Effect of Some Egyptian Natural Products Against Highly Pathogenic Avian Influenza Virus H5N1

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ABSTRACT

BACKGROUND: New prophylactic and therapeutic tools are needed for the treatment of the highly pathogenic avian influenza HPAI-H5N1 virus. Several natural products as seaweeds, plants and isolated compounds have shown to possess antiviral activity against a wide spectrum of viruses.

AIM: The present study was assessed to investigate the effect of some Egyptian natural products of marine algae (Egyptian isolates), plant extracts and isolated compounds against HPAI-H5N1 virus. CONCLUSION: Eighteen Egyptian natural product samples of marine algae, plants as well as isolated compounds were screened for their inhibitory activity against HPAI-H5N1 virus. Marine algae extracts of *Jania rubens* L. (red alga) and *Sargassum subrepandum* (brown alga) showed an activity against HPAI-H5N1 virus but there is no activity was recorded for *Ulva lactuca* Linn. (green algae). The aqueous or ethanol extracts of *Amphilophium paniculatum* L. Kunth (Bignoniaceae), *Artemisia cina* O. Berg (Asteraceae), *Azadirachta indica* A. Juss (Meliaceae), *Ipomoea batatas* L. Lam (Convolvulaceae), *Polygonum salicifolium* (Polygonaceae) and *Thunbergia erecta* (Acanthaceae) showed anti-influenza virus effects. Of these plants; Phytochemical investigation of ethanol extract of *Polygonum salicifolium* leaves had afforded the isolation of one anthraquinone; emodin and four flavonoid; quercetin 3-*O*-β-rutinoside (rutin), apigenin 8-*C*-glucopyranoside (vitexin), kaempferol 3-*O*-rhamnoside, and quercetin. Their structures were elucidated by spectroscopic methods, as well as comparison with reference compounds and published data. Of the isolated compounds; emodin, vitexin, and quercetin have shown anti-H5N1 avian influenza virus activity. This study is the first record in Egypt concerning the activity of Egyptian marine algae, medicinal plants, isolated anthraquinone and flavonoid compounds against HPAI-H5N1.

INTRODUCTION

During the past ten years a series of avian epidemics; a significant reemergence of highly pathogenic avian influenza (HPAI) H5N1; has been reported in several Asian countries including Hong kung, Korea, Japan, Taipei, Cambodia, Laos, Pakistan, China, Indonesia and Thailand, with additionally confirmed cases of human death in Vietnam *(1)*. During 2005 and early 2006 the HPAI H5N1 virus continued to circulate world-wide reaching Europe, Turkey, Iran Israel then transmitted to Nigeria and finally Egypt at February 2006.

At February 2006 HPAI -H5N1 virus has emerged in Egypt causing severe epidemic in poultry industry that resulted in a major economic losses due to the high mortalities among infected poultry farms besides the cost of eradication of poultry culling policy that had been granted to stop the epidemic *(2)*.

Also in Egypt the H5N1 AI virus has killed 23 human cases up to date. In spite of vaccination programs against AI; using inactivated oil emulsion H5N1 and/or H5N2 vaccines; a second and third wave of AI outbreaks had been recorded in Egypt at 2007 and early 2008 *(3)*. With continued outbreaks of the H5N1virus in poultry and wild birds, further human cases are likely, and the potential for the emergence of a human adapted H5 virus, either by reassortment or mutation, is a threat to public health worldwide.

Limitations in our current HPAI treatment options and the continuing outbreaks of the H5N1 virus have contributed to a growing need for new and effective chemotherapeutic agents to treat viral diseases.

Recently, marine algae have provide a rich source of natural bioactive compounds with different activities as antiviral *(4-8)*.

The control and treatment of influenza depends mainly on chemical or biochemical agents and, to date, some anti-influenza agents have been isolated from plants as a result of chemical and pharmacological studies. There are many chemical constituents (termed phytochemicals) found in plant medicines that have beneficial pharmacological effects in humans. Some bioactive phytochemicals include tannins, resins, polysaccharides, saponins, glycosides, and volatile oils among others. These agents include also a variety of polyphenols, anthraquinones, and flavonoids *(9)*.

Interest in a large number of traditional natural products has increased *(10-12)*. It has been suggested that aqueous and ethanolic extracts from plants used in allopathic medicine are potential sources of antiviral Furthermore; the selection of crude plant extracts for screening programs has the potential being more successful in its initial steps than screening of pure compounds isolated from products *(10)*.

The aim of this study was to search for anti avian influenza agents from Egyptian natural sources. Eighteen natural products of the extract of three seaweeds, ten plants as well as one anthraquinone; emodin **3** and four flavonoid compounds; rutin **1**, vitexin **2**, kaempferol 3-rhamnoside **4** and quercetin **5** isolated from the bioactive extract of *Polygonum salicifolium* (Polygonaceae) leaves were studied attempting to explore their effects against HPAI -H5N1.

EXPERIMENTAL

Materials and Methods

Natural products material

1. Marine Algal source

Jania rubens L. (red alga) and *Ulva lactuca* Linnaeus (green alga) seaweed samples were collected from El kantara, military region, Suez Canal, Ismalia, Egypt (3-5 meter depth). *Sargassum subrepandum* (brown alga) sample was collected from Preserve Nabk, Gar' ana region, Sharm El-Sheikh, Red Sea, Egypt (depth of 4 meter). All the seaweeds were collected in August, 2006. They were kindly identified by Dr. Mohammed Masoud Hijazi, Ass. Prof. marine plants, Faculty of Science, Suez Canal University.

2. Plants source

Plant leaves samples of *Amphilophium paniculatum* L. Kunth (Bignoniaceae), *Thunbergia erecta* (Acanthaceae) were collected from El-Orman public garden, Giza. *Azadirachta indica* A. Juss (Meliaceae) leaves were collected from the Ministry of Agriculture, Giza, Egypt. *Putranjiva roxburghii* (Euphorbiaceae) and *Sapium sebiferum* (Euphorbiaceae) were collected from Giza Zoo, Egypt. *Ipomoea batatas* L. Lam (Convolvulaceae) and *Polygonum salicifolium* (Polygonaceae) leaves were collected from the desert and cultivated fields in Bani Suef, respectively. Herbs of *Apium graveolens* L. (Umbelliferae); known as Celery or Karafs and flower buds of *Artemisia cina* O. Berg (Asteraceae); known as Tarragon or Shih balady as well as leaves of *Cymbopogon citratus* (D. C. ex. Nees) Stapf (Gramineae); known as Lemon grass or Hashishet el-lymoon were obtained from local markets. Plant identification was confirmed by T. Labib, head specialist for plant identification in El-Orman public garden, Cairo, Egypt. A voucher specimen was deposited at the Department of Chemistry of Natural Compounds, NRC. All plant samples were collected during the period 2006-2007.

Virus

Highly pathogenic avian influenza (HPAI) H5N1 virus A/Chicken/ Egypt/9402 NAMRU 3-CLEVB 213/ 2007 (H5N1) of accession No. EU 623467, was used at titre of 10^6 EID_{50}/ml (embryo infective dose fifty per ml).

Specific pathogen free eggs (SPF eggs)

Four hundred and eighty SPF embryonated chicken eggs were used at nine days old and inoculated *via* the allantoic sac route. SPF eggs were obtained from Nile SPF eggs, Koom Oshiem, Fayoum, Egypt.

Material for Chromatography

Precoated silica gel 60 F_{254} plates (20x20 cm, 0.2 cm thickness, Merck, Dermstadt, Germany) were used for thin layer chromatography (TLC). For column chromatography, Sephadex LH-20 (Pharmacia Fine Chemicals AB, Uppsala, Sweden), microcrystalline cellulose (Merk, Dermstadt, Germany), Polyamide S (Fluka, Steinheim, Switzerland) were used. For paper chromatography: two dimension paper chromatography (2D-PC) and comparative paper chromatography (CO-PC); Whatman No. 1 sheets (Whatman ltd., Maidstone, Kent, England) were used.

Solvent systems

S_1: *n*-BuOH/HOAc/H_2O (4:1:5 v/v/v, top layer); S_2: 15 % aqueous HOAc; S_3: $CHCl_3$/ MeOH (8: 2 v/v); S_4: Hexane/EtOAc (8: 2 v/v); S_5: MeOH/ EtOAc/ $CHCl_3$/H_2O 35: 32: 28: 7 v/v/v/v solvent systems were used for analytical grade.

Spray reagents

I. Naturstoff reagent (NA/PE) [1% diphenyl boryloxyethanolamine in ethanol (a), 5% polyethylene glycol 400 in methanol (b), heating the dry chromatogram at 120 °C for 10 min. and showed under UV light (365 nm)]. II. $FeCl_3$ (1% ethanol), III. $AlCl_3$ (1% ethanol), and III. *p*-Anisaldehyde-sulphuric acid.

Apparatus

Koffler's heating stage microscope. UV analyses for pure samples were recorded, separately, as MeOH solutions and with different diagnostic UV shift reagents on a Shimadzu UV 240 (P/N 240 –58000). The NMR spectra were recorded at 300 (^{1}H) and 75 (^{13}C) MHz, on a Varian Mercury 300 NMR spectrometer and δ-values are reported as ppm relative to TMS in the convenient solvent.

Extraction of seaweed samples

After elimination of epiphytes and stones, the algal samples were thoroughly washed with running water and rinsed many times in distilled water. A fresh sample (50 g) of each of the whole alga of *Jania rubens*, *Ulva lactuca* and *Sargassum subrepandum* was homogenized in a mixer with dichloromethane / methanol (400 ml; 2:1, v/v), followed by exhaustive extraction by maceration at room temperature with the same solvent. The combined extracts of each alga sample were evaporated under vacuum at a temperature not exceeding 40°C. The yield of extraction was 5.6, 7.4 and 3.4 g, represented 11.1, 14.8 and 6.8 % on wet weight basis, respectively, which was the average of three replicate. The dried extracts were kept at -10°C when not in use.

Extraction of plant samples

The air dried sample (50 g) of leaves of each of *Amphilophium paniculatum* L. Kunth, *Azadirachta indica* A. Juss, *Cymbopogon citrates* (D. C. ex. Nees) Stapf, *Ipomoea batatas* L. Lam, *Polygonum salicifolium*, *Putranjiva roxburghii*, *Sapium sebiferum*, and *Thunbergia erecta*, as well as the herb of *Apium graveolens* L. and flower buds of *Artemisia cina* L. were exhaustively extracted by Soxhlet apparatus with ethanol 95 %. Each extract was concentrated and evaporated to dryness on a rotary evaporator. All the crude extracts were saved in -10°C. The plant samples yield were; 4.2, 5.4, 2.8, 3.2, 3.9, 2.8, 1.9, 5.1, 2.9, and 3.3g, respectively represented 8.4, 10.8, 5.6, 6.4, 7.8, 5.6, 3.8, 10.2, 5.8, and 6.6 % on dry weight basis, respectively.

Extraction and isolation of compounds

The powdered *Polygonum salicifolium* leaves (1 kg) were exhaustively extracted by Soxhlet apparatus with ethanol 95 %. The extract was concentrated and evaporated to dryness on a rotary evaporator. The crude extracts to give 78.5 g (7.85 % dry weight basis). The residue obtained was preliminary fractionated on a polyamide column (300 g, 110 X 7 cm). Elution was carried out using water, water-methanol mixture with step gradient decreasing polarity till methanol. The elution was monitored by comparative paper chromatography (Co-PC) using solvent systems S_1 and S_2, UV-light, and spray reagent I or II for detection. Fractions of similar chromatographic profile were pooled together, concentrated and dried under vacuum to yield four major collective fractions.

These fractions were chromatographed on different columns viz., microcrystalline cellulose (20-40 % aqueous ethanol as an eluent) and Sephadex LH-20 (*n*-BuOH-isopropanol-H_2O, 4:1:5 v/v/v, top layer), methanol or methanol-water (9:1) as different eluents). All separation processes were followed by TLC with solvent system S_3-S_5 or 2D-PC with S_1 and S_2 solvent systems. Five compounds were isolated. These compounds were subjected to physical, chemical, chromatographic and spectral analysis of UV and ^{1}H NMR.

Characterization of the isolated compounds

Compound 1: Yellow powder, m.p. 185-187 °C. **R_f values:** 0.33 (S_1); 0.52 (S_2). **UV spectral data:** λ_{max}, nm MeOH 257, 360. +NaOMe: 272, 329, 409. +NaOAc: 270, 327, 337, 388. NaOAc+H_3BO_3: 262, 311, 314, 382; +$AlCl_3$: 275, 426; +$AlCl_3$/HCl: 268, 299, 366, 400. **^{1}H NMR** (300 MHz, DMSO-d_6) δ ppm 7.5 (2H, d, *J*=8.0 Hz, H-2'/6'), 6.8 (1H, d, 8.0 Hz, H-5'), 6.3 (1H, d, *J*=2.2 Hz, H-8), 6.1 (1H, d, *J*=2.2 Hz, H-6), 5.3 (1H, d, *J*=7.5 Hz, H-1''), 4.4 (1H, d, *J*=1.2 Hz, H-1'''), 3.1-3.9 (m, two sugar protons) and 1.05 (3 H, d, *J*=6.0, CH_3-rhamnosyl).

Compound 2: Yellow amorphous powder, **R_f values:** 0.41 (S_1), 0.16 (S_2). **UV spectral data:** λ_{max}, nm MeOH 271, 305, 338; +NaOMe: 278, 301, 345, 385; +NaOAc: 280, 330, 395; NaOAc+H_3BO_3: 279, 303, 379; +$AlCl_3$: 271, 301, 337; +$AlCl_3$/HCl 278, 302, 352, 390. **^{1}H NMR** (300 MHz, DMSO-d_6): δ ppm 6.77 (s, H-3); 6.27 (s, H-6), 8.02 (d, *J*=8.4, H-2'/6'), 6.89 (d, *J*=8.4, H-3'/5'), 4.69 (d, *J*=9.6, H-1''), 3.8-3.7 (m, H-2''), remaining sugar protons hidden by H_2O-signal.

Compound 3: Yellow-brown solid, **m.p.** 255-256 °C, **R_f values:** 0.72 (S_5). **UV spectral data:** λ_{max}, nm MeOH: 291 and 450. **^{1}H-NMR** (300 MHz, $CDCl_3$): δ ppm 12.30 (s, OH-8), 12.12 (s, OH-1), 7.63 (1H, d, *J* =1.2 Hz, H-4), 7.29 (1 H, d, *J* = 1.2 Hz, H-5), 7.10 (1 H, d, *J* =1.2 Hz, H-2), 6.68 (1H, d, *J* =2.4 Hz, H-7), 2.46 (3 H, s, CH_3-3).

Compound 4: Yellow powder, **m.p.** 172-175 °C, **R_f values**: 0.75 (S_1), 0.50 (S_2); **UV spectral data:** λ_{max}, nm MeOH: 263, 345; +NaOMe: 268, 223, 378; +NaOAc: 270, 302, 355; NaOAc+H_3BO_3: 270, 344; +$AlCl_3$: 272, 346, 397; +$AlCl_3$/HCl: 270, 340, 396. **^{1}H NMR** (Acetone): δ ppm 7.81 (2H, d, *J* = 8.5 Hz, H-2'/ H-6'), 6.8 (2H, d, *J* = 8.5 Hz, H-3'/ H-5'), 6.43 (1H, d, *J* = 2 Hz, H-8), 6.15 (1H, d, *J* = 2 Hz, H-6), 5.40 (1H, d, *J* = 1.5 Hz, H-1''), 3.80 (1H, dd, *J* =9.8, 3.4 Hz, H-3'', 3.1-3.4 (2 H, m, H-4'' and H-5''), 0.80 (d, *J* = 5.98 Hz, CH_3-rhamnose).

Compound 5: Yellow needle crystals, **m.p.** 316-318 °C, **R_f values:** 0.56 (S_4). **^{1}H NMR** (DMSO-d_6, 300 MHz): δ ppm 12.50 (brs, 5-OH), 7.65 (1 H, d, *J* = 2 Hz, H-2'), 7.55 (1 H, d, *J* = 8 Hz, H-6'), 6.90 (1 H, d, *J* = 8 Hz, H-5'), 6.41 (1 H, d, *J* = 2.5 Hz, H-8), 6.15 (1 H, d, *J* = 2.2 Hz, H-6)

Preparation of natural products for biological activity

Natural product samples of the prepared extracts of three seaweeds and ten plants as well as the isolated five compounds **1-5** were used. Each natural product sample (30 mg) were dissolved in 2 ml dimethylsulfoxide and filtered through a sterile Millipore filter (0.22 m). Each extract was used at three different concentrations. In the first, each extract was used in undiluted form. In the second, each

extract was diluted to 1/5 in sterile saline and in the third, at a dilution of 1/10 in sterile saline.

Haemagglutinating activity Assay

Haemagglutinating activity of the allantoic fluids of the inoculated eggs as measured by micro technique of haemagglutination test (HA) *(13).*

Experimental Design for biological activity

Three experiments were conducted.

***Experiment I*:** Two hundred and eighty SPF embryonating chicken eggs (ECE_s) were used in this experiment. Each volume of HPAI H5N1 virus was mixed with an equal volume of the original extracts at three levels:

Level I: equal volume of the HPAI H5N1 virus was mixed with equal volume of the original undiluted natural product sample and inoculated for one hour at room temperature then inoculated into the allantoic sac of five ECE_s for each natural product sample at dose of 0.2 ml/ECE.

Level II: equal volume of the HPAI H5N1 virus was mixed with equal volume of the 1/5 dilution of each natural product sample and were proceeded as level I.

Level III: equal volume of the virus was mixed with equal volume of the 1/10 dilution of each natural product sample and were proceeded as level I.

Beside five ECE_s were inoculated with the HPAI H5N1 virus that mixed with equal volume of saline at a dose of 0.2 ml/ECE (positive control) and five ECE_s were inoculated with 0.2 ml/ECE of saline alone (negative control). The ECE_s are incubated at 37 °C and candled every two hours till all the positive control ECE_s are died.

***Experiment II*:** Hundred SPF ECE_s of nine days old were used in this experiment. Equal volume of the HPAI H5N1 virus was mixed with equal volume of the original natural product sample and inoculated directly into the allantoic sac of five ECE for each natural product sample at a dose of 0.2 ml/ECE. Five ECE_s were inoculated with equal volume from the HPAI H5N1 virus and saline at dose of 0.2 ml/ECE (positive control). Other five ECE_s were inoculated with 0.2 ml/ECE of saline alone (negative control). All the ECE_s were incubated at 37°C and candled every two hours till the ECE of the positive control are died.

***Experiment III*:** Hundred SPF ECE_s of nine days old were used in this experiment. 0.1 ml of the HPAI H5N1 virus was inoculated *via* the allantoic sac of each ECE into 90 ECE_s, and then the ECE_s were incubated for one hour at 37 °C. The original natural product sample was inoculated into five ECE_s; that previously inoculated with the virus; at a dose of 0.1 ml. Five ECE_s were inoculated with 0.2 ml/ECE of the mixed HPAI H5N1 virus and saline. Other five were inoculated with 0.2 ml/ECE of saline alone. the ECE_s were incubated at 37 °C and candled every two hours till the ECE of the positive control are died.

RESULTS AND DISCUSSION

All the embryos of the positive controls were died and the allantoic fluid of each was positive for haemagglutination assay (HA), while all the embryos of negative controls were not died and the allantoic fluid of each was negative for HA.

The selected medicinal plants have various traditional uses including cold and flu (Table 1).

Eleven samples of code number 1, 3, 4, 6, 7, 8, 10, 12, 15, 16 and 18 of natural product *Amphilophium paniculatum*, *Artemisia cina*, *Azadirachta indica*, emodin, *Ipomoea batatas*, *Jania rubens*, *Polygonum salicifolium*, quercetin, *Sargassum subrepandum*, *Thunbergia erecta and* vitexin, respectively showed antiviral against HPIA –H5N1 (Table 2). Each of these samples showed antiviral effect when inoculated with H5N1 virus one hour before inoculation into nine days old ECE_s. While, they had not any effect on the virus when inoculated simultaneously with the virus just after mixing or after the virus inoculation by one hour.

The results of investigation of activity of some Egyptian natural products against the HPAI H5N1 (Table 2) showed that marine algal extracts of red alga; *Jania rubens* (sample code no. 8) and brown alga; *Sargassum subrepandum* (sample code no.15) possessed activity against HPAI-H5N1 but there is no activity was recorded for green alga (*Ulva lactuca*). The plant extracts of *Amphilophium paniculatum* L. Kunth (Bignoniaceae), *Artemisia cina* O. Berg (Asteraceae), *Azadirachta indica* A. Juss (Meliaceae), *Ipomoea batatas* L. Lam (Convolvulaceae), *Polygonum salicifolium* (Polygonaceae), and *Thunbergia erecta* (Acanthaceae) of sample code no. 1, 3, 4, 7 10, and 16, respectively.

Phytochemical investigation of bioactive *Polygonum salicifolium* leaves extract (sample code no.10) resulted in the isolation of four flavonoid compounds; rutin, vitexin, kaempferol 3-*O*-rhamnoside and quercetin along with one anthraquinone; emodin.

Emodin **3** (code sample no. 6), quercetin **5** (code sample no. 12) and vitexin **2** (code sample no. 18) were the compounds showed activity against HPAI H5N1 These products showed antiviral effect when incubated with the HPAI H5N1 virus one hour before incubation into the nine days old ECE_s, while they had not any effect on the virus when inoculated simultaneously with the virus just after mixing or after the virus inoculation by one hour.

This study showed that simultaneous inoculation of the natural product or even after infection was of no value. In spite of that it may be useful if the study was repeated and inoculate the natural product samples before the virus by enough time at least six hours for ECE or 24 hrs in chickens ***(14)***. Finally, the outcome of this study is the possibility of using of such natural products of marine algae, total crude plant extracts and isolated bioactive compounds in the production of effective antiviral products after applying the known roles concerning the use of these natural products.

This is a first record of the activity of Egyptian seaweeds and crude plant extracts against the highly pathogenic avian influenza virus H5N1 causing "bird flu"

Table 1. Egyptian medicinal seaweeds and plants under investigation

Sample Code number	Natural product	Part used	Folk medicine
1	*Amphilophium paniculatum* (L.) Kunth (Bignoniaceae)	leaves	Stomach troubles, cold, flu
2	*Apium graveolens* L.(Umbelliferae)	herb	Flu, Antispasmodic diuretic, carminative
3	*Artemisia cina* O. Berg (Asteraceae)	flower buds	Anthelmentic
4	*Azadirachta indica* A. Juss (Meliaceae)	aqueous extract leaves	A paste made with leaves is used for the cure of chicken pox, smallpox and warts. Insect repellent, remove toxins purified blood
5	*Cymbopogon citratus* (D. C. ex. Nees) Stapf (Gramineae)	leaves	Treatment of cough and flu, Antispasmodic, antiseptic, insect repellent
7	*Ipomoea batatas* L. Lam (Convolvulaceae)	leaves	leaf decoction used in tumors of the mouth and throat. Tonic demulcent, fungicide, bactericide
8	*Jania rubens* (Linnaeus) Lamouroux. (red algae)	whole red algae	-
10	*Polygonum salicifolium* (Polygonaceae)	leaves	Burned ashes are licked as a cure for sore throat. and extract from fresh leaves is used for skin troubles
11	*Putranjiva roxburghii* (Euphorbiaceae)	leaves	cold, fever, and rheumatism
14	*Sapium sebiferum* (Euphorbiaceae)	leaves	Skin problem, cold
15	*Sargassum subrepandum* (brown algae)	whole brown algae	-
16	*Thunbergia erecta* (Acanthaceae)	leaves	Remove toxins, detoxicant actions, in cases of alcohol abuse (hangover).
17	*Ulva lactuca* Linnaeus (green algae)	whole green algae	-

Table 2. Effect of various natural products on HPA1 H5N1 virus at different concentrations

Code of sample	Nature of Product	Natural Product	Experiment I						Experiment II		Experiment III	
			L I		L II		L III					
			NDE	+ HA	NDE	+ HA	NDE	+ HA	NDE	+ HA	NDE	+HA
1	Plant extract	*Amphilophium paniculatum*	0/5	0	4/5	4	5/5	5	5/5	5	5/5	5
2	Plant extract	*Apium graveolens.*	0/5	0	5/5	5	5/5	5	5/5	5	5/5	5
3	Plant extract	*Artemisia cina*	0/5	0	5/5	5	5/5	5	5/5	5	5/5	5
4	Plant extract	*Azadirachta indica*	0/5	0	4/5	4	5/5	5	5/5	5	5/5	5
5	Plant extract	*Cymbopogon citratus*	5/5	5	5/5	5	5/5	5	5/5	5	5/5	5
6	Isolated anthraquinone (compound **3**)	Emodin	0/5	0	5/5	5	5/5	5	5/5	5	5/5	5
7	Plant extract	*Ipomoea batatas*	0/5	0	5/5	5	5/5	5	5/5	5	5/5	5
8	Seaweed extract	*Jania rubens*	0/5	0	5/5	5	5/5	5	5/5	5	5/5	5
9	Isolated flavonoid (compound **4**)	Kaempferol 3-*O*-rhamnoside	5/5	5	5/5	5	5/5	5	5/5	5	5/5	5
10	Plant crude extract	*Polygonum salicifolium*	0/5	0	5/5	5	5/5	5	5/5	5	5/5	5
11	Plant crude extract	*Putranjiva roxburghii*	5/5	5	5/5	5	5/5	5	5/5	5	5/5	5
12	Isolated flavonoid (compound **5**)	Quercetin	0/5	0	5/5	5	5/5	5	5/5	5	5/5	5
13	Isolated flavonoid (compound **1**)	Rutin	5/5	5	5/5	5	5/5	5	5/5	5	5/5	5
14	Plant extract	*Sapium sebiferum*	5/5	5	5/5	5	5/5	5	5/5	5	5/5	5
15	Seaweed extract	*Sargassum subrepandum*	0/5	0	5/5	5	5/5	5	5/5	5	5/5	5
16	Plant crude extract	*Thunbergia erecta*	0/5	0	5/5	5	5/5	5	5/5	5	5/5	5
17	Seaweed extract	*Ulva lactuca* Linnaeus	5/5	5	5/5	5	5/5	5	5/5	5	5/5	5
18	Isolated flavonoid (compound **2**)	Vitexin	0/5	0	5/5	5	5/5	5	5/5	5	5/5	5

(no. **1**): Compound number **1**; (no. **2**): Compound number **2**; (no. **3**): Compound number **3**; (no. **4**): Compound number **4**; (no. **5**): Compound number **5**; NDE: Number of died; + HA: Haemagglutination Assay; L I: level I; L II: level II; L III: level III;

Phytoconstituents

Compound **1** showed dark purple color under UV change to yellow with ammonia or $AlCl_3$. UV spectrum of the compound showed the presence of free hydroxyl groups at positions 3', 4', 5, and 7. The *O*-glycosylation at position 3 was confirmed by complete acid hydrolysis which gave quercetin, glucose and rhamnose identified by Co-chromatography. The ^{1}H NMR spectrum showed two overlapping doublets at δ 7.50 for H-2' and H-6', a doublet at δ 6.8 with *J*=8.0 Hz for H-5' due to *ortho* coupling with 6' and two aromatic protons at δ 6.3 and 6.10 each appear as a doublet with *J*=2 hz due to meta coupling assigned to H-8 and H-6, respectively. Also, the ^{1}H NMR spectrum revealed the anomeric proton of the glucose moiety as a doublet at δ 5.3 with *J*=7.5 Hz indicated by its downfield shift, the linkage between signal at δ 4.40 appeared as a doublet with *J*=1.2 Hz and methyl rhammnosyl protons signal at δ 1.05 as a doublet with *J*=6.0 Hz. The configuration of the glucose and rhamnose moieties was evidenced by their respective coupling constants 7.5 Hz and 1.2 Hz as β-glucose and α-rhamnose. Compound **1** was identified as rutin (quercetin 3-*O*-β-rutinoside) =quercetin 3-*O*-α-L-rhamnosyl- (1'''-6'')-*O*-β-D-glucoside by comparison with the published data *(15)*.

Compound **2** gave deep purple color under UV. A dull yellow was obtained when exposed to ammonia vapor.With $FeCl_3$ it gave green color. NA/PE spray reagent (365 nm) gave greenish yellow color spot. It was expected to be apigenin *C*-glycoside like structure on the basis of their UV spectral data in methanol and different diagnostic shift reagents *(16)*.

On complete hydrolysis, it remains without any change (Co-PC) to support the evidence of their C-glycoside structure. ^{1}H NMR spectrum exhibit spin coupling system of the two *ortho* doublets, each integrated for two equivalent protons, at δ values of about 7.90 and 6.88 ppm, assigned to H-2'/6' and H-3'/5' of 1, 4 distributed B-ring. Absence of H-8 resonance and, was indicative to 8-*C*-glycoside structure of the compound. The presence of one *C*-glycoside in the structure was evidenced from the one anomeric proton at δ 4.69 with large *J* value (>9 Hz); which is characteristic for β-*C*-Glycoside. Accordingly the compound was identified as vitexin (apigenin 8-*C*-β-D-$^4C_1$-glucopyranoside) as compared with authentic sample and published data *(17)*.

^{1}H NMR spectrum of compound **3** showed two singlets each of one H at δ 12.30 and 12.12 pointing to two *peri*-hydroxy protons present in C-8 and C-1, respectively, around the quinone system. The spectrum displayed also two aromatic systems bear individually two *meta*-coupled protons and one of them bear an aromatic bounded methyl group at δ 2.46. The compound was identified as emodin, which was further recognised during comparison the data with literature *(18)* as well as authentic sample.

Compound **4** gave purple under UV lamp changing to yellow on exposure to ammonia or spraying with spray reagent III. The chromatographic properties of the compound and UV spectral data similar to those of kaempferol-3-*O*-rhamnoside *(18)*. Complete acid hydrolysis yielded kaempferol as aglycone and rhamnose (as detected by Co-PC with sugar authentic sample. The compound was identified as kaempferol-3-*O*-rhamnoside by comparison with authentic sample and with published data *(18)*.

The chromatographic properties of compound **5** using Co-PC and, UV spectral data with shift reagents and ^{1}H NMR was identical with flavonol aglycone nature of 3, 4, 7, 3', 4'- pentahydroxy-flavone which related to the previously discussed compound (1) aglycone that of quercetin *(17)*.

So, phytochemical investigation of bioactive *Polygonum salicifolium* leaves extract resulted in the isolation of four flavonoid compounds; rutin, vitexin, kaempferol 3-*O*-rhamnoside and quercetin as well as one anthraquinone; emodin. The structure of these isolated compounds was established applying physicochemical and spectral techniques (PC, TLC, UV and NMR analyses), as well as comparison with

reference compounds and published data *(15-18)*.

Investigation activity of isolated compounds activity

The five isolated compounds were tested for their activity against the high pathogenic influenza virus H5N1. The tested anthraquinone; emodin showed activity against HPAI H5N1. Also, quercetin and vitexin were the flavonoid compounds showed activity These products showed antiviral effect when incubated with the HPAI H5N1 virus one hour before incubation into the nine days old ECE_s, while they had not any effect on the virus when inoculated simultaneously with the virus just after mixing or after the virus inoculation by one hour.

Anthraquinone are bioactive compounds of various activities. Recently a Chinese patent was recorded concerning the use of anthraquinone derivatives for preparation of medicaments against influenza and 'bird flu' virus. Emodin was one of the bioactive anthraquinone derivatives comprised in patent. The dosage forms of which are capsules, tablets, granules and injections *(19)*.

Flavonoids are polyphenolic compounds that comprise a vast array of biologically active compounds ubiquitous in plants.

Different bioactivities were recorded for flavonoids as anti influenza activity *(20)*. Droebner and coworkers at 2007 reported antiviral activity of a polyphenol-rich plant extract against a highly pathogenic avian influenza A virus (H7N7) in cell culture and in a mouse infection model *(21)*.

Some compounds which possess antiviral activities may be derived from the flavonoid of quercetin. The mode of action of quercetin against HSV-1 and ADV-3 was found to be at the early stage of multiplication *(22)*. Another study showed the combination of quercetin with shikimic acid (which is used for the synthesis of Tamiflu), even at low doses, may be effective for the modulation of innate immunity in antiviral terms *(23)*.

Quercetin was introduced as nutritional supplement formula taken prophylactically, and throughout the duration and recovery of an H5N1 infection *(24)*.

The role of quercetin may return to its effect on monooxygenase activities by restores oxidative damage mostly in the target organ of the infection and inducing the level of cytochrome P-450 *(25)*.

During influenza virus infection, there is "oxidative stress." Because quercetin restored the concentrations of many antioxidants, it is proposed that it may be useful as a drug in protecting the lung from the deleterious effects of oxygen derived free radicals released during influenza virus infection *(26, 27)*.

There is growing evidence of an anti-viral activity of quercetin, both *in vivo* & *in vitro*, most marked against *para*-influenza 3, herpes type 1, polio virus type 1 and respiratory syncytial virus *(28)*.

Vitexin, identified as apigenin-8-*C*-β-D-glucopyranoside. It is a flavones flavonoid compounds. vitexin demonstrated antiviral activity against parainfluenza type 3 (Para 3) virus *(29)*.

Flavone flavonoid, which have potent influenza virus sialidase inhibitory activity, have anti-influenza virus activity *in vivo (30)*.

Certain type of flavone flavonoid reduces the replication of mouse-adapted influenza virus by inhibiting the fusion of the virus with endosome/lysosome membrane which occurs at early stage of virus infection cycle *(31)*. Vitexin attenuated the protein level of HIF-1α in PC12 cells *(32)*. HIF-1α inhibitors may be useful for treating various angiogenesis-related diseases associated with the over-activation of HIF-1α. The angiogenesis and metastasis frequently observed in different diseases might be blocked by vitexin. So, they suggested the potential use of vitexin as a treatment for many diseases.

Recently, the study of structure–activity relationship of flavonoids as influenza virus neuraminidase inhibitors and their anti-viral activities recorded high potency of flavone and

flavonol class of flavonoids as anti-influenza ***(14, 19)***.

Investigation of marine algae activity

Marine seaweed extracts of *Jania rubens* L. (red algae) and *Sargassum subrepandum* (brown algae) showed an activity against HPAI-H5N1 virus.

Jania rubens extract products were reported to contain many of the biological active compounds as poly unsaturated fatty acids, glycolipids, steroids, carotenoids, phenolics and terpenoids ***(33,34)***. In our current study; the extraction process with organic solvent (dichloromethane/methanol; 1:1, v/v) was able to extract the lipophilic previously mentioned bioactive compounds.

Brominated diterpenes were previously isolated from *Jania rubens* collected from Red Sea coast of Egypt ***(33)***.

According to marine alga chemical structure, most of isolated compounds belong to sulfated polysaccharides, phenolics, terpenoids, lactones, sterols and fatty acids ***(8, 34, 35)***. The fact that there are many algae that can convert simple polyunsaturated fatty acids such as arachidonic acids into complex eicosanoids and related oxylipins has been an exiting development ***(36)***.

Certain red marine algae extract was reported to inhibit the reproduction of influenza A *in vitro* and *in vivo* ***(4)***. The virus induced cytopathogenic effect, infectious virus yields and the production of hemagglutinin were all reduced at non toxic levels of the extract. The inhibition affected absorption as well as intracellular stages of viral replication.

Recently, high antiviral activity was also recorded for the seaweed *Sargassum* ***(37)***.

Phlorotannins, phenolic compounds and diterpenediol are reported to be produced by brown algae *Sargassum* and was characterized by having high amount of iodine ***(38)***.

Investigation of medicinal plant extracts activity

The aqueous or ethanol extracts of *Amphilophium paniculatum* L. Kunth (Bignoniaceae), *Artemisia cina* O. Berg (Asteraceae), *Azadirachta indica* A. Juss (Meliaceae), *Ipomoea batatas* L. Lam (Convolvulaceae), *Polygonum salicifolium* (Polygonaceae) and *Thunbergia erecta* (Acanthaceae) showed anti-influenza effects.

Artemisia cina O. Berg (Asteraceae); known as Tarragon or Shih balady showed activity against HPAI-H5N1. *Artemisia* was reported to have antiviral activity by its ability to inactivate the virus and to inhibit the cell-to-cell virus ***(39)***.

Azadirachta indica A. Juss. (Meliaceae) Neem is a nature's pharmacy (Vietmeyer, 1992). Today, researchers are saying that neem could be called "a wonder tree" and eventually expect it to benefit everyone on the planet. Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally complex ***(40-42)***.

The main reported secondary metabolites of *Ipomoea batatas* (L.) Lam (Convolvulaceae) are proved to be flavonoids, anthocyanins pigments, resin glycosides (jalapins), polyphenolic acids, phospholipids, coumarins and lignans ***(43, 44)***. Bioactive compounds (as lignans) effect as antiviral were reported and their mode of action were shown. Lignans isolated from certain *Ipomoea* species inhibit strongly replication of human immunodeficiency virus type 1 by prevent the increase of topoisomerase II activity, involved in virus replication, after infection of cells with virus ***(45)***.

In this study; one of the medicinal plant extract showed anti-H5N1 activity was *Ipomoea batatas*. High pecentage of cyanoglucoside compounds in raw *Ipomoea batatas* was recorded ***(46)***. Recently, the bioactive cyanoglucoside isolated from *Codiaeum variegatum* showed anti-influenza virus activity ***(47)***.

Polygonum genus is the richest known source of resveratol which is reported to inhibit of influenza A virus replication ***(48)***. Several representative classes have been isolated from *Polygonum salicifolium* including quinines, stilben, phenol, tannins, flavonoids, terpenoids and catechol compounds ***(49-51)***. *Polygonum salicifolium* is rich of bioactive compounds as flavonoids.

Thunbergia belongs to the botanical family of Acanthaceae. Different *Thunbergia* plant species may be consumed as herbal tea. It is traditional medicine for protection against dietary and environmental toxicants with little substantiation. They were reported to have antimicrobial activity and used for reducing toxicity of insecticide and the treatment for drug addiction *(52)*.

Different *Thunbergia* species showed antiviral activity *(12, 53, 54)*. Chemical constituents of *Thunbergia* species were reported to be of iridoid glucosides, flavonoids and phenolic acids compounds class *(52, 55)*.

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الملخص العربى

تأثير بعض المنتجات الطبيعية المصرية المضاد لفيروس أنفلونزا الطيور عالي الضراوة أتش ٥ ان ١

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يسهم العديد من المنتجات الطبيعية مثل الكائنات البحرية و النباتات الطبية وكذا المركبات الطبيعية الفعالة المفصولة من أصل نباتى فى علاج الفيروسات المختلفة واسعة الانتشار.

تهدف هذه الدراسة لاستبيان تأثير منتجات طبيعية مصرية ضد فيروس أنفلونزا الطيور من عتره H5N1 عالى الضراوة . تشتمل المصادر الطبيعية على نوع من الطحالب البحرية البنية (سرجاسم سوبريباندم) و مصدره شرم الشيخ, ونوع من الطحالب الحمراء (جانيا روبنز) وأخر من الخضراء (أولفا لاكتيوسا) و مصدرهما الإسماعيلية . كما تم دراسة تأثير المستخلصات المائية أو الكحولية لعشرة نباتات طبية تنتمي إلى عائلات نباتية مختلفة منها المعروف في الطب التقليدي بفائدتها لعلاج البرد و الرشح أو مطهره أو لم يسبق اختبارها حيويا لاكتشاف مصادر جديدة ضد الفيروسات.

و كما تم اختبار تأثير خمس مركبات طبيعية تم فصلها من نبات بوليجونيم سالسيفوليم (عائلة البطباطيات) و الذي أعطى تأثيرا مضادا للفيروس موضع الدراسة. تشتمل المركبات على مركب من الأنثراكينونات هو ايمودين و أربع مركبات فلافونيدية و هى مركب روتين و فيتكسين و كامفيرول ٣- رامنوزايد.

تم فصل هذه المركبات باستخدام الطرق الكروماتوجرافية المختلفة مثل كروماتوجرافيا العامود وكروماتوجرافيا الورق و الطبقة الرقيقة و قد تم التعرف على الصيغ البنائية للمركبات مفردة باستقراء نتائج القياسات الطيفية المختلفة مثل الأشعة فوق البنفسجية و الرنين النووي المغناطيسي و مقارنتها بالعينات المرجعية و المراجع المنشورة السابقة.

أعطى الطحلب البني و الأحمر تأثيرا مضادا للفيروس المسبب لأنفلونزا الطيور من عتره H5N1 عالي الضراوة.

كما أعطت مستخلصات النباتات الطبية التالية تأثيرا مضادا للفيروس و هى:

نبات أمفيبيم بانكيولاتم (عائلة بيجنونيسى), نبات سيمبيرجيا بانكيولاتم (عائلة أكانسيسى), نبات أزدراختا انديكا(عائلة ماليسى), نبات ايبوميا بطاطس (عائلة ايفوربيسى), نبات أرتيميزيا سنا (عائلة أستريسى), نبات بوليجونيم سالسيفوليم (عائلة بوليجونيسى). كذلك أعطى ثلاث من المركبات المفصولة : ايمودين و أيزوفيتكسن و كيرستين تأثيرا مضادا لفيروس أنفلونزا الطيور من عترة H5N1 عالى الضراوة.

تعتبر هذه الدراسة من الدراسات الأولى لتقييم مصادر طبيعية مصرية كمنتجات مضادة لأنفلونزا الطيور من عتره H5N1 عالي الضراوة.