus* in Vero cell culture and they were also able to disrupt the virus adsorption step in MDBK (Fig. 2) and Vero cells (Fig. 2), respectively. In this study, *G. lucidum and Lentinus* sp. mushroom extracts might have interfered with the BoHV-1 and *Morbili virus* infection process at the initial infection steps perhaps by blocking virus attachment or adsorption to MDBK and Vero cells, respectively. This indicating that these species might be an interesting source of antiviral compounds. However, the extract of the *P. djmour* did not show any inhibitory effect neither on virus nor on the host cell. In contrast, the extracts of *P. cervinus*and *M. foetidum* have inhibitory effect on host cells but not on targeted viruses indicating that these mushrooms are not producing antiviral compounds. Similar results were obtained by Santoyo *et al.* (2012) by using aqueous extract and methonal extract of *Ganoderma lucidium* and *Lentinus edodes* against *Herpes simplex virus-1*. Crude dichloromethane, ethanol, water and polysaccharide extracts of *Ganoderma lucidum* all suppressed HPV 16 E6 (Lai *et al*., 2010).

Identification of mushrooms by phenotypic / ITS region sequence: Mushrooms are the objects of much curiosity, speculation since time immemorial and also one of the most important components of the ecosystem. Their edibility, poisonous nature psychotropic properties, medicinal properties draw the attention of the researchers. The WG-1 was identified by its phenotypic characterswhile consulting the book *Mushroom Demystified* (Arora, 1986). The pileus of this mushroom was reddish brown in colour, kidney shaped and the border surrounded by white tissue. Stipe was brown in colour, off centric and tapered upward. Texture of the fruiting body was corky and tough. Thus, the mushroom was identified as *Ganoderma lucidum* (Table I). Identification of mushrooms through morphological characters in the field is advantageous for mushroom collection. Similarly Meera and Veena, (2012) identified 45 species of mushrooms from Kodagu district of Western ghats by studying their phenotypic characters.

The other four mushrooms were identified by ITS region sequence homology available at NCBI Gen Bank. The ITS sequence of the mushroom designated as WG-2 (684bp) showed 99 per cent homology with *Lentinus* sp., (Fig.3) WG-3 (622bp) showed 99 per cent sequence homology with *Pluteus cervinus*, WG-4 (683bp) showed 99 per cent sequence homology with *Pleurotus djamour* and WG-5 (715bp) showed 97 per cent sequence homology with *Micromphale foetidum.* Molecular identification has precise and avoid confusion while speciation. Therefore, in this study, four mushrooms were identified by ITS region sequence. Ranjaratnam and Thiagarajan (2012) identified *Perenniporia* sp. by amplification of ITS region and aligned by using Jukes-Cantor Corrected Distance model. Similarly, eighteen species

of Termitomyces collected from Ondo and Ekiti States of Nigeria were identified using ITS region of rDNA (Oyetayo, 2012).

This study revealed antimicrobial property of two mushrooms on two bovine viruses *viz*., DNA and RNA viruses. The other three mushrooms did not possess the antimicrobial propertybut characterized for reporting. However, further study required to isolate and identify the antiviral compound.

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(*Received :* May, 2017 *Accepted :* June, 2017**)**