

## Antiviral activity of extract and purified compound from red macroalgae *Asparagopsis taxiformis* against H5N1 virus

### Abstract

The discovering and development of new antiviral agents with diverse kinds of antiviral actions is required. The search for new antiviral agents focuses on not only synthetic compounds but also natural products such as plants, the aim of the current work was to evaluate the potential use of red macroalgae *Asparagopsis taxiformis* as a new source of anti H5N1 agent. The red macroalgae *Asparagopsis taxiformis* was collected from Marsa Matrouh, Mediterranean Sea, Egypt during spring season, the effects of successive extracts and pure compound from algal sp on H5N1 virus were determined using plaque reduction assay in addition to determine the mode of action of promising extract on adsorption and replication of this virus. The chemical structure of active ingredients isolated from alga was identified using chromatographic and spectroscopic analysis. The results showed that pet.ether and water extracts from this species showed high antiviral activity >99.9% and from the obtained data we can reported that the mode of action of this extract not appear any effect on virus replication. However, extracts inhibited the cell-associated infectivity by 100% of the control levels (on adsorption process). The obtained pure compound was identified as 6-methyl- $\Delta^2$ -stigmasterol-2, 3 diacetate and tested for H5N1 virus activity. The result showed that this pure compound had antiviral activity by 56% at 100 ug/ml. Our results suggest that extract and active ingredients from *Asparagopsis* sp has potential to protect against pandemic H5N1 in the event of its cross over to the human host and recommend testing these extracts as potential inhibitor of COVID-19.

**Key-words:** *Asparagopsis taxiformis* – Successive extracts - Anti H5N1 virus – Mode of action

### Introduction

In general, IAV or Influenza A virus is a healthy threat to the human community. This virus has high ability for infects various host e.g: horse's waterfowl, dogs, cats, humans, and other mammals. The highly pathogenic avian influenza (HPAI) H5N1 viruses cause different public health and economic problems and other side effects, the virus transmits from birds to humans through direct contact with indirect contact in a contaminated media (Lu et al., 2020).

Although at present time, the transmission of these viruses among humans still very little, the humans need to find new anti-viral compounds to prevent the mortality percentage of this infection. Neuraminidase inhibitors (NAIs) drugs have been widely used for the treatment of IAV infection. However, use of NAIs drugs led to viruses less sensitive to NAIs even at a low frequency (Tamura et al., 2011 and Takashita et al., 2013). Therefore, the findings or development of natural anti-influenza virus drugs is recommended ASAP,

Macroalgae species well known or recognized as ecosystem engineers and/or foundation organisms in different environment or habitats since they convert the simple surfaces into structured environments that support many of living species ( Thomsen et al., 2017).

Also, the data reported by Cardozo et al. (2006) revealed that algal products have been used in different fields such as agriculture, pharmaceuticals, food and cosmetic industries. The traditional market for algal products is a fact and the main challenge is growing macroalgae on a large scale without any problems to other living organisms. Moreover, Algae also contain multitude of bioactive compounds that might have antiviral, antibacterial, antifungal, antioxidant, , anticarcinogenic, etc. (Plaza, et al., 2008).

In relation to the activity of antiviral and marine macroalgae species, the algal species have high ability for produced and provide novel leads against various viruses e.g: H5N1, H1N1, hepatitis, HSV ---etc that are evolving and developing resistance to existing drugs as reported by Vo and Kim (2010). Thus, Algal species (especially seaweeds or macroalgae) are regarded as a promising source for antiviral drugs.

Thus, the aim of the current study is to evaluate the effect of successive extracts and pure compound isolated from *Asparagopsis taxiformis* red macroalga on H5N1 virus and identify the mode of action.

## **Materials and Methods**

### **Solvents**

Pure hexane, petrolum ether, chloroform, ethyl ether, ethyl acetate, methylene chloride, ethanol, methanol, acetone, acetic acid, tween (20 and 40) and DMSO. were obtained from Merch (Germany). All solvent and distilled before use.

### **Collection of alga**

The alga was collected from El-Garam beach at Marsa Matrouh City. The alga sp belong to Bonnemaisoniaceae (*Asparagopsis* sp, super littoral and intertidal zones, 11-13 cm), Thallus of algae was cleaned from sand and foreign materials by washing with sea water followed by fresh water. After prepared of herbarium specimens of the algae, the algal sp was identified by prof. Dr. Sanaa M. Shanab, Professor of Phycology, Botany and microbiology Department, Faculty of Science, Cairo University.

### **Quantitative analysis of alga secondary metabolites**

#### **Total Glycosides**

The total glycosides content in ASP sp was extracted and spectrophotometrically determined (as glucose) using the phenol-sulfuric acid reagent at 490 nm (Dubois *et al.* 1956).

#### **Total saponin**

Total saponin content of ASP sp was determined according to Ebrahimzadeh and Niknam, (1998) method.

#### **Total Alkaloids**

Total alkaloids content was extracted according to Sabri *et al.* (1973).

#### **Total organic acids**

Plant acids in macroalga were determined using titratable acidity method according to Harborne (1973).

#### **Total phenols**

Total phenols contents of alga was determined by the Folin-Ciocalteu method (Meda *et al.*, 2005). Ferulic acid was used for preparation of standard curve.

### **Preparation of algal extracts**

Fifty grams of the alga sample was subjected to extraction with successive selective solvents: Hexane, petroleum ether (40-60), ethyl acetate, methylene chloride: methanol (1:1, v/v) and distilled water were used. The polarity was increased from non-polar to highly polar. Each solvent extract was evaporated to dryness and weighed according to Rosenthaler (1930).

**Antiviral activity** (Silva *et al.* 1997):

Preparation of algal samples for antiviral bioassay:

1. A known weight of each algal extract was dissolved in one milliter of 10 % DMSO, to give a final concentration 100 µg/µl and served as Stock solution.
2. The stock solutions were sterilized by addition of commercial antibiotic antimycotic mixture (10,000 U Penicillin sodium or 25 µg amphotericin B, 10.000 µg streptomycin sulphates).
3. Sterility test was carried out in nutrient agar.

**Cells:**

### **MDBK cells**

The cell lines of MDBK were obtained and propagated in Virology Laboratory, National Research Center (NRC).

**Media and supplements:**

**Media:**

Minimum essential medium and RBMI 1640 medium were prepared from powdered stock and pH was adjusted to 7.3 with NaHCO<sub>3</sub>. The prepared media were sterilized by filtration through nitrocellulose membrane filter (0.2 µm pore size). Sterility test was carried out on nutrient agar plates.

**Supplements:**

1- Cell dissociation solution.(0.15% Trypsin, 0.04% versene mixture)

a- Phosphate buffered saline (0.15 M, pH 7.5, PBS).

The buffer was prepared as the following:

NaCl            0.9 g/L

KCl             0.2 g/L

KH<sub>2</sub>PO<sub>4</sub>       0.12 g/L

Na<sub>2</sub>HPO<sub>4</sub>      0.91 g/L

Deionized H<sub>2</sub>O up to 1L

The above Ingredients were mixed gently in the order shown above and pH value was adjusted to 7.5 then, the buffer was sterilized by filtration through 0.22 µm nitrocellulose membranes, and their solution was used in washing of cell monolayer sheets and in preparation of cell dissociation solution as follow:

b- Trypsin 1:250 (Sigma-Aldrich).

1.5 g of trypsin powder was dissolved in 500 ml PBS and incubated overnight at 4°C with steering.

c- Versene solution (0.04 %).

A 0.04 gram tetra sodium salt of ethylenediamine tetraacetic acid (EDTA) was dissolved in 500 ml of 1.5 M PBS pH 7.5 and mixed with equal volume of trypsin-versene mixture, this solution was adjusted to pH 8.4 by 7.5% NaHCO<sub>3</sub> solutions and sterilized by filtration through nitrocellulose membrane (0.22 µm pore size). All the reagent were stored at -20 °C until used.

2- Fetal bovine serum (Sigma-Aldrich)

3-Antibiotic-antimycotic mixture. (10,000 U Penicillin sodium or 25 µg amphotericin B, 10.000 µg streptomycin sulphates) (Sigma-Aldrich).

**Reference viruses:**

**Avian virus (H5N1)**

The viruses were supplied from Virology Laboratory, NRC. Viruses were propagated and titrated on MDBK cells as reported by Silva *et al.* (1997).

**Materials for plaque infectivity assays:**

a) Over layer medium:

Double strength concentration of both types of media was prepared and sterilized by filtration. Supplements were added to concentration of 2 % antibiotic-antimycotic.

b) Agarose solution 2 %:

The solution was prepared by cooking 2 % agarose in deionized water and sterilization by autoclaving.

c) Fixation solution:

10% formalin in H<sub>2</sub>O

d) Staining solution:

1% crystal violet in 20% methanol (w/v) was prepared and filtered through Whatman no.1 paper.

**Plaque infectivity reduction assay:**

**Anti-H5N1 assay:**

1- A 6-well plate was cultivated with MDBK culture (10<sup>5</sup>cell/ml) and incubated for 2 days at 37°C.

2-the culture of H5N1 virus was diluted to give 10<sup>7</sup> PFU/ml as final concentrations and mixed with the algal extract and incubated overnight at 4°C.

3- Growth medium was removed from the multiwell plate and virus- compound mixture was inoculated (100 µl/well).

4- After 1h contact time, the inoculums were aspirated on MDBK culture and 3ml of MEM with 1% agarose were overlaid the cell sheets.

5- The plates were left to solidity and incubated at 37 °C until the development of virus plaques.

6- Cell sheets were fixed in 10% formalin solution for 2h and stained with crystal violet solution.

7- Control virus and cells were treated identically without chemical compound.

8- Virus plaques were counted and the percentage of inhibition was calculated (Silva *et al.*, 1997).

***Mode of action***

Virus inhibited mechanism was studied for the crude algal extracts in two categories:

**First one : Viral Réplications (Amoros *et al.*, 1994)**

1-A 6 well plate was cultivated with the specific cell type (10<sup>5</sup> cell / ml) and incubated for 1- 2 days at 37°C

2- Virus was diluted at 10<sup>7</sup> PFU / ml then A 50 µl was applied to cells, and then incubated for 1 h at 37°C.

3-Unadsorbed viral particles were removed by washing the cells for several times by medium (RPMI) without supplements.

4-Extracts were applied at different concentrations starting from that one giving >99.9% inhibition.

- 5-one hour after contact time, 3 ml of concentrated medium (2x) with 2% agarose was added to the cell monolayer.
- 6-The plates were left to solidify and incubated at 37°C until the development of the viral plaques (48-72 h).
- 7-Cell sheets contained plaques were fixed in 10% formalin solution for 2 hours, and stained with crystal violet staining solution.
- 8-Viral plaques were counted and the percentage of viral reduction was calculated.
- 9- In control: the test was run as mentioned above without addition of algal extract.

### **Second one: Viral Adsorption (Zhang *et al.*, 1995).**

This mechanism was run as mentioned before except the algal extract was added before virus inoculums as follows:

- 1- A 6 well plate was cultivated with susceptible cell type ( $10^5$  / ml) and incubated for 1-2 days at 37°C.
- 2- Algal extracts were added at concentrations giving >99.9% viral inhibition with 200  $\mu$ l medium without supplements and incubated for 2 h at 4°C.
- 3- Unadsorbed extract was removed by washing the cells three successive times by media.
- 4- Virus was diluted at  $10^7$  PFU / ml then 50  $\mu$ l was applied to cells.
- 5-one hour after contact time, 3 ml of concentrated medium (2x) with 2% agarose was added to the cell monolayer.
- 6- The plates were left to solidify and incubated at 37°C until the development of the viral plaques.
- 7- Cell sheets were fixed in 10% formalin solution for 2 h and stained with crystal violet staining solution.
- 8- Virus inoculated to cells and treated identically without addition of algal extract which served as control.
- 9- Viral plaques were counted and the percentage of virus reduction was calculated.

### **Separation of active gradient**

Ten grams of the promising *Asparagopsis taxiformis* crude Pet.ether extract was fractionated over a Vacuum Liquid Chromatographic Column (VLC, 15 x10 cm, i.d packed with VLC silica gel H (100g). Gradient elution was carried out with hexane, chloroform and their mixture with an increased polarity pattern (100% hexane to 100% chloroform and finally with 80% chloroform: 20% ethyl acetate)). Fractions (200 ml of each) were collected (as shown in Table 11). Each was separated, evaporated under reduced pressure to dryness, redissolved in 5 ml of ethanol and monitored by TLC, using the solvents system (toluene: ethyl acetate, 97: 3, v/v). The TLC chromatogram was visualized under U.V light at 365nm and 245nm before exposure to anisaldehyde reagent. The major yielded fraction No 10 (3500 mg) was produced sub-fractions (pure compound). The most potent fractions were chosen for further identification using the chromatographic and spectroscopic methods as following:

#### **a. Liquid chromatography–mass spectrometry (LC–MS)**

LC–MS and LC–MS/MS (LCQ Advantage Max, Thermo Finnegan, USA) are well-established methods for steroid and sterol analysis (Griffiths *et al.*, 2005). However, as the proteomics community has demonstrated maximum sensitivity



is achieved by combining low-flow-rate LC (49.41) with electrospray ionization (ESI) - MS, spray voltage (4.53 kv), spray current (0.29  $\mu$ A), capillary voltage (14.42 v), capillary temp. (299.30 °C) and tube lens (20.0 v, sp). Performance improves as column internal diameter (i.d.) is reduced as the concentration of sample in an eluting peak follows an inverse square law with respect to i.d. as ES is also a concentration dependent process.

#### **b. UV-Visible spectrophotometer**

Shimadzu UV240 (P/N204-58000) was used for recording UV spectra and measuring the absorbance in UV range (180-360 nm).

#### **c. FTIR**

Perkin Elmer FTIR spectra (system 2000) USA was used for identification about the active groups in active gradients.

#### **d. NMR**

The identification of compounds was confirmed by carried out H-NMR analysis using NMR Joel GIM, EX 270 (400 Hz).

#### **e. Determination of carbon, hydrogen and nitrogen in different active gradients**

Carbon, hydrogen and nitrogen (CHN) in different active gradient were determined in Central lab., Faculty of Science, Cairo University by 2400 CHN Elemental Analyzer, Perkin-Elmer, USA.

### **Results and Discussion**

#### **Secondary metabolites**

The total contents of alkaloids, terpenoids, glycosides, phenolic compounds, flavonoids and organic acids are shown in Figure (1). It could be noticed *Asparagopsis* sp collected from Marsa Matrouh contained the high amount from secondary metabolites as Alkaloids followed by Glycosides, Plant acids, Terpenoids and Phenolic compounds which were 2.66, 2.15, 0.48, 0.13 and 0.11% respectively.

#### **Antiviral activity**

The antiviral activity of successive *Asparagopsis* algal extracts from Marsa Matrouh location was evaluated against avian virus (H5N1) virus which used as a model of RNA virus. Table (1) and Figure (2) show the antiviral activity of different extracts against H5N1 by using plaque reduction assay. The obtained results showed that the treatment of H5N1 with different extracts at concentration 20 and 40  $\mu$ g/ml significantly inhibited % of H5N1 virus (ranged 0.0-100%). These means that successive extracts of alga extract exhibited remarkable antiviral activity. Also, the obtained data revealed that the extracts affected viral inhibition in a dose and chemical composition dependent manner (Table 1). Results illustrated that the activity was variable between the extracts according to the polarity of these extracts. In which the maximum inhibition (virus reduction) were occurred in the following extracts: pet ether and water extracts of ASP by 100% ethyl acetate by 555 at 40  $\mu$ g/ml. these results go parallel with the results obtained by Bouhlal et al. (2010) Who reported that the aqueous extracts of *Asparagopsis armata*, *Ceramium rubrum*, *Gelidium pulchellum*, *Gelidium spinulosum*, *Halopitys incurvus*, *H. musciformis*, *Plocamium cartilagineum*, *Boergeseniella thuyoides*, *Pterosiphonia complanata*, and *Sphaerococcus coronopifolius* have been shown to inhibit antiviral activity of Herpes simplex virus type 1 with EC<sub>50</sub>

(effective concentration 50%) by mechanism of inhibition of virus replication in vitro, in the range from 2.5 to 75.9 µg/mL

Different extracts of air-dried *Ulva lactuca* (methanol, ethanol, chloroform, ethyl acetate and diethyl ether) were tested for biological activity and analysed by TLC. A complex of 6 components was tested for antiviral activity of influenza virus (H1N1). An inhibitory effect was recorded on both viral reproduction and infectious capacity. (Ivanova *et al.*, 1991). Antiviral activity of *Spirulina maxima* against herpes simplex virus type 2. was determined by Hernandez-Corona *et al.* (2002) reported that the highest antiviral activity was detected in the methanol-water (3:1) extract, which suggests that the antiviral activity is properly due to the highly polar compounds in the extract. A number of biological and synthetic sulphated polyanions, such as heparin, inhibit the replication of various mammalian viruses (Witvrouw and De Clercq, 1997). It has been suggested that these negatively charged molecules, including the sulfated algal polysaccharides, exert their inhibitory effect by interacting with the positive charges on the virus or on the cell surface and thereby prevent the penetration of the virus into the host cells (Ehresmann *et al.*, 1979).

### **Mechanism of algal extracts as antiviral activity**

#### **The effect of algal extract on virus replication**

In these experiments the activities of algal extracts against H5N1 and the clinical strain were evaluated by the plaque reduction assay. The Figures (3 and 4) show that the alga extract does not appear any effect on virus replication

#### **The effect of algal extract on virus adsorption**

The inhibitory effect of algal extracts on virus adsorption to host cell was measured by monitoring the attachment of infectious H5N1 virions on to host cells in the presence of extracts. As shown in Fig. (3 and 4), extracts inhibited the cell-associated infectivity by 100% of the control levels. These results were agreement with the results obtained by Carlucci *et al.* (1997) they reported that a sulphated galactan isolated from extracts of red alga was a selective inhibitor of herpes simplex virus (HSV-1 and HSV-2). The mode of action of sulphated galactan could be ascribed to an inhibitory action on virus adsorption. Found that the antiviral activity of cyanovirin-N (CV-N) isolated from *Nostoc sp* against HIV-2 is due, at least in part, to unique, high-affinity interaction of CV-N with the viral surface envelope glycoprotein gp120.

### **Isolation and identification of the bioactive compounds**

During the isolation of the active compounds from *Asparagopsis taxiformis* alga, the non-polar extract (Pet.ether extract) was more effective than other organic solvent extracts as antiviral activity as shown in Table 1. Further fractionation of Pet. ether extract yielded pure compound; these compounds obtained was tested for antiviral activity against H5N1 virus. The result showed that this pure compound had antiviral activity by 56% at 100 µg/ml as shown in Figure 5. These results may be due to the presence of different active groups in the isolated compound (**6-methyl- $\Delta^{22}$ -stigmasterol-2, 3 di acetate**) such as Acetate group, double bonds in the chemical structure of this compound and its conformational structure that increase from the ability of this compound to react and bind with virus protein and prevent its adsorption into specific receptor.

### **The chemical structure of active ingredients isolated from *Asparagopsis taxiformis***

Figure (6) presents the suggested chemical structure configuration of the active constituents of the algal pet. ether. The proposed configuration satisfies and complies with the analytical identification characteristics shown by the CHN Elemental Analyzer, UV, IR, <sup>1</sup>H-NMR and LC-MS/MS.

Sub-fraction with TLC R<sub>f</sub> value of 0.13, was analyzed by HPLC, LC-MS and GC-MS. The results revealed the presence of 3 compounds of which, one major constituent was found as main compounds (> 96%). Applying the mass spectral rule "13", (Bright and Chem, 1983). As can be seen in the IR spectra (Fig.7), the intense bands in region between 2935 and 2850 and at 1660 cm<sup>-1</sup> was shows due to presence of –CH<sub>2</sub>- and –CH<sub>3</sub> groups and double bond. The –OH group of steroid has and intense band in region between 3000 and 3360 cm<sup>-1</sup>. The compound has an intense band at 1725 cm<sup>-1</sup>, characteristic of the carbonyl group, and the C-O stretching band at 1265 cm<sup>-1</sup> and a second IR C-O band at 1032 was found. The band at 1032 is special for the cholesteryl acetate (Hattab et al. 2006). Also, IR spectrum showed band at 1626 and 823 cm<sup>-1</sup> (Δ ethylidene sterol). The mass spectrum of compound exhibited the molecular ion peak at m/z 506.8 corresponding to molecular formula C<sub>33</sub>H<sub>47</sub>O<sub>4</sub>. The mass spectrum showed the intense ion peak at m/z 490 (M-OH)<sup>+</sup>, 460 (M<sup>+</sup>-OH-CH<sub>3</sub>CH<sub>2</sub>), 447 (M<sup>+</sup>-OH-CH<sub>3</sub>COO<sup>-</sup>), 387 (M<sup>+</sup>- CH<sub>3</sub>COO<sup>-</sup>). The other intense peaks appeared at m/z 354 (M-H<sub>2</sub>O-C<sub>3</sub>H<sub>7</sub>)<sup>+</sup>, 294 (M-H<sub>2</sub>O-C<sub>7</sub>H<sub>15</sub>)<sup>+</sup>, 245 (M-side chain (C<sub>10</sub>H<sub>21</sub>))<sup>+</sup>, 206 (M-H<sub>2</sub>O-side chain (C<sub>13</sub> H<sub>25</sub>))<sup>+</sup>, 168 (C<sub>12</sub> H<sub>24</sub>)<sup>+</sup>, 138 (C<sub>12</sub> H<sub>18</sub>), 107 (C<sub>8</sub> H<sub>11</sub>) and 79 (C<sub>6</sub> H<sub>7</sub>). This showed the presence of steroidal skeleton. According the obtained data the chemical structure of isolated compound was elucidated as **6-methyl-Δ<sup>22</sup>-stigmasterol-2, 3 di acetate**.

## Conclusion

The red alga *Asparagopsis taxiformis* was evaluated in this study as a new source of antivirus against H5N1. Successive extractions with organic solvents of different polarities were performed (hexane, pet.ether, ethyl acetate, methylene chloride: methanol (1:1v/v), water) using concentrations 20 and 40 ug/ml. Pet .ether and Water extracts showed the highest antiviral activity (>99.9%) using plaque reduction assay. Fractionation of the nonpolar pet .ether extract yielded a pure active compound of steroidal skeleton with antiviral activity against H5N1. It may be due to the presence of different active groups as acetate group and double bonds in the chemical structure of the compound (6-methyl-Δ<sup>22</sup>-stigmasterol-2,3di acetate) which increase the ability of the compound to react and bind with the virus protein and so prevent its adsorption on specific receptors. The mod of action of algal extract and the active compound was shown to be through inhibition of virus adsorption and not its replication.

## Competing interests

The authors declare that they have no competing interests.



## References

Amoros, M.; Lurton, E.; Boustie, J. and Girre, L. (1994). Comparison of the anti-herpes simplex virus activities of propolis and 3-methyl-But-2-Enyl cafferate, J. Natural products, 57(5):644-647.

Bouhlal, R.; Riadi, H. Bourgougnon, N. 2010. Antiviral activity of the extracts of Rhodophyceae from Morocco. In African Journal of Biotechnology, 9, 7968–7975.

Bright, J. W. and Chen, E. C. M. (1983). Mass spectral interpretation using the rule of "13". J. Chem. Ed. 60: 557-558.

Cardozo, K. H. M.; Guaratini, T.; Barros, M. P.; Falcao, V. R.; Tonon, A. P.; Lopes, N. P.; Campos, S.; Torres, M. A.; Souza, A. O.; Colepicolo, P. and Pinto, E. (2006). Metabolites from algae with economical impact. Comparative Biochemistry and Physiology, 146: (1-2): 60-78.

Carlucci, M. J.; Pujol, C. A.; Ciancia, M.; Nosedá, M. D.; Matulewicz, M. C.; Damonte, E. B. and Cerezo, A. S. (1997). Antiherpetic and anticoagulant properties of carrageenans from the red seaweed *Gigartina skottsbergii* and their cyclized derivatives: correlation between structure and biological activity. International J. Biological Macromolecules, 20: 97-105.

DuBois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers and Fred. Smith (1956). Colorimetric Method for Determination of Sugars and Related Substances, Anal. Chem. 1956, 28, 3, 350-356.

Ebrahimzadeh, H. and Niknam, V. (1998). A revised spectrophotometric method for determination of triterpenoid saponin. Indian Drugs, 35: 379-381.

Ehresmann, D.; Deig, E. F. and Hatch, M. T. (1979). Antiviral properties of algal polysaccharides and related compounds. In: "Marine Algae in Pharmaceutical Science" (Eds. Hoppe, H.; Levring, T. and Tanaka, Y.). Walter de Gruyter, Berlin. New York. 293-302.

Griffiths, W. J.; Shackleton, C.; Sjövall, J.; (2005). Steroid analysis. In: Caprioli, R. (Ed.), Encyclopedia of Mass Spectrometry, vol. 3. Elsevier, Oxford. Harborne, J.B. (1973) Phytochemical Methods. Chapman and Hall Ltd., London, 49-188.

Hernández-Corona A1, Nieves I, Meckes M, Chamorro G, Barron BL. (2002). Antiviral activity of *Spirulina maxima* against herpes simplex virus type 2. Antiviral Res. 2002 Dec;56(3):279-85.

- Ivanova, V.; Rouseva, R.; Kolarova, M.; Serkedjieva, J.; Rachev, R. and Manolova, N. (1991). Isolation of a polysaccharid with antiviral effect from *Ulva lactuca*. *Preparative Biochemistry*, 24: 83-97.
- Lu, Y.; Landreth, S.; Liu, G.; Brownlie, R.; Gaba, A.; Hurk, S.; Gerdts, V. and Zhou, Y. (2020). Innate immunemodulator containing adjuvant formulated HA based vaccine protects mice from lethal infection of highly pathogenic avian influenza H5N1 virus, *Vaccine*, 38 (2020) 2387–2395.
- Meda, A.; Lamien, C. E.; Romito, M.; Millogo, J. and Nacoulma, O. G. (2005). Determination of the total phenolic, flavonoid and praline contents in Burkina Fasan honey, as well as their radical scavenging activity. *Food Chemistry*, 91: 571-577.
- Plaza, M.; Cifuentes, A. and Ibañez, E. (2008). In the search of new functional food ingredients from algae. *Food Science and Technology*, 19: 31-39.
- Rossenthaler, L. (1930). *The chemical investigation of plants*. Translated into English by Sudhamoy Ghosh from the Third German edition. Bell and Sons. Ltd London.
- Sabri, N. N.; El-Masry, S. and Khafagy, S. M. (1973). Phytochemical investigation of *Hyoscyamus desertorum*. *Planta Medica*, 23: 49.
- Silva, O.; Barbose, S.; Diniz, A.; Valdeira, M. And Gomes, E. (1997). Plant extracts antiviral activity against *Herpes Simplex Virus* type 1 and *African Swine Fever* virus. *Int. J. Pharm.*, 35: 12-16.
- Takashita, E., Fujisaki, S., Kishida, N., Xu, H., Imai, M., Tashiro, M., Odagiri, T., 2013. Characterization of neuraminidase inhibitor-resistant influenza A(H1N1) pdm09 viruses isolated in four seasons during pandemic and post-pandemic periods in Japan. *Influenza Other Respir. Viruses*. 7, 1390–1399.
- Tamura, D., Sugaya, N., Ozawa, M., Takano, R., Ichikawa, M., Yamazaki, M., Kawakami, C., Shimizu, H., Uehara, R., Kiso, M., Kawakami, E., Mitamura, K., Kawaoka, Y., 2011. Frequency of drug-resistant viruses and virus shedding in pediatric influenza patients treated with neuraminidase inhibitors. *Clin. Infect. Dis.* 52, 432–437.
- Thomsen, M.S. ; T. Wernberg, P.A. Staehr, D. Schiel Ecological interactions between marine plants and alien species E. Ólafsson (Ed.), *Marine macrophytes as Foundation Species*, CRC Press, Taylor and Francis Group, Boca Raton (USA) (2017) 277pp.

Vo, T. S. and Kim, S. K. (2010). Potential anti-HIV agents from marine resources: An overview. *Mar. Drugs* 8, 2871–2892.

Witvrouw, M. and De Clercq, E. (1997). Sulfated polysaccharide extracted from Sea algae as potential antiviral drugs. *Gen. Pharmac.*, 29: 497-511.

Zhang, J.; Zhan, B.; Yao, X.; Gao, Y. and Shong, J. (1995). Antiviral activity of tannin from the pericarp of *Punica granatum L.* against genital Herpes virus *in vitro*. *Zhongguo Zhong Yao Za Zhi*, 20(9):556-8, 576.

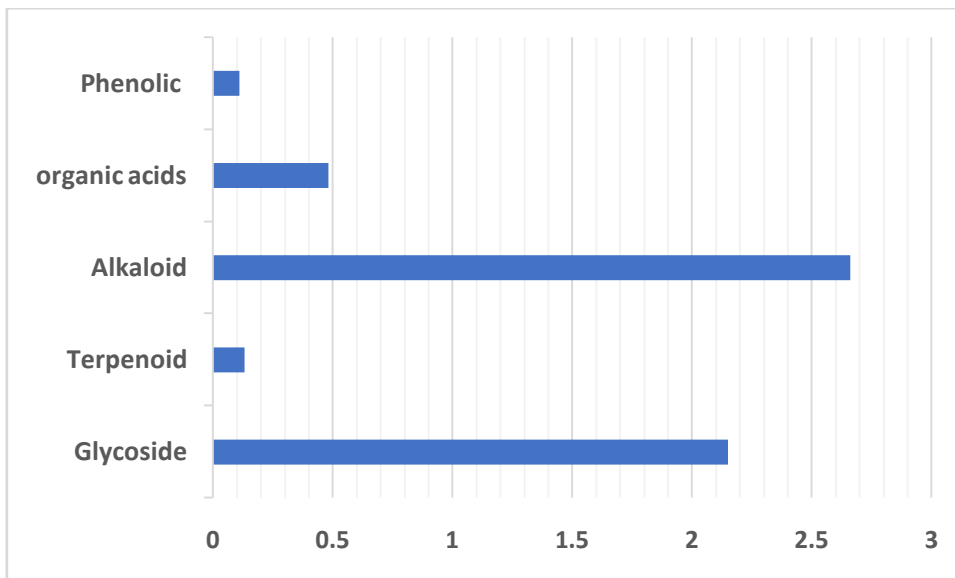
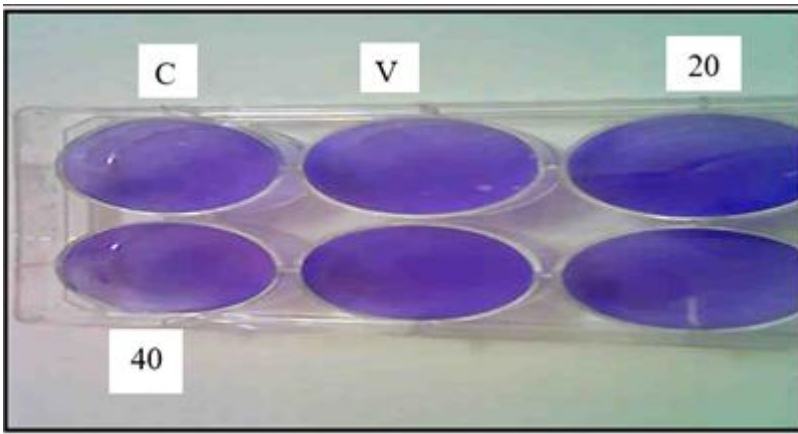


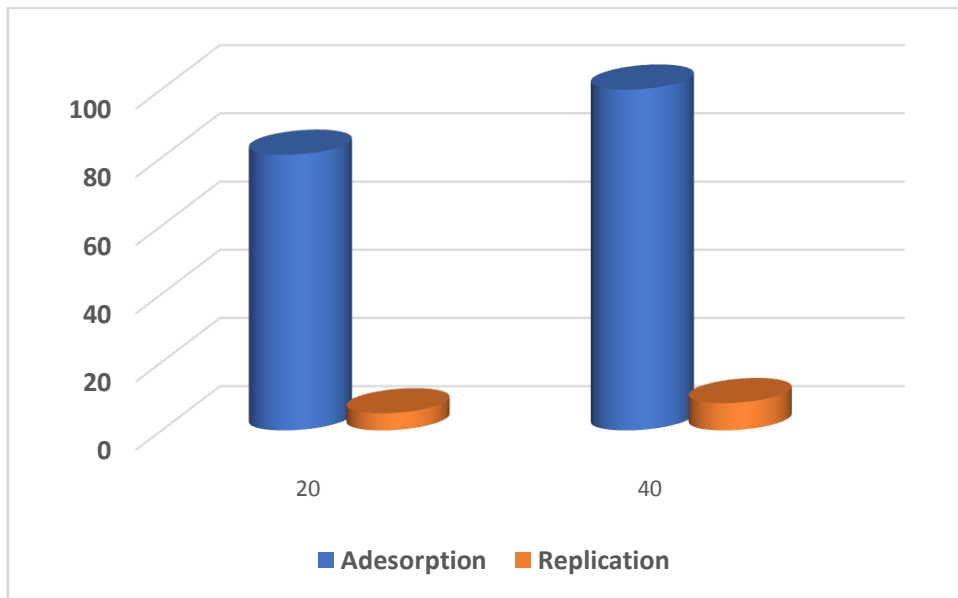
Figure 1: Secondary metabolites content (g/100g d.w) of Asp sp marine macroalgae

Table 1. Inhibitory activity of algal extracts by plaque infectivity count assay against H5N1.

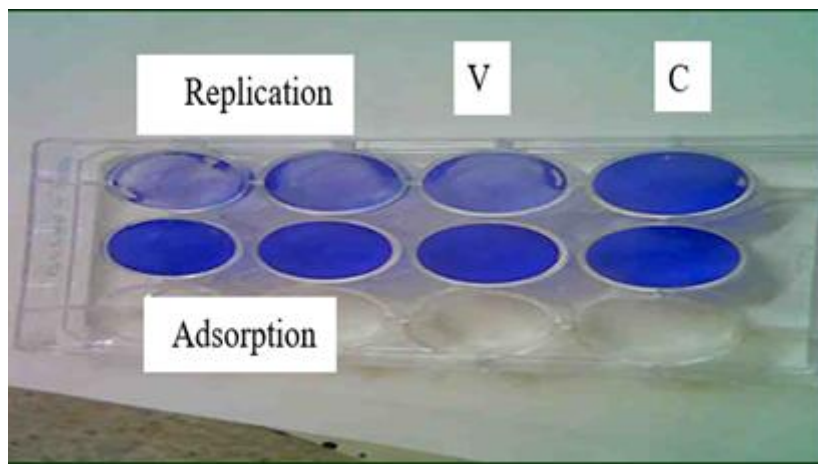
Treatments	Conc. ( $\mu\text{g/ml}$ )	Reduction %
Hexane	20	50
	40	57
Pet. ether	20	73
	40	>99.9
Ethyl acetate	20	46
	40	55
Methylene chloride : Methanol (1:1, v/v)	20	0
	40	15
Water	20	>99.9
	40	>99.9



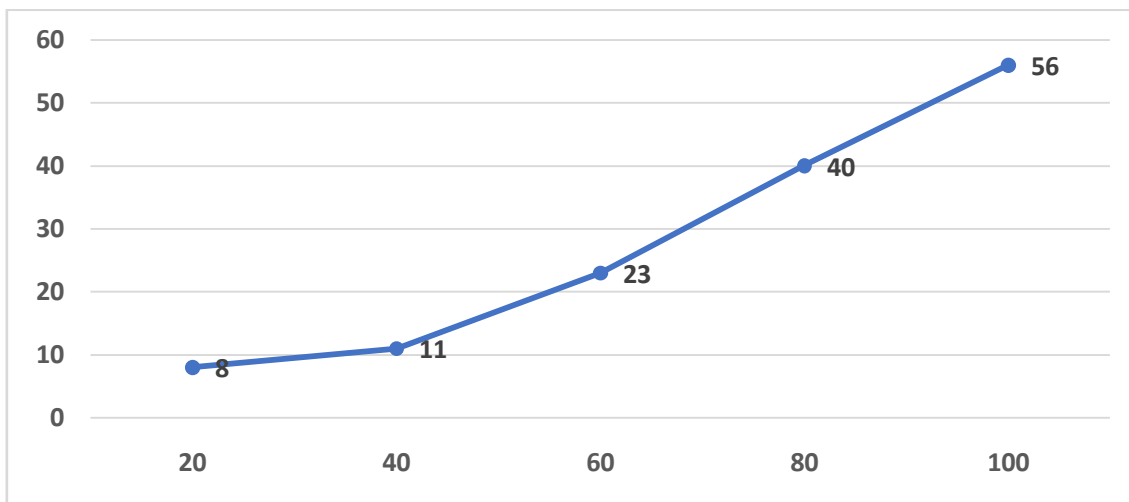
**Figure 2:**Effect of algal extract against H5N1 virus C= Cell control; V= Virus control; 20, 40 = concentration/ $\mu$ g of algal extract used in treating each well. Color wells= no viral growth; dotted wells= obvious virus growth.



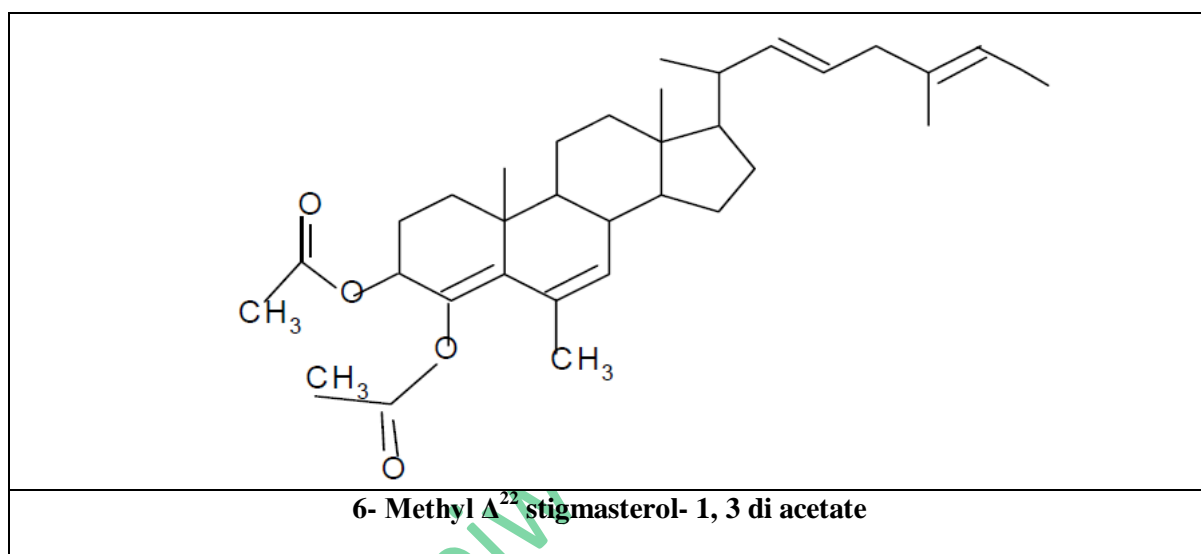
**Figure 3:** *In vitro* mode of action of ASP algal extract on H5N1 infection.



**Figure 4:***In vitro* mode of action of Algal extract on H5N1 infection. C= Cell control; V= Virus control; Color wells= no viral growth; dotted wells= obvious virus growth.



**Figure 5: The Antiviral activity of different concentration of Asparagopsis pure compounds against H5N1 virus.**



**Figure 6: Suggested chemical structure of active ingredients separated from macroalgae (*Asparagopsis taxiformis*).**



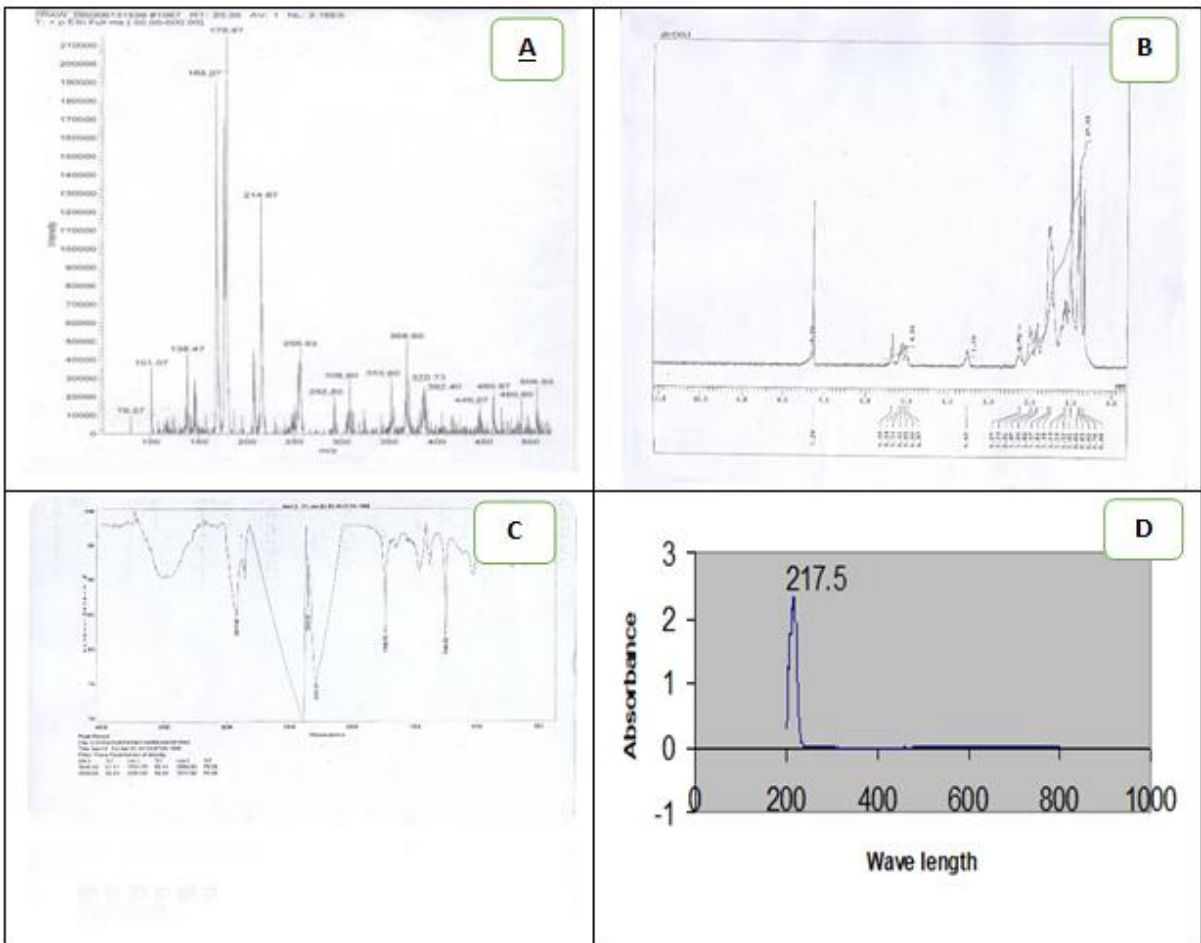


Figure 7: The spectroscopic analysis of isolated compound from Asp. A: Positive ESI/MS spectrum of compound; B: <sup>1</sup>H NMR spectrum; C: IR spectrum and D: UV spectrum

Reviewer's