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Original Article

Antioxidant and the Efficacy of *Sophora secundiflora* and Methoxyisoflavones in the Immune Function of Pigeons Vaccinated against Paramyxovirus Serotype-1

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ABSTRACT

Objective: The present work investigated the effect of oral administration of hydroalcoholic (70% aqueous ethanol) extract (TeE) of *Sophora secundiflora* leaves and its organic fractions *n*-hexane (HeE), ethyl acetate (EaE) and *n*-butanol (BuE) and major isolated methoxyisoflavones **1** and **2**, on the cellular and humoral immune responses to live attenuated avian paramyxovirus-1 (APMV-1) vaccines in pigeons. **Methods**: The structures of six isolated compounds were elucidated on the basis of chromatographic, chemical, and spectroscopic methods. The samples antioxidative and radical scavenging capabilities of 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation and ferric reducing power were determined. Total phenolic, tannin and flavonoid contents of EaE extract were evaluated. **Results:** Six compounds were isolated. Three were the methoxyisoflavones5-7-dihydroxy-4′-methoxyisoflavone (**1**), 7-hydroxy-4′methoxyisoflavone (**2**), 5,4'-dihydroxy-7-methoxy-isoflavone (**3**), along with isorhamnetin (**4**) and two quercetin derivatives quercetin 3-glucoside (**5**) and quercetin 3-rutinoside (**6**) were isolated. The hydroalcoholic extract, fractions and 4'-methoxyisoflavones showed radical scavenging effect in the order of EaE > TeE > BuE >compound **1**>compound **2**> HeE. Stimulation of both sero-responses was observed, especially this of EaE. The results showed an increase of macrophage cells, lymphocyte and antibody titers in blood. **Conclusion**: The presence of 5-hydroxyl group at A-ring may be important to show the immunostimulant and antioxidant activity of compound **1***vs* compound **2**. The present results showed the potential abilities of EaE as antioxidant and immunomodulator agent and these would impart healthy economic benefits in vaccinated birds.

Key words: *Sophora secundiflora*, Antiradical effect, Immune, Isoflavones, Paramyxovirus, Pigeons.

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INTRODUCTION

Isoflavonoids are large subgroup of flavonoids and one of the major plant secondary metabolites that mediate diverse biological activities and potential health benefits.[1,2] Isoflavones are receiving growing attention and the focus of research has been on them to promote host immune functions by augmenting cellular and humoral type.[3,4] This encourages many authors to study the plant containing isoflavones such as *Sophora* species.[2] *Sophora* species belongs to the family Fabaceae, contains about 52 species that are widely distributed in temperate regions. This genus is currently used as a traditional medicine for preventing a variety of ailments *e.g.* for dephlogistication and detoxication, and in infectious diseases[2] and some species are administered orally in classical medicinal treatises of ancient China.[5]

Genus of *Sophora* is endowed with diverse bioactive molecules, such as chromones, pterocarpans, flavonoids, polysaccharides, and alkaloids.[2] Oxymatrine, extracted from *S. alopecuraides* was reported as a strong immune-modulator has anti-hepatitis B virus immunomodulatory effect.[6] It influences signaling transduction of toll-like receptor 9 (TLR9) and improve the efficacy of immune response of the TLR9 ligand against chronic hepatitis B by a synergistic effect. In immune-compromised mice using dexamethasone, the polysaccharide of *S. subprosrate* has showed immunomodulatory effect on the production of cytokines and splenic lymphocyte proliferation.[7]

Our present study describes the isolation and structural elucidation of isoflavones. These classes of compounds were reported to affect immune functions.[8] Isoflavones are hemostatic constituents offer immunologic benefits and are known to exert pseudohormonal activity and may be used in estrogen replacement therapy.[9] Isoflavones genistein and 2′-hydroxygenistein, isolated from *S. alopecuroids*, are known act as phytoestrogens and were reported to stimulate various aspects of immune function.[10] The injection of isoflavone genistein in ovariectomized juvenile micemay be lead to affect both sero responses of immunity through either estrogen receptor (ER)-or non-ER-mediated pathways.[9] Other

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isoflavone 5,7-dihydroxy-4'-methoxyisoflavone was reported to down-regulate the expression of matrix metalloproteinases and upregulate tissue inhibitor of metalloproteinase-1 at both the mRNA and protein levels in interleukin (IL)-1β induced rabbit chondrocytes. [11]

In view of the wide continued interest in the biological activity of this important genus, one of the important sources of isoflavonoids, the current study reports, herein, on the antioxidant and immunomodulatory activities of *S. secundiflora* species grown in Egypt on immune response in pigeons vaccinated against paramyxovirus serotype-1 (APMV-1). APMV is a virus able to infect all orders of avian species. [12] In Egypt, a high mortality and morbidity was associated with the virulent strains of this virus and has a devastating effect on the poultry industry.[13] The potential role of Egyptian pigeons in the transmission and evolution dynamics of APMV-1 was also suggested.[13]

MATERIALS AND METHODS

Plant material

Leaves of *Sophora secundiflora* (Ortega) Lag. ex DC. were collected from fruited trees cultivated in El-Orman Botanical Garden (OBG), Giza, Egypt. Authentication of the plant was established by Treas Labib, Herbarium Section, OBG. A voucher specimen was kept in the Department of Chemistry of Natural Compounds, National Research Centre (Egypt).

Pigeons

The birds were purchased from local market in Fayoum, Egypt. Pigeons were reared with the history of parent unvaccinated to paramyxovirus vaccine might be at day 18 - 22. The birds were housed in self-contained isolation units that were ventilated under negative pressure with inlet and exhaust high efficiency particulate air (HEPA)-filtered air and maintained under continuous lighting. Feed and water were provided with *ad libitum* access. Birds were cared for in accordance to Central Laboratory for Evaluation of Veterinary Biologics (CLEVB) Institutional Animal Care and Use Committee approved animal use protocol.

Vaccine

Avian paramyxovirus serotype-1 (APMV-1) vaccine with a titer of $10^{6.5}$ EID_{50}/mL (embryo infective dose) was used for vaccination of the experimental birds *via* drinking water. Hemagglutination inhibition (HI) assays were used to quantify antibody responses to virus infection as previously described.[14] The vaccine was tittered by measuring the hemagglutinating activity using a microplate HA test.

Virus titration

Virulent strains of APMV-1 (10^7 EID_{50}/mL) obtained from the bank of strains of CLEVB, Abbassia, Egypt as used as challenging virus.

Phytochemical study

General experimental procedures

The NMR spectra were recorded at 400 (^{1}H) and 100 (^{13}C) MHzon Bruker High Performance Digital FT-NMR 400 Avance III spectrometer using deuterated DMSO as solvent. The chemical shifts (δ) are reported in parts per million (ppm) and coupling constants (*J*) in Hz. The UV-analyses of the pure samples were recorded, separately, as MeOH solutions and with different diagnostic UV shift reagents on a UV-visible spectrophotometer: Shimadzu UV 240 (P/N 240-5800). Sephadex LH-20 (Pharmacia, Uppsala, Sweden) and silica gel (Si) 60 mesh of 230–400 and 60-120 (E. Merck, Darmstadt, Germany) were used for column chromatography. Pre-coated silica gel plates (Kieselgel 60 F254, 0.25 mm) were used for thin layer chromatography (TLC) analyses. Folin-Ciocalteu reagent, sodium carbonate, glucose and aluminum chloride were purchased from Merck Company (Darmstadt, Germany). 2,2-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), potassium ferricyanide, and ferric chloride ($FeCl_3$) were purchased from Sigma Chemical Co., Ltd (St. Louis, MO, USA). All chemicals in the present study are of analytical grade. For paper chromatography, Whatman No. 1 paper sheets (Whatman Ltd., Maid stone, England) were used. The visualization of spots was carried out by spraying with the spray reagentR_1: $AlCl_3$ (1% in ethanol) were used to visualize the compounds. Solvent systems S_1 [*n*-BuOH/ HOAc/H_2O (4:1:5, v/v/v, top layer)], S_2 (15% aqueous HOAc) and S_3 [EtOAc/MeOH/H_2O (100:13.5:10, v/v/v)], S_4 [EtOAc/HCOOH/CH_3COOH/H_2O(100:11:11:27, v/v/v/v)] and S_5 [*n*-BuOH/C_3H_8O/H_2O (4:1:5, v/v/v, top layer)] were used.

Extraction and isolation

The air-dried (35 ºC, 24 h under air circulation) leaves (1.2 kg) were powdered. The scheme showed the extraction, fractionation and purification of 70% ethanolic extract of *S. secundiflora* (Ortega) Lag. ex DC leaves was illustrated in Figure 1.

Compound **1**: yellowish-white crystals (35 mg); R_f:0.89 (S_1) and 0.30 (S_2), m.p. 210-213 °C;UV λ_{max}(nm): (MeOH): 261 and 330 (sh), (+ NaOMe): 273 and 329, (+ $AlCl_3$) : 273, 310 (sh) and 375, (+$AlCl_3$/HCl): 273, 310 (sh) and 373, (+ NaOAc): 272 and 326,(+NaOAc/ H_3BO_3): 262 and 330(sh); ^{1}H NMR (400 MHz, DMSO-d_6): δ_{ppm} 8.37 (1H, s, H-2), 7.51 (2H, d, *J* = 8.72 Hz, H-2'/6'), 7.02 (2H, d, *J* = 8.76 Hz, H-3'/5'), 6.24 (1H, d, *J* = 2.04 Hz, H-8), 6.40 (1H, d, *J* = 2.04 Hz, H-6), 3.79 (3H, s, O-CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ_{ppm} 180.6 (C-4), 164.8 (C-7), 162.4 (C-5), 159.6 (C-4'), 158.1 (C-9),154.8 (C-2), 130.6 (C-2'/ 6'), 123.4 (C-3), 122.4 (C-1'), 114.2 (C3'/5'), 104.9 (C-10), 99.5 (C-6), 94.2 (C-8), 55.6 (O-CH_3).

Compound **2**: yellow amorphous powder (33 mg); R_f: 0.49 (S_3), 0.28 (S_4), m.p. 221 °C; UV λ_{max}(nm): (MeOH): 248, 258 (sh) and 301, (+ NaOMe): 255 and 335 (sh), (+ $AlCl_3$): 248, 261 (sh) and 301, (+$AlCl_3$/ HCl): 248, 261 (sh) and 302, (+ NaOAc): 254, 313 and 335(+NaOAc/ H_3BO_3): 264 and 303(sh). ^{1}H NMR (400 MHz, DMSO-d_6): δ_{ppm} 8.16 (1H, d, *J*= 8.8, H-5), 7.99 (1H, s, H-2),7.55 (2H, d, *J*= 8.8, H-2'/6'), 7.14 (1H, dd, *J*= 2.4 and 8.8, H-6), 7.01 (1H, d, *J*= 2.4, H-8),6.95 (2H, d, *J*= 8.8, H-3'/5'), 3.50 (3H, s, *O*-CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ_{ppm} 176.8 (C-4), 171.0 (C-7), 159.7 (C-4'), 157.3 (C-9), 152.9 (C-2), 130.04 (C-2'/6'), 126.2 (C-5), 124.8 (C-1'), 123.8 (C-3), 118.3 (C-10), 114.5 (C-6), 114.2 (C-3'/5'), 103.04 (C-8), 54.2 (O-CH_3).

Compound **3**: yellow amorphous powder (20 mg); R_f: 0.86 (S_2) and 0.31 (S_2), m.p. 240-242 °C. UV λ_{max}(nm): (MeOH) : 263 and 325 (sh), (+ NaOMe) : 272 and 353, (+ $AlCl_3$) : 273, 309 (sh) and 374 , (+$AlCl_3$/HCl): 279, 310 (sh) and 370, (+ NaOAc) : 262 and 330, (+NaOAc/H_3BO_3): 262 and 332 (sh).^{1}H NMR (400 MHz, DMSO-d_6): δ_{ppm}8.17 (1H, s, H-2), 7.34 (2H, d, *J* = 8.56 Hz, H-2'/6'), 6.80 (2H, d, *J* = 8.56Hz, H-3'/5'), 6.51 (1H, d, *J* = 2.16 Hz, H-6), 6.32 (1H, d, *J* = 2.16Hz, H-8), 3.81 (3H, s, O-CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ_{ppm}180.1 (C-4), 165.0 (C-7),161.4 (C-5), 157.4 (C-4`), 157.0 (C-9), 153.4 (C-2), 129.7 (C-2'/ 6'), 122.6 (C-3), 121.0 (C-1'),114.5 (C-3'/5'), 105.1 (C-10), 97.4 (C-6), 91.6 (C-8), 55.06 (O-CH_3).

Compound **4**: yellow microcrystalline powder, 20 mg; R_f: 0.75 (S_1); 0.28 (S_2), m.p. 294-296°C; ^{1}H NMR (400 MHz, DMSO-d_6), δ_{ppm} 7.48 (1H, d, *J* = 2.0 Hz, H-2'), 7.32 (1H, dd, *J* = 8.4 Hz, 2.0, H- 6'), 6.78 (1H, d, *J* = 8.4 Hz, H-5'), 6.25 (1H, d, *J* = 2.0 Hz, H-8), 6.23 (1H, d, *J* = 2.0 Hz, H-6), 3.71 (3H, *s*,O-CH_3);^{13}C NMR (100 MHz, DMSO-d_6), δ_{ppm} 176.4 (C-4), 167.4 (C-7), 164.8 (C-5), 161.3 (C-9), 156.8 (C-2), 148.4 (C-4'), 147.4 (C-3'), 145.7 (C-5'), 136.3 (C-3), 147.4 (C-3'), 120.6 (C-1'), 116.3 (C-6'), 115.7 (C-2'), 103.5 (C-10), 98.9 (C-6), 94.0 (C-8), 56.8 (O-CH_3).

Compound **5**: dark yellow amorphous powder, 20 mg; R_f: 0.56 (S_1) and 0.36 (S_2); m.p. 219-221 °C; UV λ_{max} (nm), (MeOH): 258 and 354;

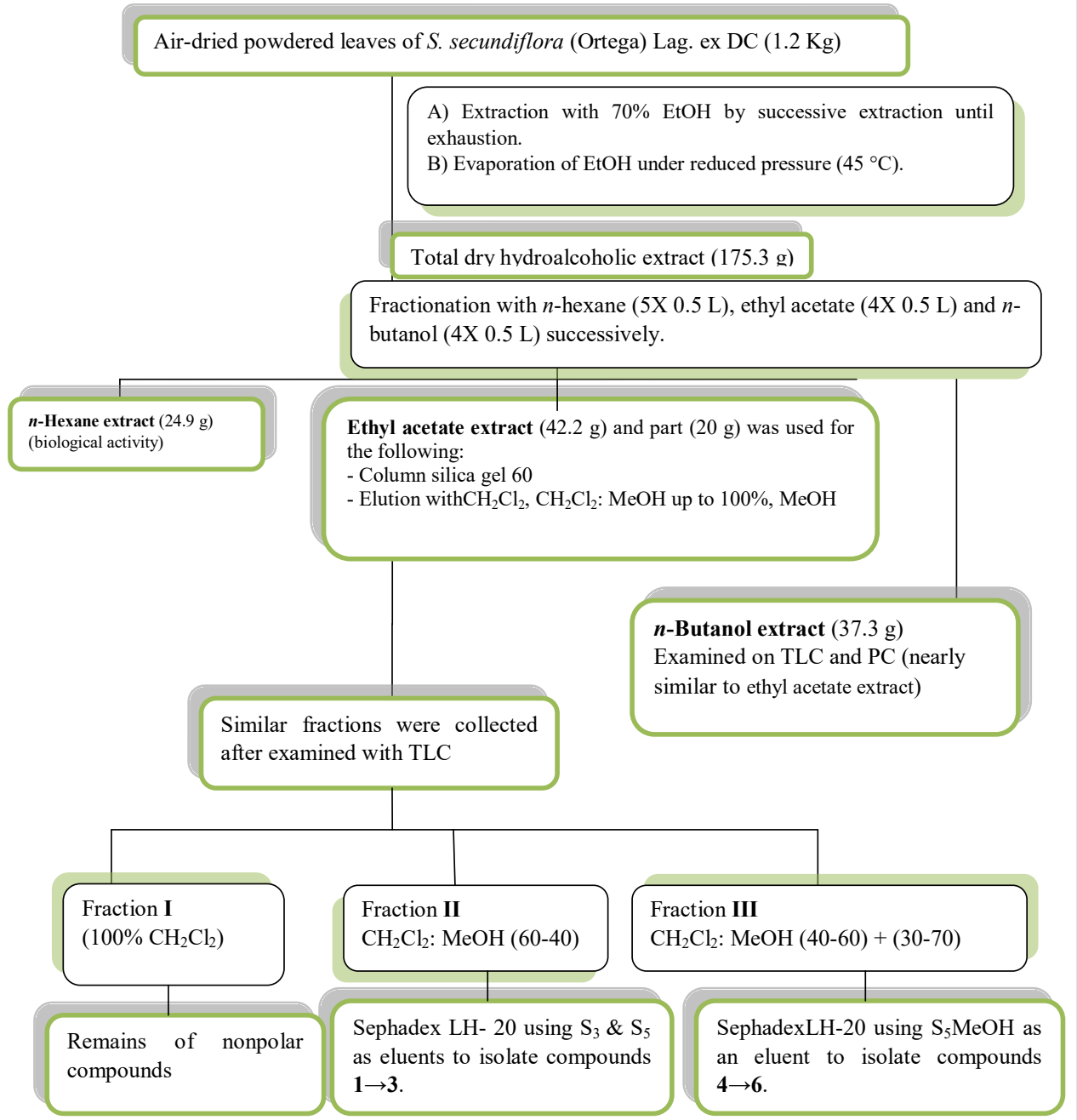

Figure 1: Extraction, fractionation and purification of 70% ethanolic extract of *S. secundiflora* (Ortega) Lag. ex DC leaves.

(+NaOMe):273, 325 and 409; (+NaOAc): 273, 321 and 373; (+NaOAc/H_3BO_3): 263 and 377; (+$AlCl_3$): 274, 302 and 427; ($AlCl_3$/HCl): 270, 302, 356 and 402; ^{1}H NMR (400 MHz, DMSO-d_6), δ_{ppm} Aglycone: 12.64 (1H, s, H- bond OH-5), 7.60 (1H, dd, H-6' hidden by H-2"), 7.58 (1H, d, *J*=6.2 Hz, H- 2'), 6.85 (1H, d, *J* = 9 Hz, H-5'), 6.39 (1H, d, *J*= 1.6 Hz, H-8), 6.19 (1H, d, *J*= 1.6 Hz, H-6), 5.47 (1H, d , *J* = 6.6 Hz ,H-1"), 3.59-3.08 (m, rest of glucose protons); ^{13}C NMR (100 MHz, DMSO-d_6), δ_{ppm}177.9 (C-4), 164.9 (C-7), 161.7 (C-5), 156.8 (C-2), 156.6 (C-9), 148.5 (C-4'), 145.3 (C-3'), 133.7 (C-3), 122.1 (C-6'), 121.6 (C-1'), 116.6 (C-5'), 115.7 (C-2'), 104.4 (C-10), 101.3 (C-1"), 99.2 (C -6), 94.0 (C-8),78.6 (C-5"),77.9 (C-3"), 74.5 (C-2"), 70.4 (C-4"), 61.4 (C-6").

Compound **6**: yellow crystals, 18 mg; R_f: 0. 46 (S_1); 0.60 (S_2), m.p. 189-191 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ_{ppm} aglycone: 12.59 (1H, s, 5-OH), 7.55 (1H, dd, *J* = 2.5, 8.5 Hz, H-2'), 7.53 (1H, d, *J* = 2.5 Hz, H-6'), 6.84 (1H, d, *J* = 8.5 Hz, H-5'), 6.37 (1H, d, *J* = 1.2 Hz, H-8), 6.18 (1H, d, *J* = 1.2 Hz, H-6), sugar: 5.34 (1H, d, *J* = 7.5 Hz, H-1"), 4.38 (1H, s, H-1'"), 3.07-3.72 (m, rest of sugar protons), 0.99 (3H, d, *J* = 6.0 Hz, Rha-CH_3); ^{13}C NMR (100 MHz, DMSO-d_6): δ_{ppm}178.1 (C-4), 164.7 (C-7), 161.9 (C-5), 157.3 (C-2), 157.1 (C-9), 149.1 (C-4'), 145.4 (C-3'), 134.0 (C-3),122.3 (C-1'),121.9 (C-6'),117.0 (C-5'), 115.9 (C-2'),104.7 (C-10), 101.9 (C-1"), 101.4 (C-1'"), 99.4 (C-6), 94.3 (C-8), 77.2 (C-3"), 76.6 (C-5"), 74.8 (C-2"), 72.6 (C-4'"), 71.3 (C-2'"), 71.1 (C-3'"), 70.7 (C-4"), 68.9 (C-5'"), 67.7 (C-6"), 18.4 (Rha-CH_3).

Quantitative determination of total phenolics content

Total phenolic content of *S. secundiflora* (Ortega) Lag. ex leaves extract was estimated by the Folin-Ciocalteu method using gallic acid as

standard.[15] Aliquots of the extract were taken in a test tube and made up to the volume of 1 mL with distilled water. The distilled water itself was used as blank. Then 0.5 mL of Folin-Ciocalteu reagent (1:1 with water) and 2.5 mL of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortex the reaction mixture, the tubes were placed in the dark for 40 min and the absorbance was recorded at 725 nm against the reagent blank. Total polyphenol contents were expressed as mg gallic acid equivalents (GAE)/g dry weight, calculated from a standard curve prepared with 00-100 mg/L gallic acid.

Quantitative determination of total flavonoids content

Total flavonoid content of ethanol extract was estimated by a colorimetric assay according to Ordonez *et al.*[16] To 0.5 mL of dry leaves extract, 0.5 mL of 2% $AlCl_3$ ethanol solution was added. After 1 h at room temperature, filtered, then the absorbance was measured at 420 nm. Total flavonoid content was expressed as mg quercetin equivalents (QE)/g dry weight, calculated from a standard curve prepared with 0-500 μg/mL quercetin.

Quantitative determination of total tannins content

Total tannin content of ethanol extract was determined according to modification to the Folin-Ciocalteu method using polyvinyl polypyrrolidone (PVPP) to separate tannin phenols from non-tannin phenols. About 100 mg of PVPP was added to 1 mL sample extracts diluted with 1 mL water and left 15 min at 4°C. After centrifugation, PVPP forms a precipitate with tannins, and the supernatant has only simple phenols. Simple phenols were determined using the Folin-Ciocalteu reagent. The difference between total and simple phenol values represents the total tannin content, expressed as mg GAE/ g dry weight.

Role of S. secundiflora on immune responses in vaccinated pigeons

A pilot experiment was carried out to choose the suitable dose which neither caused degeneration nor necrosis in livers and kidneys. Samples of 0.25, 0.5 and 1 mg/kg body weight (b.w.) of TeE, HeE, EaE, and BuE and compounds **1**and **2** were given orally to pigeons, separately. It was found that the selected dose for studying the immunomodulatory activity was 0.25 mg/kg b.w. Pigeons were reared and kept in isolators under complete hygienic measure and divided into 8 groups each of 45 pigeons. The first non-treated non-vaccinated group was considered as control. The second group was vaccinated at the 7th day with living attenuated APMV-1vaccine. The remaining six groups received 0.25 mg/kg b.w. of each extract and compounds **1** and **2** orally daily from the 2nd to 6th day of life. Then they were vaccinated at the 7th day with living attenuated APMV-1 vaccines *via* drinking water. Twenty random blood samples were collected from each group at 3, 7, 10, 14, 21, 28, and 35 dose post vaccination (DPV).

Immunomodulatory activity

Humoral immune response

Ten of the previously mentioned 20 random blood samples were used for estimation of humoral immune response after serum separation using the hemagglutination inhibition (HI) test. [16]

Cell-mediated immune response

The other ten samples were used for the evaluation of cell- mediated immune response *via* the following tests:

Assay of lymphocyte blastogenesis

The lymphocyte blastogenesis assay[17] was carried out and evaluated using the MTT test. [18] The results were expressed as Delta optical density. The change in optical densities were recorded at λ_{max} 490 nm by the aid of an automatic Titertek multiskan Reader model ELX 800 UV, INC, USA for reading ELISA plated.

Macrophage migration index

Macrophage activity was preceded and the phagocytic index[19] was determined as follows: phagocytic percentage = total no. of phagocytes which ingest more than 2 *Candida* / total no. of phagocytes which ingest *Candida*.

Avian paramyxovirus serotype-1 (APMV-1) challenge test

The humoral and cellular immune response were confirmed by the APMV-1 challenge test and were proceeded by choosing 15 pigeons randomly from each group at 3, 14, and 35 DPV and subjected to the challenge test with 0.5 mL of APMV-1 strain (10^6 EID_{50} /mL). The chickens were observed for 10 DPV or any symptoms of disease. Pigeons died within this period were collected and subjected to detailed *post mortem* examination.

Investigation of *in vitro* antioxidants activities

DPPH free radical scavenging assay

The free-radical scavenging activity using DPPH reagent was determined according to Brand-Williams *et al.* [20] The extracts and isolated compounds of *S. secundiflora* were soluble with 85:15 v/v methanol: water. To 0.5 mL of the extract sample 1.0 mL of freshly prepared ethanolic DPPH solution (20 $\mu g/mL^{-1}$) was added and stirred. The decolorizing process was recorded after 5 min of reaction at 517 nm and compared with a blank control. All samples were analyzed in triplicate. The ability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging activity (%) = [(control absorbance − sample absorbance) / control absorbance] ×100.

ABTS radical scavenging activity

ABTS radical scavenging activity was measured by the ABTS cation decolorization assay as described by Rice-Evans *et al.*[21] with some modifications. The stock solutions included 7 mM ABTS solutions and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL ABTS radical solution with 60 mL methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. ABTS radical solution was freshly prepared for each assay. The extracts and isolated compound of *S. secundiflora* (0.5 mL) was allowed to react with 2.5 mL of the ABTS reagent and the absorbance was taken at 734 nm after 7 min using the spectrophotometer. The ABTS radical cation decolorization assay capacity of the extract and percentage inhibition calculated as ABTS radical scavenging activity.

ABTS (%) = [(Abs. control − Abs. sample)] / (Abs. control)] ×100

Where Abs. controls the absorbance of ABTS radical cation methanol; Abs. sample is the absorbance of ABTS radical cation sample extract.

Reducing power assay

The method of Oyaizu and Jpn[22] was used to assess the reducing power of the extracts and isolated compounds of *Sophora secundiflora*. From each extract (0.5 mL) was added to phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 1% potassium ferricyanide (2.5 mL). The mixture was incubated at 50 °C for 20 min. Aliquots of trichloroacetic acid (2.5 mL, 10%) were added to the mixture, which was then centrifuged at 1000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and a freshly prepared $FeCl_3$ solution (0.5 mL 0.1%). The intensity of the blue-green color was measured at 700 nm.

In this assay, the yellow color of the test solution changes to be green depending on the reducing power of test specimen. The presence of reductants in the solution causes the reduction of the ferric/ferricyanide complex to the ferrous form. Therefore, ferrous can be monitored by the measurement of the absorbance at 700 nm. Increased absorbance of their action mixture indicated increased reducing power.

RESULTS

Phytochemical investigation

Identification of compounds

Chromatographic fractionation and purification of the EaE extract of the leaves of *S. secondi flora* on different columns of Si gel and Sephadex LH-20 afforded six compounds **1**–**6** (Figure 2). Three flavonoids, previously not isolated from this plant, were the isorhamnetin **4** and the two quercetin derivatives quercetin 3-*O*-β-D-$^4C_1$-glucopyranoside (isoquercitrin, **5**) and quercetin-3-*O*-α-L-$^1C_4$-rhamnopyranosyl-(1'''→6'')-*O*-β-D-$^4C_1$-glucopyranoside (quercetin 3-rutinoside, **6**) and the 5-hydroxy-isoflavones; 4'-*O*-methyl genistein **1** and 5,4'-dihydroxy-7-methoxy-isoflavone **3** as well as the 7-hydroxy-4'-methoxyisoflavone (4′-methoxy-daidzein) **2**. Compounds **1** and **2** were previously detected in plant roots while compound **3** was in the plant stems.[23]

The isolated compounds were identified by UV, ^{1}H NMR and ^{13}C NMR analyses. The analytical data were in agreement with those reported in the literature. The chromatographic properties of compounds **1** – **3** suggested the characteristics of isoflavonoid. TLC of the three compounds showed them as yellow spot in visible light and purple under UV light that remained blue after exposure to ammonia vapor. The UV spectrum of these compounds **1** – **3** in methanol exhibited two absorption maxima in the ranges 245 to 275 and 300 to 330 nm which are characteristics for isoflavones structure. Compound **1** showed the characteristic of absorption of 261 and 330 (sh) while compound **2** showed the characteristic of absorption of 248, 258 (sh) and 301.[24] Compound **3** has λ_{max} at 263 and 325 nm. Compounds 1 and **3** showed a bathochromic shift of 273, 310 (sh), 375 and 273 and 309 nm respectively on addition of $AlCl_3$ and no change with $AlCl_3$/HCl relative to methanol spectrum indicating the presence of free 5-OH group. While compound **2** showed no bathochromic shift on addition of $AlCl_3$ suggesting the absence of free 5-OH group. The presence of a free 7-OH group at C-7 of compounds **1** and **2** is evidenced by a bathochromic shift in Band II, induced by NaOAc by 270, 326(sh) and 254, 313, respectively. While nothing was observed with compound **3** on addition of NaOAc that may be attributed to the substituted C-7 in ring A. ^{1}H NMR spectrum of compound **1** showed characteristic signal at δ_H 8.37 ppm indicating a proton on C-2 which is characteristic for isoflavones, and a singlet signal at δ_H 3.79 ppm indicating one methoxy group.[24] The ^{1}H NMR resonances of compound **1** showed two coupled doublets at δ_H 6.40 and 6.24 ppm with a small coupling constant (*J*= 2.04 Hz) were characteristic of two *meta*-related H-6 and H-8 protons of ring A of an isoflavone. These chemical shifts indicate 5,7-dihydroxy substitution pattern. While compound **2** showed resonances at δ_H 7.14 (*J*= 8.8 and 2.4 Hz) and δ_H 7.01 (*J*= 2.4 Hz) which are corresponding to H-6 and H-8 protons in ring A respectively. The presence of signals at δ_H 7.51 ppm (*J* = 8.72 Hz, H-2'/6') and at δ_H 7.02 ppm (*J* = 8.76 Hz, H-3'/5') indicating the presence of the methoxy group at C-4' in ring B of compound **1**. Spectrum of compound **2** revealed characteristic patterns of isoflavone proton resonances in the aromatic region at δ_H 7.55 (*J*= 8.8 Hz, H-2'/6') and δ_H 6.95 (*J*= 8.8 Hz, H-3'/5') corresponding to B ring protons. Compound **3** exhibited one methoxy group δ_H 3.81 (s), one set of *meta*-coupled aromatic protons δ_H 6.51 (d, *J*=2.16 Hz) and δ_H 6.32 (d, *J*=2.16 Hz), two sets of *ortho*-coupled aromatic protons δ_H7.34 (*J*=8.56 Hz) and δ_H 6.80 (*J*=8.56 Hz) and a non-coupled aromatic hydrogen δ_H 8.17 (s). The low field aromatic singlet at δ_H 8.17 was assigned to H-2 due to deshielding effects of the oxygen atom in the furan ring. These features are characteristic of a 5,4'-dihydroxyisoflavone derivative[25], in addition of δ_H 3.81 (*s*, O-CH_3). Compound **1** showed characteristic resonance at δ 8.16 (*J*= 8.8, H-5), δ 7.99 (*s*, H-2) and δ 3.50 (s, *O*-CH_3).^{13}C NMR chemical shift positions of ring carbons of compound **1** and **2** were found in the range of range 94.2-180.and 103.0-176.8 ppm. Compounds**1**and**2** showed signals at δ 154.8 and 152.9, respectively of the C-2 characteristic of isoflavone type. In addition to the appearance of absorbance at δ 55.6 and 54.2 corresponding to O-CH_3 at C-4' was reported.[26,27] ^{13}C NMR spectrum of compound **3** exhibited the presence of fifteen carbons and directly proved the methoxylation on C-7 due to the appearance of the methoxy resonance at δ 55.1 and the second one was the α-downfield shift of 165.0 (Δ~+2 ppm) of C-7 and β upfield of C-6 at 97.4 (Δ~-2ppm) relative to these of genistein.[26,27] They were identified as 5-7-dihydroxy-4′-methoxyisoflavone (**1**), 7-hydroxy-4′-methoxyisoflavone (**2**), 5,4'-dihydroxy-7-methoxy-isoflavone (**3**).

Compound **4** showed yellow spot and compounds **5** and **6** showed dark purple spot under long/short UV. These compoundsturned tobright yellow fluorescent with ammonia vapour or spray reagent R_1.^{1}H NMR spectrum of compound **4** showed an ABM spin coupling system of two protons in the form of doubled at δ 7.48 (H-2'), 7.32 (H-6') and one proton *ortho*-doubled assigned to H-5' at δ 6.78 conclude a 3',4'-disubstituted B-ring. Compound **5** exhibited a spin coupling system in the form of ABX for three types of protons H-2', 6' and 5' of a 3',4'-dihdroxy B-ring. Additionally, 5,7-dihydroxy A-ring of compound **4** was deduced due to the two *brs*, one proton each, at δ 6.25 and 6.23 describable to H-8 and H-6, respectively. While in case of compound **5**, another spin coupling system was exhibited and was explained as two douplet signals of the two *meta* coupled protons H-6 and H-8 in a 5,7-dihydroxy A-ring. The large *J*-value (~7 Hz,β-configuration) and characteristic δ-value (5.47 of 3–*O*-glucoside) of H-1''were indicative for *O*- β--$^4C_1$-pyranose structure of the glucoside moiety. ^{1}H NMR spectrum of compound **6** showed a β-anomeric proton signal of inner glucoside moiety at δ 5.34 and α-anomeric proton signal atδ 4.38 for

1: R= OH, R^1= R^2= H, R^3= OCH_3
2: R= R^1= R^2= H, R^3= OCH_3
3: R= OH, R^1= CH_3, R^2= H, R^3= OH

4: R= H, R^1= OCH_3
5: R= Glc, R^1= OH
6: R= Glc-rha, R^1= OH

Figure 2: Chemical structures of the isolated compounds from *S. secundiflora* (Ortega) Lag. ex DC leaves.

the terminal α-rhamnosyl moiety with a characteristic doublet signal of CH_3-6'''at 0.99 (d, *J*= 6 Hz). The downfield location H-6'' at δ 3.72 was confirmative evidence for (1'''→6'') rhamno-glucoside linkage. Like previous quercetin compound the ABX (H2'/6', 5' B-ring) and AM (H-8, H-6, A-ring) spin coupling systems were confirmative documents in their δ and *J*-values of quercetin aglycones.[26,27] ^{13}C NMR spectrum of compound **4** was directly proved the methoxylation on C-3' due to the appearance of the methoxy resonance at δ 56.8 and the second one was the α-downfield shift of C-3' to 147.4 (Δ~+3 ppm) and β-upfield of C-2' at 115.7 (Δ~-3 ppm) relative to these of quercetin.[26] Also, fifteen typical carbon resonances for a quercetin moiety were assigned in the aromatic region of the spectrum of compound **5**, among which the characteristic position of C-3 (133.7 ppm) to confirm the *O*-glucosidation at C-3. In addition, the two key signals of quercetin aglycone were assigned at δ 148.5 (C-4') and 145.3 (C-3') ppm. Compound **5** showed characteristic six carbon resonances in the aliphatic region of ^{13}C NMR spectrum. While twelve carbon resonances of glucose and rhamnose moieties were observed in ^{13}C NMR spectrum of compound **6**. The terminal attachment of the rhamnosyl moiety to C-6'' of glucoside, was confirmed from the characteristics downfield location of C-6'' to 67.7 (~Δ+7, α effect) and up-field location of C-5'' to 76.6, (~Δ-1-1.5 ppm) due to (1'''→6'')-*O*-glycoside. They were identified as isorhamnetin (**4**) and two quercetin derivatives quercetin 3-glucoside (**5**) and quercetin 3-rutinoside (**6**).

Quantitative determination of total phenolics, flavonoids and tannins content

Phytochemical investigation of EaE of *S. secundiflora* leaves has revealed the total polyphenols content expressed as gallic acid equivalent (GAE) was 12.36±0.30 mg GAE/g dry weight (D.W.) extract. The content of flavonoids was 5.80 ± 0.15 mg expressed as quercetin equivalent (QE), while it exhibited 1.05±0.08 mg/g D.W. of tannins content. Each value represents the mean of 3 replicates (mean ± SD).

Immunomodulatory activity

The biological study was planned to assess the immunomodulatory role of a hydroalcoholic extract and its organic fractions of *S. secundiflora* leaves and the major isoflavones **1** and **2** on both cell- and antibody-mediated immune responses in APMV-vaccinated group treated with the investigated samples. Increases of the hemagglutinating antibodies in all the investigated samples were recorded. The treated groups with the Ea E, Te E and He E showed higher remarkable increase of the hemagglutinating antibodies titer. At the 7th day, each group was separately vaccinated with live attenuated virus. These groups recorded high antibody titers of $2^{11.0}$, $2^{10.8}$, and $2^{10.0}$, respectively at 28 days-post vaccination peak titer (Table 1). The vaccinated control group was that vaccinated only (at the 7th day) with live attenuated APMV-1 vaccine (Table 1). The treated groups with BuE and isoflavones**1** and **2** gave a slight increase in the antibodies titer ($2^{8.0}$, $2^{8.9}$, and $2^{8.8}$, respectively) in comparison with control which recorded $2^{8.1}$at the 28DPV.

There was remarkable and progressive increase in the macrophage activity with the ethyl acetate EaE, crude total ethanol TeE and *n*-hexane HeE treated groups .All the groups showed maximum values of lymphocyte transformation at the 14th DPV which were expressed as delta optical density (Table 2). These maximum values reached 0.179, 0.176 and 0.174 with EaE, TeE and HeE, respectively. The avian virus was considered as the antigen and the use of phyto hemagglutinating (PHA) as a mitogen in comparison. At the 14th DPV, the vaccinated non-treated group showed a PHA value of 0.134. While the vaccinated and treated groups recorded increase in values of PHA compared with the vaccinated non-treated control groups. The highest values were

Table 1: The average log2 of heamagglutination inhibition (HI) titer to avian paramyxovirus serotype-1 (APMV-1) in pigeons.

	HI				
Group	7	14	21	28	35
Control	0.0	0.0	0.0	0.0	0.0
Vaccinated control	$2^{3.4}$	$2^{6.6}$	$2^{7.6}$	$2^{8.1}$	$2^{7.5}$
TeE	$2^{3.9}$	$2^{6.4}$	$2^{9.8}$	$2^{10.8}$	$2^{8.5}$
HeE	$2^{3.1}$	$2^{5.9}$	$2^{9.3}$	$2^{10.0}$	$2^{8.0}$
EaE	$2^{4.2}$	$2^{6.9}$	$2^{10.2}$	$2^{11.0}$	$2^{9.0}$
BuE	$2^{2.4}$	$2^{3.9}$	$2^{7.1}$	$2^{8.0}$	$2^{6.9}$
Compound**1**	$2^{2.8}$	$2^{4.9}$	$2^{8.0}$	$2^{8.9}$	$2^{7.2}$
Compound**2**	$2^{2.6}$	$2^{4.7}$	$2^{7.9}$	$2^{8.8}$	$2^{7.0}$

TeE; Hydroalcoholic (70% Aqueous ethanol) extract, HeE; Hexane extract, EaE; Ethyl acetate extract; BuE, *n*-Butanol extract, Compound **1**: Genistein 4'-Methyl ether (Biochanin A), Compound **2**; 4′-Methoxy-daidzein (Biochanin B).

Table 2: Cell-mediated immune response following a vaccination of pigeons with APMV and treatment with the investigated samples.

Group	7		14		21		35	
	PHA[a]	APMV-1[b]	PHA	APMV-1	PHA	APMV-1	PHA	APMV-1
Control	0.010	0.011	0.007	0.012	0.011	0.013	0.008	0.007
Vaccinated	0.101	0.121	0.134	0.168	0.112	0.159	0.079	0.081
TeE-treated	0.108	0.130	0.140	0.176	0.115	0.164	0.084	0.093
HeE-treated	0.104	0.128	0.136	0.174	0.112	0.162	0.079	0.089
EaE-treated	0.110	0.134	0.142	0.179	0.118	0.166	0.086	0.096
BuE-treated	0.099	0.119	0.128	0.152	0.101	0.146	0.069	0.081
Compound **1**-treated	0.102	0.122	0.131	0.164	0.110	0.138	0.076	0.082
Compound **2**-treated	0.103	0.124	0.129	0.166	0.111	0.159	0.077	0.085

TeE-treated; The group treated with hydroalcoholic (70% aqueous ethanol) extract, HeE-treated; The group treated with hexane extract, EaE-treated; The group treated with ethyl acetate extract; BuE-treated; The group treated with *n*-Butanol extract, Compound **1**-treated; The group treated with genistein 4'-methyl ether (Biochanin A), Compound **2**-treated; The group treated with 4′-methoxy-daidzein (Biochanin B), [a]PHA, phytohemagglutinating. [b]APMV-1, avian paramyxovirus serotype-1.

recorded for the group treated with ethyl acetate EaE (0.142) and hydroalcoholic extract TeE (0.140) (Table 2).

On the other hand, more assertion for the former results was obtained after evaluation of the macrophage activity (Table 3). A maximum phagocytic activity was 69.60 in the 14 DPV vaccinated group, while remarkable increase in vaccinated and treated groups with EaE (74.05), HeE (73.45) and TeE (73.20). While the vaccinated and treated groups with compounds **1** and **2** showed nearly the same phagocytic activity (72.23 and 72.18, respectively). The immune status of the birds was reflected by recording the number of pigeons has symptoms of disease and the number of the protected birds (Table 4). The vaccinated groups showed a maximum protection of 66.6% at the 14 DPV. The protection percentage of the vaccinated EaE-treated groups of birds at the DPV of 3, 14, 21 and 35 were 53, 87, 100, and 80%, respectively.

Investigation of *in vitro* antioxidants activities

The crude TeE, BuE, HeE, EaE extracts and major isoflavones (compounds **1** and **2**) of *S. secundiflora* were investigated for their antioxidant capacities (Figures 3a-c). EaE extract recorded the highest radical scavenging activity using DPPH assay it found to be 85.07 ± 0.65% at the concentration of 0.5 mg followed by TeE (60.43 ± 0.97%). The lowest DPPH radical scavenging activity between examined extracts was 20.07 ± 0.28% for compound **2** (Figure 3a).

Figure 3b described the reducing power of the six investigated samples. As can be seen, EaE extract showed the highest antioxidant property (1.61 ± 0.02) at the concentration of 0.5 mg/mL, indicating that it was effective as an antioxidant. Both TeE and BuE exhibited moderate reducing power ability. They recorded 0.90 ± 0.03 and 0.83 ± 0.04, respectively.

The radical-scavenging activity of plant samples and major isoflavones (compounds **1** and **2**) which was determined by ABTS radical cation decolorization assay was shown in Figure 3c. TeE, BuE, HeE, EaE fractions, were 65.53 ± 0.41, 62.79 ± 0.38, 27.82 ± 26 and 75.77 ± 0.33% respectively (0.5 mg/mL),while compounds **1** and **2** were 29.38 ± 0.46 and 26.16 ± 0.19% ABTS radical scavenging activity at the same concentration. These results were as near as to those of DPPH and reducing power assays.Findings from this study indicated that all the investigated samples showed radical scavenging effect in the order of EaE > TeE> BuE> Compound **1**> Compound **2**> HeE.

DISCUSSION

Recently there has been an upsurge of interest in the multipronged therapeutic potential of medicinal plants containing isoflavones.[2,5] Oral administration of some medicinal herbs to improve immunization has been reported previously.[2,28] The crude extracts, herbal formulations or isolated compounds from many medicinal plants could be used as adjuvant and exhibit strong immunomodulatory function.[5,29] Adjuvants play an important role in the development of vaccines. Many plants based vaccine adjuvant were reported for their immunostimulatory activity and their potential role as an alternative adjuvant for improving and maintaining the improved immune status.[30,31] The present study was designed to investigate the effect of oral administration of different

Table 3: The activities of the investigated samples on the macrophages phagocytosis of *Candida albicans*.

Days Group	3		7		10		14		21		28		35	
	PH-%[a]	PH-I[b]	PH-%	PH-I	PH-%	PH-I	PH-%	PH-I	PH-%	PH-I	PH-%	PH-I	PH-%	PH-I
Control	16.09	0.05	11.05	0.05	11.90	0.07	14.00	0.13	13.00	0.06	10.90	0.06	16.10	0.07
Vaccinated	44.60	0.42	56.30	0.41	75.90	0.58	69.60	0.49	69.80	0.57	53.20	0.39	41.09	0.29
TeE-treated	48.90	0.44	57.90	0.43	76.10	0.63	73.20	0.58	73.70	0.61	55.80	0.44	46.80	0.33
HeE-treated	48.60	0.41	57.60	0.41	69.90	0.60	73.45	0.54	73.10	0.59	55.70	0.41	46.10	0.31
EaE-treated	49.60	0.46	58.40	0.45	78.60	0.66	74.05	0.60	74.00	0.63	56.00	0.45	47.20	0.36
BuE-treated	44.90	0.31	54.80	0.34	65.90	0.49	69.76	0.46	69.80	0.51	54.30	0.39	42.10	0.22
Compound **1**-treated	47.90	0.39	56.90	0.38	69.10	0.59	72.23	0.51	72.10	0.57	54.90	0.38	44.70	0.29
Compound **2**-treated	48.10	0.40	57.10	0.39	69.40	0.56	72.18	0.52	72.20	0.58	55.10	0.39	44.80	0.28

TeE-treated; The group treated with hydroalcoholic (70% aqueous ethanol) extract, HeE-treated; The group treated with hexane extract, EaE-treated; The group treated with ethyl acetate extract; BuE-treated; The group treated with *n*-Butanol extract, Compound **1**-treated; The group treated with genistein 4'-methyl ether (Biochanin A), Compound **2**-treated; The group treated with 4′-methoxy-daidzein (Biochanin B), [a]PH-%; phagocytic percentage,[b] PH-I; phagocytic index.

Table 4: The percentage of protection following the vaccination of pigeons with APMV at various intervals between challenges.

Groups	Number of pigeons have symptoms of disease				Numbers of protected (The protection percentage)			
days	3	14	21	35	3	14	21	35
Control	15	15	15	15	0 (0)	0 (0)	0 (0)	0 (0)
Vaccinated	9	5	2	6	6 (40.0)	10 (66.6)	13 (86.6)	9 (60.0)
TeE-treated	9	2	1	3	6 (40.0)	13 (90.0)	14 (93.3)	12 (80.0)
HeE-treated	10	5	2	4	5 (33.3)	10 (66.6)	13 (86.6)	11 (73.3)
EaE-treated	7	2	0	3	8 (53.3)	13 (86.6)	15 (100.0)	12 (80.0)
BuE-treated	14	8	5	7	1 (6.6)	7 (46.6)	10 (66.6)	8 (53.3)
Compound **1**-treated	13	7	3	5	5 (33.3)	8 (53.3)	12 (80.0)	10 (66.6)
Compound **2**-treated	6	6	3	6	2 (13.3)	9 (60.0)	12 (80.0)	9 (60.0)

TeE-treated; The group treated with hydroalcoholic (70% aqueous ethanol) extract, HeE-treated; The group treated with hexane extract, EaE-treated; The group treated with ethyl acetate extract; BuE-treated; The group treated with *n*-Butanol extract, Compound **1**-treated; The group treated with genistein 4'-methyl ether (Biochanin A), Compound **2**-treated; The group treated with 4′-methoxy-daidzein (Biochanin B). Values in parentheses represent the protection percentage.

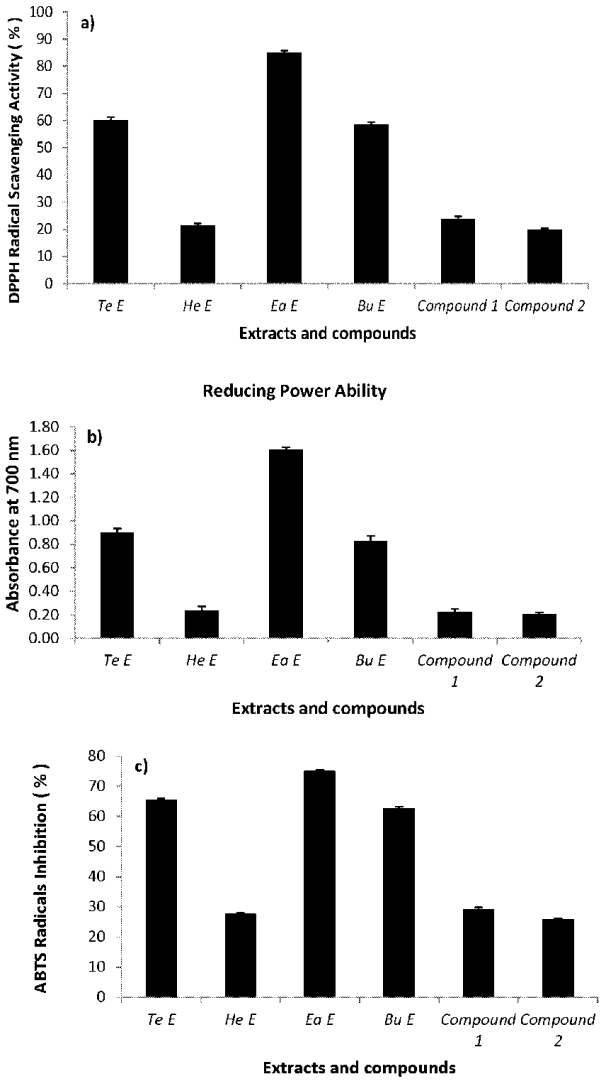

Figure 3: Antioxidant activity of the extracts and isolated compound of *S. secundiflora* (0.5 mg dried sample/mL). a) DPPH Free radical scavenging activity; b) Reducing power activity; c) Radical cation $ABTS^{+}$ scavenging activity.

samples on the cellular and humoral immune responses to live attenuated APMV vaccines in pigeons.

Various studies suggest that phenolic compounds, specifically the flavonoids promoted the secondary immune response and modulate host resistance.[29,32-34] Our current study showed the isolated and identified of three flavonoids **4** - **6** for the first time from *S. secundiflora*. Also, 5-7-dihydroxy-4′-methoxyisoflavone (biochanin A) **1** and 7-hydroxy-4′methoxyisoflavone (formononetin) **2** were isolated and identified. Mansoori *et al.* [35] reported the immunomodulatory activity of the methoxy isoflavones formononetin and isoformononetin which were suggested to translate into improved skeletal parameters, thereby preventing ovariectomized -induced bone loss. The activity of the TeE of the plant under investigation may be due to polyphenols component as tannins and isoflavones. Our previous studies have shown that another isoflavone (genistein-8-*C*-glucoside), isolated from *Retama raetam* seeds, had an inhibitory activity against reactive nitrogen species derived from nitric oxide.[3] These components may reduce lipid peroxidation products and enhancing contents of antioxidants and activities of relevant antioxidase enzymes and increased contents of cytokine.[5]

As a result of the present study the stimulation of both seroresponses in the vaccinated group treated with the investigated sample, especially this of EaE has been confirmed. The antibody titers showed a remarkable increase as well as the lymphocyte and macrophage cells in blood. Our results showed the identification of 4′-methoxy-daidzein **2**. One of the common metabolites of daidzein and formononetol is equol. It is a nonsteroidal estrogen of the isoflavone class.[36] This metabolite was reported to inhibit lipopolysaccharide induced-oxidative stress and reduces lipid peroxidation products. Equol is shown to protect intestinal epithelial cells from oxidative damage and enhances the immune response in HD11 macrophages of chicken.[36]

EaE of *S. secundiflora* leaves has revealed the highest total polyphenols, flavonoids and tannins content. Antioxidant-rich plants exhibited the prevention of oxidative-stress-related diseases. The present results demonstrated a linear relationship of antioxidant activities of the investigated samples (such as total crude extract and its organic fractions) with their phenolics, flavonoids and tannins content. The TeE extract and its organic fractions of *S. secundiflora* and two methoxyisoflavones showed antioxidant capacity (Figure 3). The pronounced radical scavenging activities of TeE and EaE extracts compared to other samples may have attributed to their higher lipid peroxidation reducing capabilities by acting as antioxidants. This effect may be aid the endogenous antioxidant enzymes involved in the inactivation of ROS before lipid peroxidation takes place.[5]

Genus of *Sophora* is endowed with antiradical molecules.[3,5] The high reducing power of the TeE and EaE extracts of *S. secundiflora* leaves (Figure 3) is probably because of their hydrogen donating ability.[37] They act as electron donors and could alleviate the number of oxidative stress. The donor reacts with free radicals to convert them into more stable products and then terminate the free radical chain reaction. Both extracts demonstrated antioxidant capacity and immune modulating activity at the present study. Other bioactive metabolites as resveratrol oligomers have been isolated from the genus *Sophora*.[38] Stilbenoids including resveratrol promoted rapid and transient release of free radicals/ reactive oxygen species (ROS).[39] The anti-herpetic activity of oligomeric stilbenoids to innate immunity was reported.[40] They inhibit herpes simplex virus infection through free radicals/ROS generation. Effect of diethylstilboestrol (DES) compounds on the phagocytic activity of the reticulo-endothelial system was reported.[39] DES neonatal treatment in male and female mice affected the immune cell percentage. Pterocarpan glycosides isolated from *S. tonkinensis* were reported to inhibit the production of nitric oxide induced by lipopolysaccharide in RAW 264.7 macrophages.[41] Apterocarpan and indigocarpan from *Indigofera aspalathoides* have exhibited a strong antioxidative effect in human colorectal adenocarcinoma LS174T cells.[42]

In our work the vaccinated and treated groups with ethyl acetate EaE recorded the highest values of PHA compared with the vaccinated non-treated control groups. Currently, studies have found that the EaE fraction of *Sophora* contain other bioactive constituents such as alkaloids, saponins and polysaccharides.[5] Quinolizidine alkaloids of matrine-type were isolated from the roots and rhizomes of *S. tonkinensis* and have showed potent anti-hepatitis B virus (HBV) activity with an inhibitory potency against hepatitis B surface antigen HBsAg.[31] Wang and his co-authors[43] have suggested that the alkaloids belongs to this class when combined with thymopolypeptides, they could inhibit HBV DNA replication, and further promote the antiviral effect by promoting the expression of IFN-α.[43] Therefore, in our present study, the high activity of EaE fraction may be attributed to the synergistic in its biological activity, including *in vitro* antiviral and immunomodulatory activities.[43]

Many studies have reported that flavonoids isolated from *Sophora* root act as antibacterial, anti-inflammatory and immunoregulatory. In our present work, two *O*-methylated isoflavones; 4'-methyl ethers of genistein (**1**) and daidzein (**2**) were identified in the plant leaves. These compounds may exhibit stimulating properties on natural killer cell activity.[32] The importance of cell-mediated immune response during disease infections and its implications for the development of effective vaccines was reported.[44] The activity of APMV vaccinated groups, as a result of the treatment with successive plant extracts (TeE, EaE, and BuE), might be ascribed to the ability of polyphenolics to reduce oxidative stress, improving the membrane integrity of the cells and enhance the innate immune function.[33] Treatment of vaccinated group with EaE extract, containing the 4'-methoxyisoflavones **1** and **2**, may prevent oxidative damage by detoxifying ROS/ free radicals. An effective immune response for preventing of many diseases is related with the activities of antibody *via* the complement activation and antibody-dependent cell-mediated cytotoxicity pathway, macrophage and T-cell.[45] Table 3 showed a maximum phagocytic activity in vaccinated control group the 14 DPV. Production of oxygen radicals and nitric oxide by activated macrophages is important for their cytopathic effects. In the vaccinated and treated groups with EaE, HeE and TeE, there were remarkable macrophage activations as they recorded 74.05, 73.45, and 73.20, respectively. While vaccinated isoflavones compounds (**1** and **2**) treated groups have showed nearly the same phagocytic activity. Isoflavone daidzein can up-regulate interleukin-4 production in activated T cells and increase phagocytic response of peritoneal macrophages.[8]

The enhanced immune responses may be related to immunocompetent cells activated by treatment with the natural products before vaccination, as reported.[8] Many studies justified that the diverse bioactivities of *Sophora* species might due to the presence of high-added-value polyphenolic components, including isoprenylated flavonoids and stilbenes in *Sophora* species.[2,5] Isoprenylated flavonoids are a class of flavonoids with diverse structures and some of those showed antiviral and antioxidant bioactivities.[46] These compounds may inhibit the expression of pro-inflammatory mediators and cytokines, including NO in immune cells. These compounds possess strong antioxidant properties that enable them to scavenge free radicals, donate hydrogen, chelate metals, break radical chain reactions, and quenchsinglet oxygen *in vitro* and *in vivo*.[5,21] The six samples were evaluated as antioxidant agents using the three antioxidant bioassay tests. In the present study, The TeE and EaE extracts showed remarkable results, followed by the isoflavone **1**. There were positive relationship between the radical scavenging activity of TeE, BuE and EaE and their total phenolic, tannins and flavonoids content. Similar relationships have been widely reported in many plants.[3,33] The difference in antioxidant potential of the extracts may be related to variation in the percentage of phytoconstituents extracted in various solvents. HeE sample has exhibited good antioxidant activity, although it contains less polyphenolic content than other samples. The difference in the type of polyphenolic content may be attributed to this recorded activity or the presence of antioxidant-active nonphenolic compounds.[47]

DPPH assay constitutes a quick, simple and low cost method, which has been widely used to determine the ability of a substance to act as a radical scavenger or hydrogen donor. Our preliminary phytochemical screening of the of *S. secundiflora* (Ortega) Lag. ex DC leaves showed the presence of various polyphenols such as coumarins, flavonoids, sterols and/or triterpenes, tannins as well as alkaloids in the crude extract (TeE). These phytoconstituents might have a role to stabilize free radicals, chelating transition metals, inhibition of peroxidation or scavenging ROS and these may reflect the integrated antioxidant status.[3] The reductive capability of substances using potassium ferricyanide reduction assay is correlated with their content of electron donors such as polyphenols as isoflavones.[48] This result is in agreement with the previously reported about *S. japonica* that have high content of polyphenolic constituents including: tamarixetin, sissotrin, gallic acid, and ellagic acid 4-*O*-α-L-arabinofuranoside, and showed good antioxidant capacity.[2]

The influence of many plant-derived secondary metabolites like flavonoids on immune function has been examined extensively.[32] Quercetin glycosides have many hydroxyl groups and have been possessed strong scavenging ability for free radicals.[49] In our present work, two quercetin glycosides quercetin 3-glucoside and quercetin 3-rutinoside were isolated through bioassay-guided fractionation of the EaE fraction of *S. secundiflora* leaves. Quercetin glycosides were reported as equally effective in suppressing lipid peroxidation in 6-hydroxydopamine-induced pheochromocytoma PC-12 cells as they suppressed the malondialdehyde generation and prevented cell damage. Also, Valentová *et al.*[50] recorded that many quercetin derivatives showed significant antimutagenic activity and DNA-protective effects against oxidative damage. Isoflavones have chemical structures similar to estrogen and so they are sometimes referred to as phytoestrogens.[10] The B-ring of flavonoid is linked to the C_3 position of the C-ring instead of the C_2 position in the isoflavone molecule. Our results in the present study show compound **1** to be more biologically active as compared to compound **2**. The proposed mechanism for immuno-regulation by these isoflavones may be related to their estrogenic actions.[10] Also; the presence of 5-hydroxyl group at A-ring might be of certain importance to the immuno-regulation and antioxidant activity of compounds **1** *vs* **2**.[51]

Medicinal plants with antioxidant activity could also have immunomodulatory ability.[50] Our previous research found that a supplement of the extract made from *Jatropha curcus* leaves in commercially inactivated Newcastle disease vaccines could significantly enhance the immune responses in chickens.[28] One of the herbal plants acts as immunomodulator is *S. subprosrate.* The polysaccharides extracted from the plant stimulated proliferation of murine splenic lymphocytes in immunosuppressed mice.[7,52] The extract TeE and its fractions were administered orally to the experimental groups of pigeons. This may be related to the mucosal immunity of birds. Wang *et al.*[43] reported the potentiating intestinal mucosal immunity of miceafter oral administration of a polyphenol-enriched extract of a Chinese herbal formula. Isoflavonoids isolated from some medicinal plants have been proven to possess immunomodulatory effect.[1,3,52] It is therefore possible that the effect observed within the extract of EaE and BuE may be attributable to their isoflavonoids component that regarded as protective antioxidants based on their ability to donate hydrogen atom to free radicals. Findings from this study indicate the presence of promisingly potent phytoconstituents in EaE and these may be attributed to its capability to act as antioxidants and free radical scavengers. The aqueous extract of *S. tonkinensis* could induce the apoptosis of mouse lymphoma, and contained immune modulators for promoting the anti-lymphoma properties *in vivo.*[53] Compound **1** (5,7-dihydroxy-4'-methoxyisoflavone) contain OH group, suggesting that the hydroxyl moiety is a critical structure that increases the antioxidant and immunomodulatory activity.[3,28,30] The results reveal that, the presence of –OH, in the isoflavonoid skeleton, enhanced the immunomodulatory activity.

The presence of a variety of classes of flavonoids in the investigated samples such as flavones (apigenin derivatives), flavonols (quercetin derivatives) and isoflavones (5,7-dihydroxy- and 7-hydroxy-4'-methoxyisoflavone) may be show a synergistic effect against paramyxovirus disease and also the potent antimutagenic activity.[5,54] Flavonol quercetin was also reported to enhance natural killer cell killing activity towards mouse Cr-labelled lymphoma YAC-1 target cells.[32,51]

In our study, the isolation of quercetin derivatives as quercetin 3-glucoside (**5**) and quercetin-3-rutinoside (**6**), were reported as effective and other flavonols against viruses[34] was carried out. The most common isoflavones genistein and daidzein are also found as the 4'-methyl ethers compound **1** and formononetol, respectively in the extract of EaE.[9] Most *in vivo* studies discussed the effect of isoflavones on immune functions are those for genistein.[8] This compound affects antibody- and cell-mediated immune responses and is known to control general immune function.[8] It inhibits lymphocyte proliferation and thymocyte differentiation. The suppressive activity of isoflavonoids against lymphocyte proliferation was reported.[51]

Based on these findings, we postulate that EaE and BuE have immunomodulatory potential for the development of an effective strategy against paramyxovirus. The strategy would likely have no toxic side-effects. Therefore, these extracts may be considered as immune-stimulatory potentiators. The respective 4'-methoxy derivative of genistein biochanin A has showed remarkable activity.

Several researches suggest that the global combination of secondary metabolites produces synergistic pharmacological activity.[30,54-56] Phytochemical investigation of the EaE extract showed the presence of considerable amount of flavonoids and polyphenols like phenolic compounds and tannins. Therefore, it is possible to assert that the activities of the EaE are in suitable correlation with these chemical contents, once these classes of compounds are known as potent molecules with immune modulating and radical scavenging activity.[3,33,49] The vaccinated groups showed a maximum protection of 66.6% at the 14 DPV. The protection percentage of the vaccinated and EaE-treated groups of birds at the DPV of 3, 14, 21 and 35 were 53, 87, 100, and 80%, respectively (Table 4). Collectively, our findings indicate that oral administration of *S. secundiflora* especially the EaE fraction and the major isolated methoxyisoflavones **1** and **2** may have potential role in antibody-mediated immune response against paramyxovirus disease which is severely endangering out the poultry industry by causing economic losses.[41]

CONCLUSION

In this work, the extracts of EaE and BuE exert marked activity as immunomodulator that may be attributed to the presence of isoflavonoids in these extracts, what may provide a novel approach to the development of plant-based vaccine adjuvant. Our study should provide new insights for further pertinent investigations to establish the action mechanisms of the isolated compounds.

This can be deduced that the tested fractions from ethanolic extract of *S. secundiflora* and isoflavones have remarkable antioxidant(s), along with immunoregular activities. Our findings have suggested that they could impart health benefits in the vaccinated pigeons against APMV-1.

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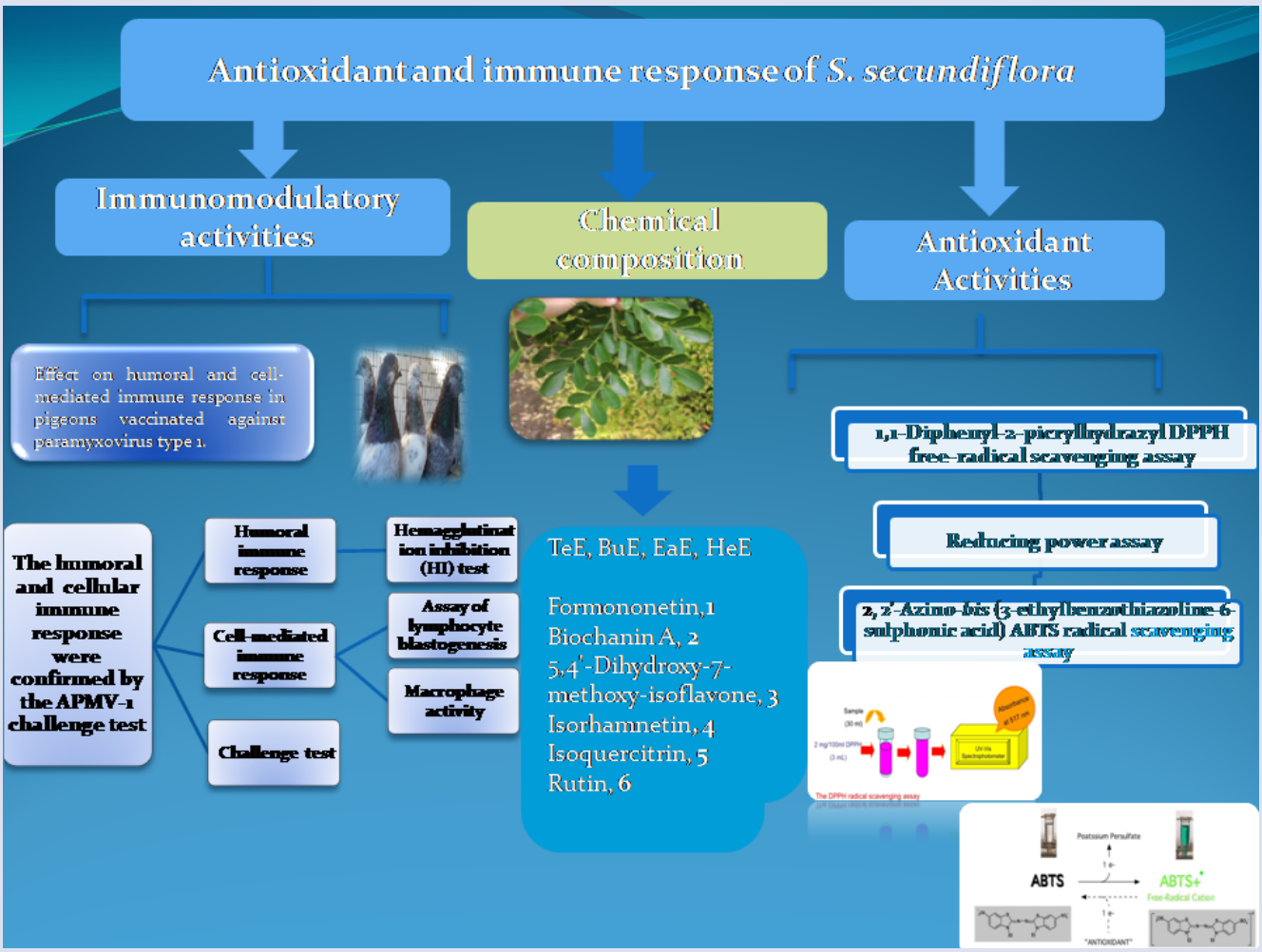
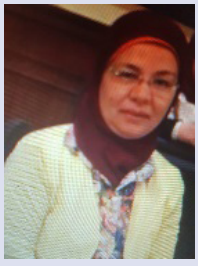
GRAPHICAL ABSTRACT



ABOUT AUTHORS



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