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ANTIVIRAL ACTIVITY OF PLANT EXTRACTS AGAINST SAC BROOD VIRUS *IN VITRO* - A PRELIMINARY REPORT

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ABSTRACT

This study aims at determining the antiviral effects of herbal extracts on the lethal Sacbrood virus of honeybees. The Sacbrood viral suspension was inoculated on BHK-21 cell line and the cytopathic effects were determined (using cell proliferation assay) after introduction of herbal extracts of *Ficus bengalensis* (Vad), *Ficus religiosa* (Pimpal), *Jasminum auriculatur* (Jaai), *Acacia catechu* (Khair), *Azadirachta indica* (Neem), *Curcuma longa* (Turmeric) and *Withania somnifera* (Ashwagandha) in suitable concentrations. It was observed in this preliminary study that pimpal root, ashwagandha root, jasmine, neem barks are effective herbal extracts against sac brood virus.

INTRODUCTION

Honey bees play a vital role in the environment by pollinating both wild flowers and many agricultural crops as they forage for nectar and pollen, in addition to producing honey and beeswax. The essential and valuable activities of bees depend upon beekeepers maintaining a healthy population of honey bees, because like other insects and livestock, honeybees are subject to many diseases and pests. The useful apiculture products are honey, wax, pollen and bee bread, propolis, royal jelly, bee venom, and application of honey bees in pollination. The inclusion of "natural" bee product in cosmetics, medicines and foods has improved consumer appeal and with the increasing multitude of possible uses for bee products, beekeepers can diversify their activities and generate greater income.

Sacbrood is a condition affecting the brood of the honeybee, resulting in larval death. Larvae with Sacbrood fail to pupate and ecdysial fluid, rich in SacBrood Virus (SBV), accumulates beneath their unshed flaccid skin and the body watery forming the sac for which the condition is named. Infected larvae change in color from pearly white to pale yellow and shortly after death they dry out forming a dark brown gondola shaped scale (Bailey L. 1975). Symptoms of sacbrood are partially uncapped cells scattered about the frame or capped cells that remain sealed after others have emerged. Diseased individual inside cells will have characteristically darkened heads which curl upward. The dead prepupa resembles a slipper inside the cell. The dark brown individual becomes a wrinkled, brittle scale that is easily removed from the cells.

Brood combs show some degree of irregularity in their pattern. Dead brood will be found scattered among healthy brood and the cappings may be discolored, sunken, perforated or removed by the bees. Larvae almost always die after the cell has been capped. Nurse bees transmit the virus when they feed larvae with brood food from their hypophareangeal glands. The virus may survive up to four weeks in larval remains or in honey or pollen. Sacbrood occurs most frequently in spring when the colony is growing most rapidly and large numbers of susceptible larvae and young adults are available (Bailey L. 1969). SBV is one of many insect viruses generally referred to as picornavirus-like. This presumed similarity has been largely on biophysical properties and the presence of RNA genome (Moore et. al., 1985). SBV particles are 28 nm in diameter, nonenveloped, round, and featureless in appearance (Bailey L. 1968 and Brcak and Kralik 1965).

Furthermore, the consequences of virus infections are becoming more significant, since infections today have repercussions beyond their direct impact on honey production. Environmental pollution has dramatically reduced (or even eradicated) the populations of many insect species, and the role of bees as essential pollinators for plants has become paramount. Virus-induced population decrease among honeybees thus affects not only the bee-farming economy but also other aspects of agriculture (especially fruit

production) and plant ecology. At least three distinct genetic lineages of SBV could be identified: a European genotype (with Central European and British subtypes), a Far Eastern genotype representing the Thai SBVs and consisting of strains from India and Nepal and a distinct third genotype originating from South Africa (Grabensteiner *et al*., 2001). This particular problem of sacbrood infection was chosen because the infection causes tremendous economic as well as environmental losses. If infected with this virus, the larvae die and fail to pupate and leads to a general weakening of the colony. In Mahabaleshwar, this virus infected the brood of *Apis cerana* and caused destruction of 21 colonies out of a total of 44 colonies in summer of 2008. Since the major income source of 85% honeybee keepers is honey, destruction of colonies caused a huge economic setback. There is no chemical treatment for Sacbrood disease. Some beekeepers have been known to protect the colonies by requeening with a young vigorous queen or smoking of the hives using mixtures of chillies and tobacco. Some farmers even fed the colonies with sugar syrup mixed with antibiotic tetracycline and neem oil. These methods had a knockout effect on the honeybees but they were not very effective in clearing out of the infection. Thus, this project was initiated with the objective of finding an effective remedy against the Sacbrood virus. Identification of effective herbal extracts might lead to the formulation of a medicine against the sacbrood virus that might act as a preventive measure as well as a clearing agent in hives with established infection. In this project, the main aim was to obtain a crude viral suspension from infected honeybee samples, infecting a cell line with the crude viral suspension, checking for the antiviral effects of herbal extracts on the infected cells. Earlier, the susceptibility of arboviruses on BHK-21 was reported (Karabastos and Buckley 1967). *Apis cerana* colonies are infected by Thai sac brood virus (Verma et al., 1990). These two viral diseases are simiar in symptomology but virus strains slightly differed serologically (Rana and Rana 2008 and Rana et. al., 1986). Sacbrood disease occurrence in *Apis cerana indica* was studied (Kshirsagar et. al., 1981). Strain of sac brood virus in *Apis cerana* was studied (Bailey et. al., 1982) and Sacbrood disease in India was reported (Phadke 1983).

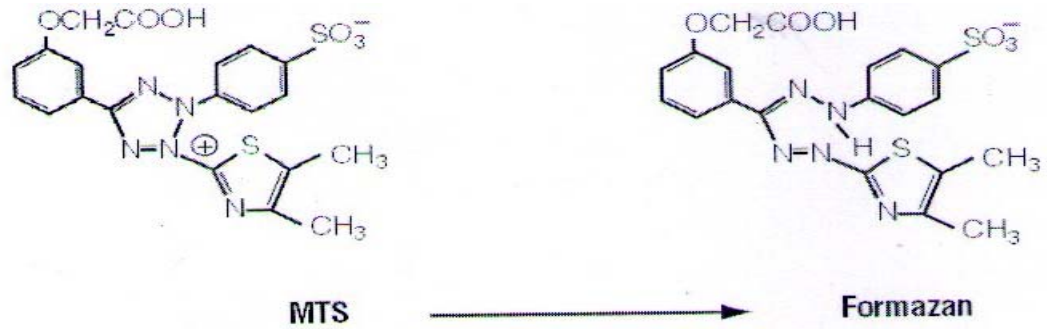
MATERIALS AND METHODS

Plant materials, reagents, cell line and virus: The medicinally important plants that were used for preparation of extracts were herbal extracts were *Ficus bengalensis* (Vad), *Ficus religiosa* (Pimpal), *Jasminum auriculatur* (Jaai), *Acacia catechu* (Khair), *Azadirachta indica* (Neem), *Curcuma longa* (Turmeric) and *Withania somnifera* (Ashwagandha). The cell line used for the in vitro propagation of the virus was BHK 21(clone 13) procured from National Centre for Cell Science (NCCS), Pune. Cell line description is Subclone of parent line derived from 5 1-day-old unsexed hamster kidneys (Macpherson and Stoker 1962). Used extensively for virus replication studies i.e. poliovirus, rabies, foot and mouth disease,

VSV (Indiana strain), herpes simplex, AD25, arboviruses.. Its specifications are : Morphology Fibroblast Species Hamster Tissue Kidney Growth mode adherent Karyotype:2n = 44, pseudodiploid (Macpherson 1963). Dulbecco's Modified Eagle's Medium (DMEM), Trypsin Phosphate Versene Glucose (TPVG), antibiotic, antimycotic solution, Trypan Blue, Fetal Calf Serum (FCS) were purchased from Himedia, Mumbai, India and the CellTitre 96^R AQ_{ueous} One Solution Cell Proliferation Assay MTS was purchased from Promega.

Cell Proliferation assay:

The CellTitre 96^R AQ_{ueous} One Solution Cell Proliferation Assay (Promega) is a colorimetric method for determining the number of viable cells in proliferation or cytotoxicity assays. The CellTitre 96^R AQ_{ueous} One Solution Reagent contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (Phenazine EthoSulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable solution. The MTS tetrazolium compound (Owen's reagent) is bioreduced by cells into a colored formazan product that is soluble in tissue culture medium Barltrop, J.A. *et al.* (1991). This conversion is presumably accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells Berridge, M.V. and Tan, A.S. (1993). Assays are performed by adding a small amount of CellTitre 96^R AQ_{ueous} One Solution directly to culture wells, incubating for 1- 4 hours and then recording the absorbance at 490 nm with a 96 well plate reader Cory, A.H. *et al.* (1991) and Riss, T.L. and Moravec, R.A. (1992). The quantity of formazan product as measured by the absorbance at 490 nm is directly proportional to the number of live cells in the culture.



Obtaining infected honeybee samples:

Combs containing a mixture of healthy and infected brood of *Apis cerana* were obtained from the Khadi Village Industries Commission located in Mahabaleshwar, Maharashtra, India. The combs were transported to the lab at normal room temperature and stored in polythene bags.

Separation of infected honeybee samples : The infected larvae were identified by visual observation of the comb. These larvae were curled up in their respective cells and showed a black spot at the head region. They were picked up using sterile forceps and care was taken so as not to rupture the sac (filled with ecdysial fluid containing virus particles) that surrounded the larvae. The samples were collected in a sterile petriplate and stored at -80^{0}C for further use.

Isolation of virus particles from infected honeybee samples:

One gram of the infected larval tissue was crushed in a mortar and pestle using liquid nitrogen and the powder thus obtained was suspended in 5 ml of DiEthylPyroCarbonate (DEPC) treated water. This suspension was centrifuged at 5000 rpm for 5 min and the pellet discarded. This step was repeated a total of 3 times and the supernatant thus obtained was collected. The supernatant was filter sterilized using a syringe filter having cellulose acetate membrane of pore size 0.22 µm. Aliquots were made of the filtrate and stored at -80^{0}C for further use.

Observations: The supernatant obtained after centrifugation was turbid even after repeated rounds of centrifugation and hence a number of changes of the filter membrane had to be effected in order to get a clear supernatant. The turbidity of the supernatant often led to clogging of the membrane filter. Once filter sterilized the crude viral suspensions were stable at -80^{0}C (i.e. maintained their infectivity). Plating the viral suspension on sterile agar plates showed no bacterial contamination.

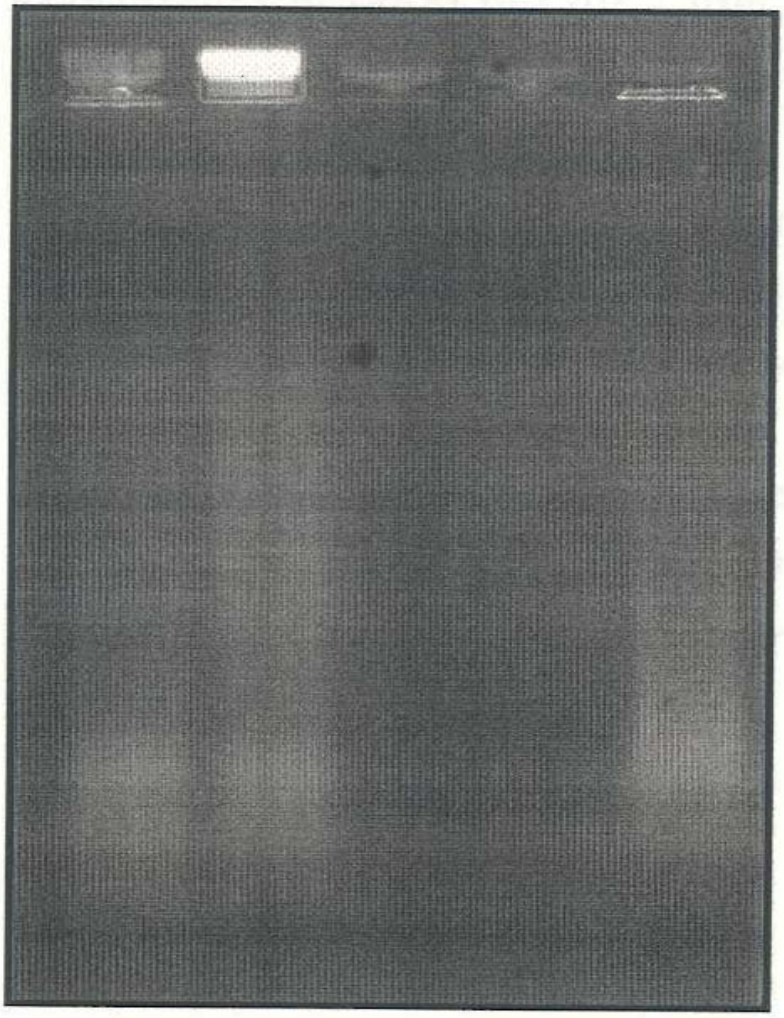
Isolation of RNA from viral suspension: Polyethylene glycol (PEG) solution (16%) was added to 1ml of the viral suspension and mixed well. This mixture was incubated on ice for 1 hour. The mixture was then centrifuged at 12,000 rpm for 8 minutes and the pellet so obtained was retained. Another aliquot was taken and incubated on ice for 1 hour but no PEG solution was added to it. Both the samples were processed in the same way after this initial differentiation step. 1ml of Tri Reagent (MRC-Molecular Research Centre) solution was added to both the samples (pellet and filtrate respectively) and mixed well. The pellet was dissolved in Trizol by vortexing. The mixtures were incubated at room temperature for 5 minutes. 0.2 ml chloroform (per 1ml of Trizol) was added and the mixtures vortexed for 15-30 seconds. The samples were then centrifuged at 12,000 rpm for 10 minutes and the aqueous phase collected. 0.5 ml of isopropanol was added for each initial ml of Trizol. The mixture was then centrifuged at 12,000 rpm for 8 minutes. The pellet (RNA) was obtained, was air dried and dissolved in 30 µl DEPC treated water. This sample was stored at -80^{0}C for further use.

Modifications in this protocol included crushing the infected larval sample in 0.25 M NaCl and Phosphate buffer respectively without the use of liquid nitrogen.

Gel electrophoresis of RNA samples: The samples were run on a formaldehyde gel that was prepared as follows: 0.8 gm of agarose was weighed and dissolved in 28.8 ml DEPC treated water. This mixture was then cooled to 55^0C using water bath. 4 ml formaldehyde agarose electrophoresis buffer was added to it along with 7.2 ml of 37% formaldehyde and swirled well. The gel was cast in casting apparatus and allowed to polymerize for 30-45 minutes.

Loading sample: RNA loading buffer (7 µl) was taken in sterile PCR vial and RNA sample (8 µl) was added to it. Formaldehyde (2 µl) was then added to it. This mixture was incubated at 55^0C for 15 minutes. RNA loading dye (1 µl) was added and the mixture mixed gently by pipetting up and down. The samples were then loaded into the wells of formaldehyde gel and allowed to run at voltage of 85 mV. The electrophoresis buffer used was 1X formaldehyde agarose electrophoresis buffer.

OBSERVATIONS



Lane 1: RNA obtained after pelleting virus with PEG

Lane 2: RNA obtained without pelleting virus with PEG

Lane 3 and 4: No RNA was obtained after crushing the infected larval sample in phosphate buffer

Lane 5: RNA obtained after crushing the infected larval sample in 0.25M NaCl.

Results: Viral RNA was successfully isolated using the Trizol reagent. It was distinguished from genomic RNA by the presence of one clear band as opposed to genomic RNA that produced two distinct bands of 18S and 28S RNA.

Maintenance of BHK-21 cell line: The BHK-21 cell line was split in the ratio 1:4 according to the instructions given by the above mentioned institute initially and was allowed to get acclimatized to DMEM medium. After sufficient acclimatization, the cell line was split in the ratio 1:2 to obtain confluent monolayer of cells. This was done as follows: All the culture medium was removed from the flask with the help of 1 ml micropipette. 500 µl of TPVG (Trypsin Phosphate Versine Glucose solution) was added to the flask and the flask rocked gently. Detachment of cells from the substratum was observed against the light. This treatment was given for a period of 2-5 minutes. 5 ml of DMEM containing 10 % fetal calf serum and antibiotics was then added to flask and the cell suspension centrifuged at 5000 rpm for 10 minutes The supernatant was decanted and the pellet redissolved in 1ml DMEM (containing 10 % serum and antibiotics and antibiotic solution). The cell count was done using Improved Neubauer chamber. For cell density $1X10^6$ cells/ml, 0.5 ml of the cell suspension was taken and transferred to a fresh culture flask (1^{st} passage). The total volume in the flask was made up to 10 ml using DMEM. The flasks were observed under 4X, 10X and 40X magnification of inverted microscope and placed in CO_2 incubator (37^0C and 5% CO_2). **Observations:** The cells were observed to be floating in the culture medium immediately after transfer to new flask. Confluent monolayer was obtained in the flasks after 48 hours incubation. **Checking the cell viability:** 0.4 % Trypan blue solution was mixed with equal volume of cell suspension. The cell suspension was observed on Neubauer chamber under 10X magnification of inverted microscope.

Preparation of microtitre plates for cell proliferation assay:

A single cell culture flask showing 100% confluency (complete monolayer formation) was taken and the medium removed completely with the help of micropipette. 500 µl of TPVG was added to the flask and the flask rocked gently. Detachment of cells from surface was observed against the light. This treatment was given for a period of 2-5 minutes 5 ml of DMEM containing 10 % FCS was then added to flask and the culture centrifuged at 5000 rpm for 10 minutes The supernatant was decanted and the pellet redissolved in 2 ml DMEM (containing 10 % serum and antibiotic antimycotic solution). 1ml of the cell suspension was

transferred back to the original flask and the volume made up to 10 ml using DMEM. To the remaining 1ml cell suspension, 9 ml DMEM was added and the suspension mixed well. Each well of the 96 well microtitre plate was filled with 100 µl of this cell suspension. The plate was covered with the lid and sealed with parafilm. The plate was then incubated at 37^0C and 5% CO_2 in CO_2 incubator. The cells were observed routinely.

Observations : The cells in each well of the 96 well microtitre plate achieved confluence after 48 hours of incubation.

Preparation of herbal extracts: Preparation of aqueous extracts was done from *Ficus bengalensis* (Vad), *Ficus religiosa* (Pimpal*)*, *Jasminum auriculatur* (Jaai), *Acacia catechu* (Khair), *Azadirachta indica* (Neem), *Curcuma longa* (Turmeric) and *Withania somnifera* (Ashwagandha). Samples were collected from different parts of the plants such as leaves, stem, bark, roots and flowers. The samples were allowed to air dry and were then crushed in a grinder to obtain a fine powder. One gram of this powder was added to 20 ml milliQ water and the sample boiled for 10 minutes to obtain the aqueous extract. The extracts were allowed to cool and then filtered through filter paper. The filtrate that was obtained was again filtered using Whatman filter paper Number 1. The filtrate thus obtained was divided into 2 aliquots. One aliquot was autoclaved at 121^0C at 15 psi for 15 minutes and the other was used directly. The herbal extracts were serially diluted for use in the cell proliferation assay. The dilution scheme was as follows: 2 ml herbal extract (neat) was added to 2 ml sterile distilled water (1:1 dilution- solution A). 2ml of solution A was added to 2 ml sterile distilled water (1:2 dilution-solution B). 2 ml of solution B was added to 2 ml sterile distilled water (1:4 dilution-solution C). Thus, 3 dilutions and 1 neat solution were ready for use.

Inoculation of virus in cell line and addition of herbal extracts: Plate 1:

The virus suspension was serially diluted for use in the assay. The dilution scheme was as follows: 2 ml virus suspension (neat) was added to 2 ml sterile distilled water (1:1 dilution-solution 1V). 2 ml of solution A was added to 2 ml sterile distilled water (1:2 dilution-solution 2V). 2ml of solution B was added to 2 ml sterile distilled water (1:4 dilution-solution 3V). Thus, 3 dilutions and 1 neat solution were ready for use.

The culture medium was completely removed from the wells (having confluent monolayer) and discarded. 50 µl of the filtered virus suspension was added to each of the wells except those which represented the negative control. The plates were allowed to stand for 2 hours to allow the virus to adsorb to the cells. Two sets of such microtitre plates were prepared to test the effectiveness of autoclaved and unautoclaved herbal extracts. On completion of the incubation period, the viral suspension was removed and 50 µl each of the

herbal extracts were added to the designated wells in accordance with the designed template. The volume in each well was made up to 200 µl with sterile medium.

Addition of viral sample: Plate 1

	1	2	3	4	5	6	7	8	9	10
A	D/W	NV	PC	NV	NV	NV	NV	NV	NV	NV
B	D/W	NV	PC	NV	NV	NV	NV	NV	NV	NV
C	D/W	1V	PC	1V	1V	1V	1V	1V	1V	1V
D	D/W	1V	PC	1V	1V	1V	1V	1V	1V	1V
E	D/W	2V	PC	2V	2V	2V	2V	2V	2V	2V
F	D/W	2V	PC	2V	2V	2V	2V	2V	2V	2V
G	D/W	3V	PC	3V	3V	3V	3V	3V	3V	3V
H	D/W	3V	PC	3V	3V	3V	3V	3V	3V	3V

Where,

D/W = sterile Distilled Water, NV = undiluted virus sample (neat), IV = 1:1 dilution of viral sample, 2V = 1:2 dilution of viral sample 3V = 1:4 dilution of viral sample PC = Positive Control

Addition of herbal extracts: Plate 1

	1	2	3	4	5	6	7	8	9	10
A	D/W	NV	PC	TN	NBN	VRN	NLN	ABN	TLN	PN
B	D/W	NV	PC	TN	NBN	VRN	NLN	ABN	TLN	PN
C	D/W	1V	PC	T1	NB1	VR1	NL1	AB1	TL1	P1
D	D/W	1V	PC	T1	NB1	VR1	NL1	AB1	TL1	P1
E	D/W	2V	PC	T2	NB2	VR2	NL2	AB2	TL2	P2
F	D/W	2V	PC	T2	NB2	VR2	NL2	AB2	TL2	P2
G	D/W	3V	PC	T3	NB3	VR3	NL3	AB3	TL3	P3
H	D/W	3V	PC	T3	NB3	VR3	NL3	AB3	TL3	P3

Where,

TN = Turmeric (neat), T1 = Turmeric dilution (1:1), T2 = Turmeric dilution (1:2), T3 = Turmeric dilution (1:4), NBN = Neem bark (neat), NB1 = (1:1) dilution, NB2 = (1:2) dilution, NB3 = (1:4) dilution; VRN = Vad root (neat), VR1 = (1:1) dilution,VR2 = (1:2) dilution,VR3 = (1:4) dilution, NLN = Neem leaves (neat), NL1 = (1:1) dilution, NL2 = (1:2) dilution, NL3 = (1:4) dilution, ABN = Acacia bark (neat), AB1 = (1:1) dilution, AB2 = (1:2) dilution, AB3 = (1:4) dilution, TLN = Tulsi leaves (neat), TL1 = (1:1) dilution, TL2 = (1:2) dilution, TL3 = (1:4) dilution, PN = Pimpal (neat), P1 = (1:1) dilution, P2 = (1:2) dilution,P3 = (1:4) dilution

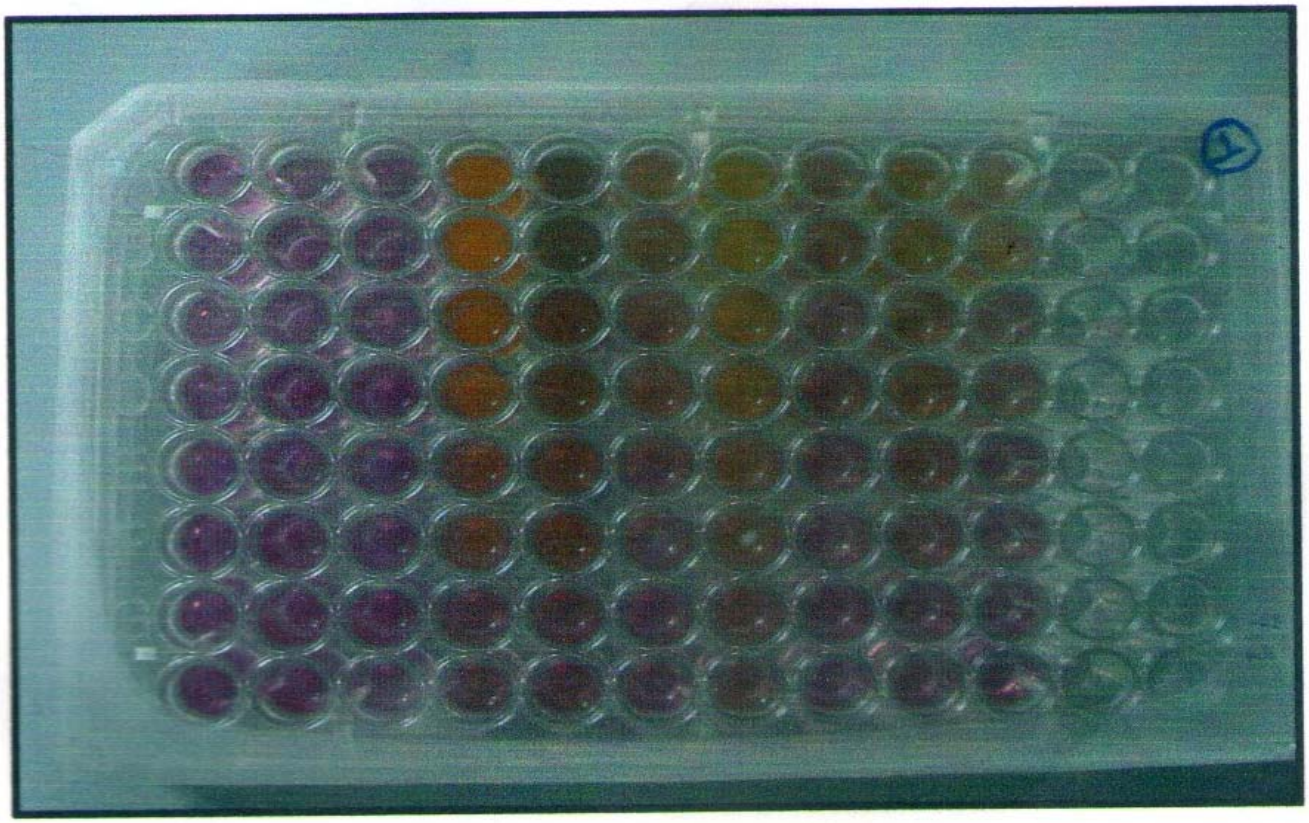


Plate 2: The virus and herbal extract addition template was same as that of template 1, the only difference being that the herbal extracts were autoclaved.

Observations on the basis of microscopic observations: Plate 1

S. No.	Well No	Sample present in wells with cells	Activity of plant extract	Activity of viral sample
1	A1-H1	Distilled water + cells	-	-
2	G2	1:4 dilution of virus sample + cells	-	+
3	E2	1:2 dilution of virus sample + cells	-	++
4	C2	1:1 dilution of virus sample + cells	-	+++
5	A2	Undiluted virus sample + cells	-	++++
6	A4/B4	Undiluted Turmeric + cells + neat virus	-	++++
	C4/D4	1:1 diluted Turmeric + cells + 1:1 virus	-	+++
	E4/F4	1:2 diluted Turmeric + cells + 1:2 virus	-	++
	G4/H4	1:4 diluted Turmeric + cells + 1:4 virus	-	+
7	A5/B5	Undiluted Neem bark + cells + neat virus	+++	++++
	C5/D5	1:1 diluted Neem bark + cells + 1:1 virus	++	+++
	E5/F5	1:2 diluted Neem bark + cells + 1:2 virus	+	++
	G5/H5	1:4 diluted Neem bark + cells + 1:4 virus	+	+
8	A6/B6	Undiluted Vad root + cells + neat virus	+++	++++
	C6/D6	1:1 diluted Vad root + cells + 1:1 virus	++	+++
	E6/F6	1:2 diluted Vad root + cells + 1:2 virus	+	++

	G6/H6	1:4 diluted Vad root + cells + 1:4 virus	+	+
9	A7/B7	Undiluted Neem leaves + cells + neat virus	+	++++
	C7/D7	1:1 diluted Neem leaves + cells + 1:1 virus	+	+++
	E7/F7	1:2 diluted Neem leaves + cells + 1:2 virus	+	++
	G7/H7	1:4 diluted Neem leaves + cells + 1:4 virus	+	+
10	A8/B8	Undiluted Acacia bark + cells + neat virus	++	++++
	C8/D8	1:1 diluted Acacia bark + cells + 1:1 virus	++	+++
	E8/F8	1:2 diluted Acacia bark + cells + 1:2 virus	++	++
	G8/H8	1:4 diluted Acacia bark + cells + 1:4 virus	+	+
11	A9/B9	Undiluted Tulsi leaves + cells + neat virus	+	++++
	C9.D9	1:1 diluted Tulsi leaves + cells + 1:1 virus	+	+++
	E9/F9	1:2 diluted Tulsi leaves + cells + 1:2 virus	+	++
	G9/H9	1:4 diluted Tulsi leaves + cells + 1:4 virus	+	+
12	A10/B10	Undiluted Pimpal + cells + neat virus	+++	++++
	C10/D10	1:1 diluted Pimpal + cells + 1:1 virus	++	+++
	E10/F10	1:2 diluted Pimpal + cells + 1:2 virus	+	++
	G10/H10	1:4 diluted Pimpal + cells + 1:4 virus	+	+

Observations on the basis of microscopic observations: Plate 2 (autoclaved extracts)

S. No.	Well No	Sample present in wells with cells	Activity of plant extract	Activity of viral sample
1	A1-H1	Distilled water + cells	-	-
2	G2	1:4 dilution of virus sample + cells	-	+
3	E2	1:2 dilution of virus sample + cells	-	++
4	C2	1:1 dilution of virus sample + cells	-	+++
5	A2	Undiluted virus sample + cells	-	++++
6	A4/B4	Undiluted Turmeric + cells + neat virus	-	++++
	C4/D4	1:1 diluted Turmeric + cells + 1:1 virus	-	+++
	E4/F4	1:2 diluted Turmeric + cells + 1:2 virus	-	++
	G4/H4	1:4 diluted Turmeric + cells + 1:4 virus	-	+
7	A5/B5	Undiluted Neem bark + cells + neat virus	++	++++
	C5/D5	1:1 diluted Neem bark + cells + 1:1 virus	++	+++
	E5/F5	1:2 diluted Neem bark + cells + 1:2 virus	-	++
	G5/H5	1:4 diluted Neem bark + cells + 1:4 virus	-	+
8	A6/B6	Undiluted Vad root + cells + neat virus	-	++++
	C6/D6	1:1 diluted Vad root + cells + 1:1 virus	-	+++
	E6/F6	1:2 diluted Vad root + cells + 1:2 virus	-	++
	G6/H6	1:4 diluted Vad root + cells + 1:4 virus	-	+
9	A7/B7	Undiluted Neem leaves + cells + neat virus	-	++++
	C7/D7	1:1 diluted Neem leaves + cells + 1:1 virus	-	+++
	E7/F7	1:2 diluted Neem leaves + cells + 1:2 virus	-	++
	G7/H7	1:4 diluted Neem leaves + cells + 1:4 virus	-	+
10	A8/B8	Undiluted Acacia bark + cells + neat virus	+	++++
	C8/D8	1:1 diluted Acacia bark + cells + 1:1 virus	+	+++
	E8/F8	1:2 diluted Acacia bark + cells + 1:2 virus	+	++
	G8/H8	1:4 diluted Acacia bark + cells + 1:4 virus	+	+

11	A9/B9	Undiluted Tulsi leaves + cells + neat virus	-	++++
	C9.D9	1:1 diluted Tulsi leaves + cells + 1:1 virus	-	+++
	E9/F9	1:2 diluted Tulsi leaves + cells + 1:2 virus	-	++
	G9/H9	1:4 diluted Tulsi leaves + cells + 1:4 virus	-	+
12	A10/B10	Undiluted Pimpal + cells + neat virus	-	++++
	C10/D10	1:1 diluted Pimpal + cells + 1:1 virus	-	+++
	E10/F10	1:2 diluted Pimpal + cells + 1:2 virus	-	++
	G10/H10	1:4 diluted Pimpal + cells + 1:4 virus	-	+

Plate 3: On the basis of observations conducted on plates 1 and 2 it was found that the undiluted viral sample and 1:1 diluted sample showed maximum infectivity. Hence, in this plate, the undiluted viral sample was used in all the wells (kept constant) and different concentrations of the herbal extracts were tested on the viral sample.

The culture medium was completely removed from the wells (having confluent monolayer) and discarded. 50 µl of the filtered virus suspension was added to each of the wells except those which represented the negative control. The plates were allowed to stand for 1 hour to allow the virus to adsorb to the cells. Two sets of such microtitre plates were prepared to test the effectiveness of autoclaved and unautoclaved herbal extracts. On completion of the incubation period, the viral suspension was not removed. 50 µl each of herbal extracts were added to the designated wells in accordance with the designed template. The volume in each well was made up to 200 µl with sterile medium.

Addition of viral sample: Plate 3

	1	2	3	4	5	6	7	9	10	11	12
A	CM	NV	TN	NV	NV	NV	NV	NV	NV	NV	NV
B	CM	NV	NBN	NV	NV	NV	NV	NV	NV	NV	NV
C	CM	NV	VRN	NV	NV	NV	NV	NV	NV	NV	NV
D	CM	NV	NLN	NV	NV	NV	NV	NV	NV	NV	NV
E	CM	NV	ABN	NV	NV	NV	NV	NV	NV	NV	NV
F	CM	NV	TLN	NV	NV	NV	NV	NV	NV	NV	NV
G	CM	NV	PN	NV	NV	NV	NV	NV	NV	NV	NV
H	CM	NV	JN	NV	NV	NV	NV	NV	NV	NV	NV

Where

CM =Culture Medium;NV = Undiluted viral sample (neat),TN = Turmeric Neat,NBN = Neem bark neat, VRN = Vad root neat, NLN= Neem Leaves Neat,ABN = Acacia bark neat,TLN = Tulsi leaves neat, PN = Pimpal neat, JN = Jasmine neat

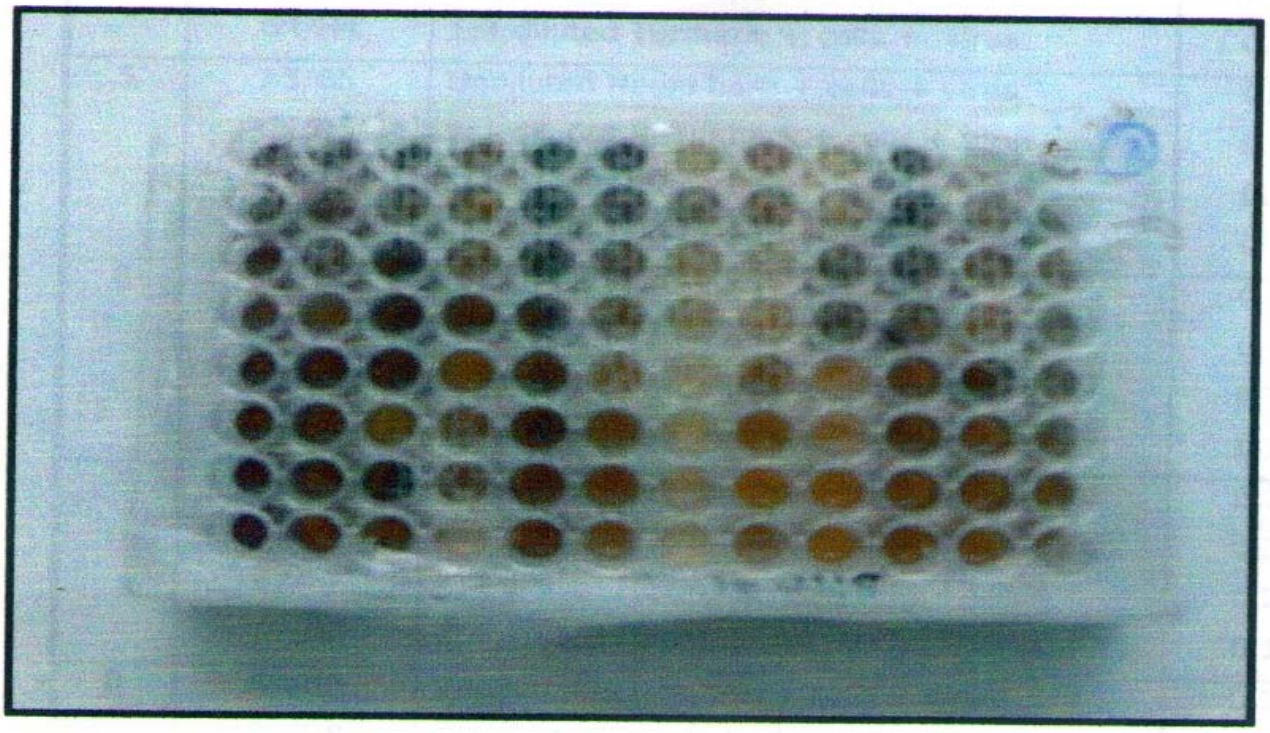
Addition of unautoclaved herbal extracts: Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	CM	NV	TN	TN	NBN	VRN	NLN	ABN	TLN	PN	JN	ARN
B	CM	NV	NBN	TN	NBN	VRN	NLN	ABN	TLN	PN	JN	ARN
C	CM	NV	VRN	T1	NB1	VR1	NL1	ABN1	TL1	P1	J1	AR1
D	CM	NV	NLN	T1	NB1	VR1	NL1	ABN1	TL1	P1	J1	AR1
E	CM	NV	ABN	T2	NB2	VR2	NL2	ABN2	TL2	P2	J2	AR2
F	CM	NV	TLN	T2	NB2	VR2	NL2	ABN2	TL2	P2	J2	AR2
G	CM	NV	PN	T3	NB3	VR3	NL3	ABN3	TL3	P3	J3	AR3
H	CM	NV	JN	T3	NB3	VR3	NL3	ABN3	TL3	P3	J3	AR3

Where

TN = Turmeric (neat),T1 = Turmeric dilution (1:1), T2 = Turmeric dilution (1:2), T3 = Turmeric dilution (1:4), NBN = Neem bark (neat) NB1 = (1:1) dilution, NB2 = (1:2) dilution, NB3 = (1:4) dilution; VRN = Vad root (neat),VR1 = (1:1) dilution,VR2 = (1:2) dilution,VR3 = (1:4) dilution,NLN = Neem leaves (neat),

NL1 = (1:1) dilution,NL2 = (1:2) dilution, NL3 = (1:4) dilution ABN = Acacia bark (neat), AB1 = (1:1) dilution, AB2 = (1:2) dilution, AB3 = (1:4) dilution,TLN = Tulsi leaves (neat),TL1 = (1:1) dilution,TL2 = (1:2) dilution, TL3 = (1:4) dilution, PN = Pimpal (neat), P1 = (1:1) dilution, P2 = (1:2) dilution, P3 = (1:4) dilution. JN=Jasmine (neat), J1=(1:1) dilution, J3=(1:4) dilution; ARN=Ashwagandha root (neat), AR1=(1:1) dilution, AR2=(1:2) dilution, AR3=(1:4) dilution.



After an incubation of 48 hours, 10 µl ul of CellTitre 96^R AQ_{ueous} One Solution was added to each wells. The microtitre plate was kept on shaker incubator (70 rpm) for 10 minutes to facilitate mixing of the assay solution with the culture medium. The plates were then transferred to the CO_2 incubator and incubated for 4 hours. After end of the incubation period, 10 µl ul of 10% SDS was added to each of the wells and plates kept overnight.

Spectrophotometric analysis :

200 µl ul of each sample was added to 3 ml distilled water in a cuvette. The solution was mixed well and the reading taken at 490 nm. The instrument was auto zeroed using distilled water. The blank was 200 µl culture medium in 3 ml distilled water.

Observations:

S.No.	Well no	Name of sample	O.D. at 490 nm
1	A1-H1	Culture medium + cells	0.7133
2	A2-H2	Undiluted virus sample + cells + CM	0.1256
3	A3-H3	Undiluted plant extracts + cells	0.6813
4	A4/B4	Undiluted Turmeric + cells + virus	0.2184
	C4/D4	1:1 diluted Turmeric + cells + virus	0.1919
	E4/F4	1:2 diluted Turmeric + cells + virus	0.1830
	G4/H4	1:4 diluted Turmeric + cells + virus	0.1539
5	A5/B5	Undiluted Neem bark + cells + virus	0.6924
	C5/D5	1:1 diluted Neem bark + cells + virus	0.4645
	E5/F5	1:2 diluted Neem bark + cells + virus	0.3637
	G5/H5	1:4 diluted Neem bark + cells + virus	0.3186
6	A6/B6	Undiluted Vad root + cells + virus	0.5088
	C6/D6	1:1 diluted Vad root + cells + virus	0.2283
	E6/F6	1:2 diluted Vad root + cells + virus	0.2249
	G6/H6	1:4 diluted Vad root + cells + virus	0.1845
7	A7/B7	Undiluted Neem leaves + cells + virus	0.3300
	C7/D7	1:1 diluted Neem leaves + cells + virus	0.3192
	E7/F7	1:2 diluted Neem leaves + cells + virus	0.2625
	G7/H7	1:4 diluted Neem leaves + cells + virus	0.1802
8	A8/B8	Undiluted Acacia bark + cells + virus	0.2425
	C8/D8	1:1 diluted Acacia bark + cells + virus	0.2218
	E8/F8	1:2 diluted Acacia bark + cells + virus	0.1509
	G8/H8	1:4 diluted Acacia bark + cells + virus	0.1447
9	A9/B9	Undiluted Tulsi leaves + cells + virus	0.3042
	C9/D9	1:1 diluted Tulsi leaves + cells + virus	0.1678

	E9/F9	1:2 diluted Tulsi leaves + cells + virus	0.1599
	G9/H9	1:4 diluted Tulsi leaves + cells + virus	0.1592
10	A10/B10	Undiluted Pimpal + cells + virus	0.6922
	C10/D10	1:1 diluted Pimpal + cells + virus	0.4554
	E10/F10	1:2 diluted Pimpal + cells + virus	0.3027
	G10/H10	1:4 diluted Pimpal + cells + virus	0.2202
11	A11/B11	Undiluted Jasmine + cells + virus	0.6367
	C11/D11	1:1 diluted Jasmine + cells + virus	0.4813
	E11/F11	1:2 diluted Jasmine + cells + virus	0.3602
	G11/H11	1:4 diluted Jasmine + cells + virus	0.2522
12	A12/B12	Undiluted Ashwagandha root + cells + virus	0.6066
	C12/D12	1:1 diluted Ashwagandha root + cells + virus	0.4118
	E12/F12	1:2 diluted Ashwagandha root + cells + virus	0.3629
	G12/H12	1:4 diluted Ashwagandha root + cells + virus	0.2482
13	Blank	Only culture medium	0.0685

The percentage viability of the cells in each well was calculated using the formula:

$$\% \text{ viability of cells} = \frac{O.D._{490} \text{ of sample} - O.D._{490} \text{ of blank} \times 100}{O.D._{490} \text{ of sample}}$$

Ex. % viability of cells in positive control wells (cells containing only culture medium)

$$= \frac{0.7133-0.0685 \times 100}{0.7133}$$

$$= 90.39\%$$

Ex. % viability of cells in negative control wells (cells containing virus sample and culture medium)

$$= \frac{0.1089-0.0685 \times 100}{0.1089}$$

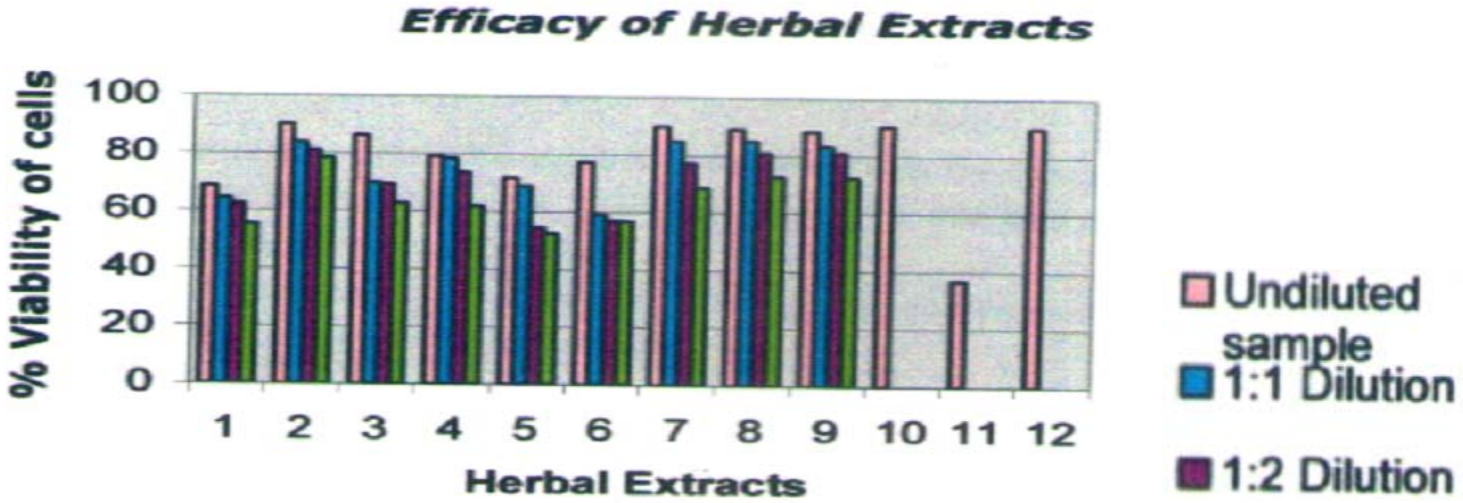
$$= 37\%$$

Similarly, values were calculated for all herbal extracts to find their effectivity.

S. No.	Herbal extract	% Viability of cells
1	Undiluted Turmeric	68.63
	1:1 diluted Turmeric	64.30
	1:2 diluted Turmeric	62.56
	1:4 diluted Turmeric	55.49
2	Undiluted Neem bark	90.19
	1:1 diluted Neem bark	83.86
	1:2 diluted Neem bark	81.16
	1:4 diluted Neem bark	78.49
3	Undiluted Vad root	86.53
	1:1 diluted Vad root	69.99
	1:2 diluted Vad root	69.54

	1:4 diluted Vad root	62.87
4	Undiluted Neem leaves	79.24
	1:1 diluted Neem leaves	78.54
	1:2 diluted Neem leaves	73.90
	1:4 diluted Neem leaves	61.90
5	Undiluted Acacia bark	71.75
	1:1 diluted Acacia bark	69.11
	1:2 diluted Acacia bark	54.60
	1:4 diluted Acacia bark	52.66
6	Undiluted Tulsi leaves	77.48
	1:1 diluted Tulsi leaves	59.17
	1:2 diluted Tulsi leaves	57.17
	1:4 diluted Tulsi leaves	56.97
7	Undiluted Pimpal	90.10
	1:1 diluted Pimpal	84.95
	1:2 diluted Pimpal	77.37
	1:4 diluted Pimpal	68.89
8	Undiluted Jasmine	89.24
	1:1 diluted Jasmine	85.21
	1:2 diluted Jasmine	80.98
	1:4 diluted Jasmine	72.83
9	Undiluted Ashwagandha	88.70
	1:1 diluted Ashwagandha	83.36
	1:2 diluted Ashwagandha	81.12
	1:4 diluted Ashwagandha	72.40

Results : Virus was successfully isolated from the infected honeybee samples. Viral RNA was successfully isolated using the Trizol reagent. The cell line BHK-21 was successfully infected with the isolated Sacbrood virus. The cytopathic effects (rounding of cells in culture, loss of adherence, dislodgement of monolayer) were clearly observed under the inverted microscope. Better infectivity was observed when the viral sample was not removed after the end of incubation period. This could be because the virus got more time to adapt to the cell line. With reference to the graph given below, we can see that the extracts that showed maximum effectiviy against inhibiting the virus infection were Neem bark, Pimpal root, Jasmine, Ashwagandha root.



Neem bark showed % viability of cells 90.19, 83.86, 81.16, 78.49 corresponding to undiluted, 1:1, 1:2 and 1:4 dilution of herbal samples respectively. Thus, the average % viability was **83.42%.**

Pimpal root showed % viability of cells 90.10, 84.95, 77.37, 68.89 corresponding to undiluted, 1:1, 1:2 and 1:4 dilution of herbal samples respectively. Thus, the average % viability was **80.32%.**

Jasmine showed % viability of cells 89.24, 85.21, 80.98, 72.83 corresponding to undiluted, 1:1, 1:2 and 1:4 dilution of herbal samples respectively. Thus, the average % viability was **82.06 %.**

Ashwagandha root showed % viability of cells 88.70, 83.36, 81.12, 72.40 corresponding to undiluted, 1:1, 1:2 and 1:4 dilution of herbal samples respectively. Thus, the average % viability was **81.39%.**

Thus, the effectivity of the herbal extracts in increasing order is

Pimpal root>Ashwagandha root>Jasmine>Neem bark

Also, the effectiveness of the herbal extracts was highest when the samples were undiluted.

DISCUSSION

The cell line BHK-21 was successfully infected with the isolated Sacbrood virus. The cytopathic effects (rounding of cells in culture, loss of adherence, dislodgement of monolayer) were clearly observed under the inverted microscope. Better infectivity was observed when the viral sample was not removed after the end of incubation period. This could be because the virus got more time to adapt to the cell line. The extracts that showed maximum effectivity against inhibiting the virus infection were Neem bark, Pimpal root, Jasmine and Ashwagandha root. Thus, the effect of the herbal extracts in increasing order is Pimpal root>Ashwagandha root>Jasmine>Neem bark. Also, the effectiveness of the herbal extracts was highest when the samples were undiluted. The *Acacia* genus has presented various secondary metabolites, such as tannins, flavonoids, alkaloids and gums (Duarte et. al., 2005). Plants described in ayurvedic literature namely, *Ficus bengalensis*, *Ficus glomerata* possess significant amount of flavonoids as secondary metabolites. tannins, saponins, flavonoids, alkaloids and glycolsides (Manimozhi et. al., 2012). The stem bark of *F. religiosa* are reported phytoconstituents of phenols, tannins, steroids, alkaloids and flavonoids, β-sitosteryl-D-glucoside, vitamin K, n-octacosanol, methyl oleanolate, lanosterol, stigmasterol, lupen-3-one (Chandrasekar et. al., 2010). A total of 62 metabolites identified in *Withania somnifera* included 32 withanamides, 22 withanolides, 3 steroidal saponins, 2 lignanamides, feruloyl tyramine, methoxy feruloyl tyramine and a diglucoside of hydroxyl palmitic acid (Bolleddula et. al., 2012). Turmeric (*Curcuma longa* L., Zingiberaceae) rhizomes contain two classes of secondary metabolites, curcuminoids and the less well-studied essential oils (Funk et. al., 2010).

Three known flavone glycosides, (1) quercetin 3-O-rutinoside, (2) kaempferol 3-O-rutinoside and (3) kaempferol 3-O-robinobioside from *Ficus benjamina*, showing highest antiviral efficiency were selected and their structure was determined by spectroscopic analyses including NMR and mass spectrometry (MS). These three flavones were highly effective against HSV-1 reaching a selectivity index (SI) of 266, 100 and 666 for compound 1, 2 and 3, respectively, while the SI of their aglycons, quercetin and kaempferol amounted only in 7.1 and 3.2, respectively. Kaempferol 3-O-robinobioside showed similar SI to that of acyclovir (ACV), the standard anti-HSV drug (Yarmolinsky et. al., 2012). The effective ingredient was extracted from the leaves of Ficus carica, and the anti-virus effect was observed on Hep-2, BHK21 and PRK cells. The water extract from the leaves of Ficus carica possessed distinct anti-HSV-1 effect. It possessed low toxicity and directly killing-virus effect on HSV-1 (Wang et. al., 2004). Oleuropein (Ole) from *Jasminum officinale* blocks effectively HBsAg secretion in Hep G2 2.2.15 cells in a dose-dependent manner (IC(50)=23.2 microg/ml. Ole (80 mg/kg, intraperitoneally, twice daily) also reduced viremia in DHBV-infected ducks. Ole therefore warrants further investigation as a potential therapeutic agent for HBV infection (Zhao et. al., 2009). The activity of two polysaccharides (P1 and P2) isolated from the leaves of *Azadirachta indica* and their chemical sulfated derivatives (P1S and P2S) against poliovirus type 1 (PV-1) was evaluated (Faccin et. al., 2012). The polysaccharides did not show any cytotoxic effects on HEp-2 cells at the highest tested concentration (200 µg/ml) and exhibited significant antiviral activity with inhibitory concentrations (IC_{50}) of 80 µg/ml, 37.5 µg/ml, 77.5 µg/ml, and 12.1 µg/ml for P1, P1S, P2 and P2S, respectively and the selectivity indexes (SI) ranged from 18 to 131.9. The compounds demonstrated better inhibitory effect when added concomitantly with the virus infection with a dose-dependent curve inhibition. The polysaccharides obtained from *Azadirachta indica* act against PV-1 by inhibiting the initial stage of viral replication. Importantly, original polysaccharides showed better virucidal effect than their sulfated derivatives at all tested concentrations. This study provides a scientific basis for the past and present ethnomedical uses of this plant Galhardi et. al.,2012).

The present study was carried out to test the antiviral activity of hydro-alcoholic root extract of *Withania somnifera* /Ashwagandha against Infectious Bursal Disease Virus using Cytopathic Effect Reduction Assay (Pant et al. 2012). Antiviral activity of *Withania somnifera* extract has been reported earlier on Herpes Simplex Virus Type-1 (Kambizi et. al., 2007). The inhibitory action of Withaferin A, a steroidal compound present in *Withania somnifera* against Herpes Simplex Virus has also been reported (Grover et al., 2011). In an in-vivo experiment polyherbal drug (Immu-25), containing *Withania somnifera* has shown antiretroviral

activity against HIV infection (Usha et al., 2003). In their preliminary study, a well known medicinal plant i.e. *Withania somnifera* showed antiviral properties against IBD Virus. Further studies are required to know the mechanism of action (Pant et al., 2012). From this preliminary study, it is implicated that the secondary metabolites of Pimpal, Ashwagandha Jasmine, Neem may have antiviral activity against the sacbrood virus. The antiviral effects of these herbal extracts could probably due to one or a combination of the actions such as 1) Prevention of adsorption of virus particles to the host cells 2) Prevention of viral penetration into the cells 3) Prevention of viral replication inside the cells 4) Lysis of viral particles.

Thus, a formulation effective against the virus can be devised on the basis of these results. Furthermore, different combinations of the extracts can also be tried out to check their synergistic activity. Research can also be carried out to identify the components of the herbal extracts which lead to specific antiviral effects. With these advances and suitable protective measures, the aim of eradicating the Sacbrood virus from honey bee colonies could be successfully achieved.

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