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Utilization of *Dianthus superbus L* and its bioactive compounds for antioxidant, anti-influenza and toxicological effects

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ABSTRACT

Dianthus superbus (DS) is a traditional medicinal herb well known for its medicinal and therapeutic potential and widely distributed in various Asian countries. The ethyl acetate (EA), butanol (Bu) and distilled water (DW) extracts of DS assessed for extraction of bioactive compounds and their biological activities. The chemical analysis was done using LC-MS/MS and antioxidant, anticancer and antiviral activities were determined. EA extracts showed strong anticancer activity with IC_{50} of 9.5, 13.8 and 69.9 μg/mL on SKOV, NCL-H1299 and Caski cancer cell lines, respectively. The Bu extracts exhibited strongest antiviral activity with respect to both influenza A and B viruses with IC_{50} values of 4.97 and 3.9 μg/mL, respectively. Also the metabolic profile for EA, Bu and DW extracts shows high variations and influence precisely the antioxidant, anticancer and antiviral properties. The quercetin 3- rutinoside and isorhamnetin 3- glucoside showed higher neuraminidase inhibition activity in dose dependent manner. Molecular docking study revealed that flavonol glycosides have higher binding activities towards influenza polymerase membrane glycoprotein. Correlation study showed that flavonol glycosides were linked to anti-influenza activity and cyclic peptides with anticancer activities. This study provides vital information for effective utilization of DS for medicinal, food and therapeutic purposes.

1. Introduction

In recent years cancer and viral diseases predominantly leading cause of human death. About 12.7 million cancer patients were detected in 2008, and approximately 7.98 million people died in 2010 due to cancer (Jemal et al., 2011; Lozano et al., 2012). The various factors such as chewing of tobacco, obesity, a poor diet, stress, and lack of physical exercise, infections, ionizing radiation, and environmental pollutants leading to development of cancer in humans (Anand et al., 2008). Occurrence of cancer risk is gaining significantly with age, food habit and region wise as many cancers occurring more frequently in developed countries (Jemal et al., 2011). In the 20th century, many anticancer treating treatments were developed such as chemotherapy and radiation therapy, but these methods have strange side effects leading to the loss of healthy cell count and body weight (Corrie and Pippa, 2008; Lee et al., 2012). Hence, research is important for screening of natural drugs or compounds from medicinal plants for therapeutic applications against cancer and other diseases gained paramount importance. Influenza virus referred to as “flu”, plays a dangerous impact on children and elder people. Influenza vaccine protection is extremely less or missing and gives only 35% protection (Lozano et al., 2012). Additionally, influenza A and B viruses spread globally and generates mutations in human body which leads to development of antigenic drift and shift after administration anti influenza drugs (Stephan et al., 2009). The studies showed that the previously developed drugs such as Tamiflu (oseltamivir) couldn't inhibit infection during the 2007–2008 seasonal influenza occurrence and even couldn't recognize binding to glycoproteins of the influenza virus membrane, hemagglutinin (HA) and neuraminidase (NA) (Choi et al., 2009). Hence, scientists need to discover more drugs and scientific proof against influenza viral infections. There has been significant increase in influenza outbreaks leaving thousands either infected or dead

Abbreviations: ABTS, 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; Bu, Butanol; DMEM, Dulbecco's Modified Eagle's Medium; DPPH, 2,2 diphenyl-2-picrylhydrazyl; DS, *Dianthus superbus* var. *longicalysinus*; DW, Distilled water; EA, Ethyl acetate; Glu, glucoside; I, Isorhamnentin; K, Kaempferol; LC-MS, Liquid chromatography–mass spectrometry; MDCK, Madin-Darby Canine Kidney; MeOH, Methanol; PBS, Phosphate Buffered Saline; Q, Quercetin; RPMI-1640, Roswell Park Memorial Institute medium; Rha, Rhamnoside; Rut, Rutinoside; SRB, Sulforhodamine B; TFC, Total flavonoid content; TPC, Total polyphenol content

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and many vaccines were used as a primary prophylaxis to control influenza virus infection. However, due to genetic changes occurred in life cycle of influenza virus during infection to human being there is limitation to control influenza pandemics in many regions by using available drugs of market (Kaiser et al., 2003; Gansukh et al., 2016). Neuraminidase inhibitors such as oseltamivir and zanamivir are commonly used to control the influenza virus infections but the seasonal influenza crises revealed that some isolates of H1N1 were resistant towards these available commercial drugs (Deyde et al., 2009). Due to lack of scientific knowledge on aspects of anti-influenza drug development, safer, inexpensive, and low resistance drugs are needed for the protection of our society (Ozawa et al., 2011). Recent study reveled that the DS crude leaf extract has been proved to have potent antiviral effect against tobacco mosaic virus (Cho et al., 2000).

Plants are always utilised as best source of food and for therapeutic purposes in human life due its high nutrients and secondary metabolites composition including flavonoids, phenolics, alkaloids and others bioactive compounds. Depending on their metabolite content, different and a wide range of biological activities and mechanisms were observed in the human body (Enkhtaivan et al., 2015). In modern day's drugs from natural origin mainly from folk medicinal plants were applied to prevent various infections including viral, bacterial and fungal with promising disease control properties against cancer and diseases related to oxidation stress (Yu et al., 2012; Enkhtaivan et al., 2015). The plant bioactive compounds help to reduce free radical effect as antioxidants and because the conventional wisdom is that antioxidants should lower cancer risk by neutralizing cell-damaging, cancer-causing free radicals (Hawk et al., 2016). *Dianthus superbus* var. *longicalysinus* (DS) belongs to a genus of *Dianthus* and the family Caryophyllaceae. DS commonly grows at high altitudes and is native to European, Asian countries and widely distributed in Mongolia (Flora GrEIF. 2010). Several species of *Dianthus* have been considered medicinally important as they are widely used to treat various infections and diseases in China, Korea, Iran and Mongolia for millennia (Mutlu et al., 2016; Yun et al., 2016). It is also known as 'Qumai' in traditional Chinese medicine (TCM) and widely used to treat urethritis, diuretic, carbuncles, and carcinoma effects, further it is widely used as natural agent for anti-inflammatory and urinary infections (López-Expósito et al., 2011; Yu et al., 2012). *Dianthus* species revealed that plants belonging to this genus are a rich source of triterpenoid saponins, flavonoids, pyrane like glycosides, macrocyclic anthocyanins, and cyclopeptides (Nakano et al., 2011; Tong et al., 2012; Mutlu et al., 2016). *D. superbus* herb has been reported to contain various bioactive compounds including cyclic triterpene saponins, dianthramide and cyclic peptides (Hsieh et al., 2004; Tong et al., 2012). Many studies showed that the DS is more effective as antimicrobial, anticancer and antioxidant natural herbal agent with immunosuppressive, osteoblastic proliferative effects and prevent peanut-induced anaphylaxis (Gou et al., 2011; Tong et al., 2012; Ding et al., 2013). Recent research prove that DS extracts are effective agents for displaying potent anticancer and anti-inflammatory properties (López-Expósito et al., 2011; Yu et al., 2012). Previous researchers revealed that DS extracts showed cytotoxicity against liver cancer (HepG2, Hep 3B), breast cancer (MCF-7, MDA-MB-231) and lung cancer (A-549) cell lines (Yu et al., 2007, 2012).

Nonetheless, few studies have endeavored to highlight its therapeutic and functional potential with bioactive phytochemical composition of DS. Indeed, a comprehensive literature survey shows that there need to focus on studies related to its medicinal uses, phytochemical profile and biological activities. In light of the traditional usages of species from *Dianthus* genus, we hypothesized that *D. superbus* L would possess multi-pharmacological potential and bioactive compounds that would justify its use as a traditional herbal remedy. To this effect, the present study was designed to utilize different extracts of *D. superbus* and its bioactive compounds for antioxidant, anti-influenza and toxicological effects. In the present study this plant was investigated for its potential application towards management of influenza and cancer. Different extraction solvents and techniques were used to study its biological potential and extracts were also evaluated for possible antioxidant potential using *in vitro* assays. Also, we investigated correlations between the extracted metabolites for their anticancer, antivirus and antioxidant properties.

2. Materials and methods

2.1. Chemicals

Organic solvents used in extraction such as butanol, ethyl acetate and methanol were analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile purchased from Merk, Drmstadt, Germany. Folin-ciocalteu's phenol reagent was obtained from Junsei Chemicals (Junsei chemical, Japan). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2 diphenyl-2-picrylhydrazyl (DPPH) used for antioxidant estimation and Sulforhodamine B (SRB) used for the anticancer study and quercetin, berberine and gallic acid used as a control for studying anticancer activity were obtained from Sigma-Aldrich (Seoul, Korea). HPLC grade distilled water purchased from Duksan Pure Chemicals CO., LTD (Seoul, South Korea).

2.2. Cell lines and virus

Influenza viruses (APR/34/8 and B/LEE/40) and Madin-Darby Canine Kidney (MDCK), human ovary cancer cells (HeLa and SKOV) were provided by ATCC (Manassas, VA, USA). Lung cancer cell (NCL-H1299) and cervix cancer cell (CaSki) were purchased from KCLB (Korean Cell line Bank, Seoul, South Korea). RPMI-1640, DMEM medium (Dulbecco's Modified Eagle's Medium), fetal bovine serum, antibiotic-antimytotic and trypsin-EDTA were supplied by Gibco BRL (Grand Island, NY, USA). The cell culture dish and 96 well plates were purchased from Falcon (BD Bioscience, Franklin Lakes, NJ, USA).

2.3. Extraction and fractionation of plant material

The dried plant sample (upper part of plant excluding root) of DS was purchased from Tavin-Us-Pharm, Drug company, Ulaanbaatar, Mongolia in July 2016. Dried DS plant material 150 g was minced with a mechanical grinder (Hanil Co. Seoul, South Korea). Extracted 3 times with 600 mL of 100% methanol and once with 80% methanol and collected methanol extraction was dried using rotary evaporator (EYELA N1001S-WD, NY, USA). The methanol extract (MeOH) was dissolved in distilled water (DW) and partitioned using the order of polarity of ethyl acetate (EA), butanol (Bu) and remaining with DW residue fraction. Detail scheme and description of liquid-liquid extraction using different solvents for *D. superbus* L was explained in Fig. 1.

2.4. Estimation of total flavonoid content (TFC)

TFC of DS fractions were studied using previously described method by Enkhtaivan et al. (2015). Each DS fractions (20 μL: Total concentration was 1 mg/mL) added to 180 μL of 90% diethylene glycol and 20 μL of 1M NaOH. Mixture was kept for 30 min at room temperature and absorbance was observed at 515 nm using microplate reader (SpectraMax® Plus 384-spectrophotometer, Molecular Devices, USA). The content of flavonoid was calculated comparing values of different concentrations of quercetin with a value of the sample and expressed in mg/g of sample.

2.5. Estimation of total polyphenol content (TPC)

TPC of DS fractions was measured following methods previously described by Maria John et al. (2015). 20 μL (Total concentration was 1 mg/mL) of each fraction was added to wells with 100 μL of 0.2 M

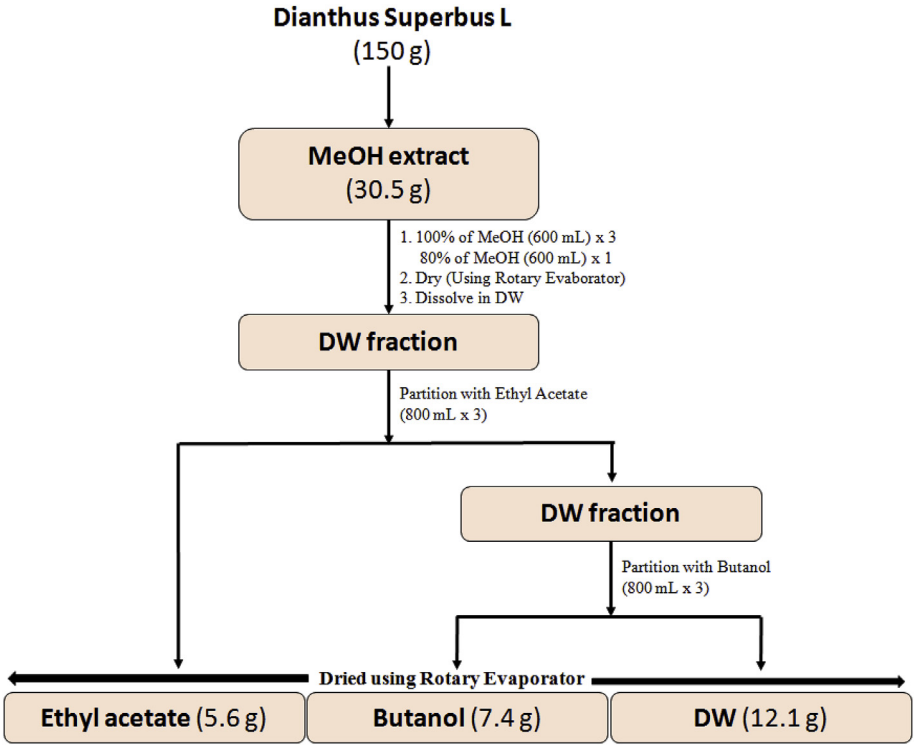

Fig. 1. Detail scheme and description of liquid-liquid extraction of different extractions of *D. superbus* L.

Folin-Ciocalteu's phenol reagent. After 30 min of incubation at room temperature, 80 µL of saturated sodium carbonate was added and kept at room temperature for 1 h. Using a microplate reader; absorbance was measured at 750 nm. The results were expressed as milligram of Gallic acid equivalent per gram (mg/g) of dried DS fractions.

2.6. Secondary metabolite identification using LC-MS/MS

LC-MS/MS analysis is performed using Bruker EVOQ LC-MS system equipped with Bruker, CTC PAL-xt autosampler (Bruker Daltonics, Bremen, Germany). Liquid chromatography analysis of DS extracts was carried out on a YMC packed C18 column (150 × 4.6 mm; Waters, Milford, USA). Samples were injected as the volume of 5 µL and at flow rate was 200 µL/min. During analysis, 2 phases as A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) were used gradually: phase B was 0% during 0–0.5 min then increased up to 50% until 5 min and then maintained 50% until 15 min. From 16 min, phase B was reduced to 0% and maintained 0% until finish experiment. The mass spectrometer was equipped with heated electrospray ionization (HESI) source. Analyzing parameters were as follows: ESI source was positive ion mode and voltage of 4000 V and scan range between 100 and 1200 *m/z*; capillary voltage, 80 V; nebulization at 40 psi (air); drying gas temperature, 350 °C; drying gas pressure, 30 psi (N_2); drying gas flow, 60 L/h; MS/MS fragmentation energy, 1.3 V. The instruments were controlled by MS Workstation System Control (Version 8.1.2; Bruker) and observed data were processed by MS Workstation MS Data Review (Version 8.1.2; Bruker).

2.7. DPPH radical scavenging activity

DPPH radical scavenging activity of DS fractions was performed using previously reported method by Maria John et al. (2015). In brief, 180 µL of 0.1 mM concentrated DPPH solution and 20 µL (Total concentration: 0.1, 1 and 10 µg/mL) of the sample was added to the wells of 96 well microplates. The mixture was incubated at room temperature for 20 min and then absorbance was observed using micro plate reader at 517 nm. Methanol was used as a control. DPPH scavenging activity was calculated using the followed formula: DPPH Scavenging activity (%) = $((A_c - A_s)/A_c) \times 100$, Where A_c: absorbance of control and A_s: absorbance of the sample.

2.8. ABTS radical scavenging activity

ABTS radical scavenging activity of DS fractions was studied using reported method by Enkhtaivan et al. (2015). Briefly, 20 µL (Total concentration: 0.1, 1 and 10 µg/mL) of DS fraction and/or 20 µL methanol was added with 180 µL of 7.4 mM ABTS solution containing 2.6 mM potassium persulfate into the wells of a 96 well microplate. The mixture was incubated at room temperature for 20 min. The absorbance of samples was measured at 734 nm using microplate reader. Methanol was used as a control and based on the difference of optical density value, the ABTS scavenging activity was calculated same formula used in DPPH scavenging activity.

2.9. Cell culture

NCL-H1299, HeLa, Caski, SKOV and MDCK cells were grown in 10% of FBS and 1% of Antibiotic-Antimycotic solution added in DMEM (MDCK cell) and RPMI-1640 (all other cancer cell lines) medium, respectively. Humidified incubator was adjusted as 37 °C with 5% of CO_2 to maintain the cells.

2.10. Antiviral activity assay

The antiviral activity assay was performed using SRB method using reduction of cytopathic effect previously reported by Choi et al. (2009). 1.5×10^4 concentration of MDCK cells was seeded onto 96 well microplates. After 1 day, the medium was removed and washed twice with phosphate buffered saline (PBS). Then, Influenza virus (A/PR/34/8 and B/LEE/40) was injected to the MDCK cells with a tissue culture infective dose of 50% ($TCID^{50}$). Anti-Influenza activity and cytotoxicity of dried fractions of DS were checked using final concentrations of 0.1 µg/

mL, 1 μg/mL, 10 μg/mL and 100 μg/mL (dissolved in 100% of DMSO and diluted into cell culture medium up to ≤ 0.1% of DMSO), in triplicates. The microplates were incubated for 48 h at 37 °C in the CO_2 incubator, the medium was removed and washed tree times with PBS. The plates were fixed with 70% of acetone overnight at - 4 °C. Then 70% of acetone was removed and dried at 60 °C. Fixed plates were subjected to SRB assay for antiviral assay.

2.11. Anticancer activity assay

NCL-H1299, HeLa, Caski and SKOV cells were cultured using 96 well microplates and maintained in humidified incubator at 37 °C and 5% of CO_2. After 24 h of incubation, cell cultured medium was removed and washed three times with PBS. Final concentrations of DS at 0.1 μg/mL, 1 μg/mL, 10 μg/mL and 100 μg/mL (dissolved in 100% of DMSO and diluted into cell culture medium up to ≤ 0.1% of DMSO) in triplicates were added on to the wells with new medium. After 2 days of incubation in CO_2 incubator at 37 °C, the medium was removed and washed three times with PBS. The microplates were fixed with 70% of acetone for overnight at – 4 °C. After removal of acetone, microplates were dried in a dry oven for 1 day. Then SRB assay was performed for determining the anticancer activity.

2.12. SRB assay

Each well of plates was added with 100 μL of Sulforhodamine B solution (0.4% w/v) (SRB) and incubated for 3 h. After incubation, the plates were washed with 1% of acetic acid 5 times and then dried in a dry oven. After plates fully dried, the morphology of cells was observed using light microscopy at 40x magnification (Olympus CKX41, Tokyo, Japan). SRB attached with fixed cells were dissolved in 100 μL of tris-base (10 mM) for overnight and then absorbance was read at 540 nm using a microplate reader. Values of inhibition concentration of 50% (IC_{50}), the cytotoxic concentration of 50% (CC_{50}) and therapeutic index (TI) were calculated.

2.13. Influenza NA inhibition assay

The effect of DS flavonol glycosides on the neuraminidase activity of influenza virus was studied using previously described NA inhibition assay by Enkhtaivan et al. (2017). NA inhibition assay was carried out by NA-Star® Influenza Neuraminidase Inhibitor Resistance Detection Kit (Applied Biosystems, Massachusetts, USA) following the manufacturer's instruction. Briefly, various concentrations of compounds such as Q 3-rut, I 3-glu and Oseltamivir (0.1, 1, 10 μg/mL dissolved in 100% of DMSO and diluted into assay buffer up to ≤ 0.1% of DMSO) were incubated with NA-Star® Assay Buffer (50 μL) treated with influenza A virus (A/PR/8/34) in 96 well plate for 30 min at 5% of CO_2 in 37 °C. Additionally, NA-Star® Substrate (10 μL) was added to wells and maintained at room temperature for 30 min in dark place. Moreover, NA-Star® Accelerator (60 μL) was added, absorbance of each sample was observed by SpectraMax L luminescent microplate reader (Molecular Device, California, USA). NA inhibition assay result was calculated as:

NA activity (%) = (T/V) × 100, T: Virus + Compound, V: Virus.

2.14. Molecular docking study on influenza targets

Computer based, ligand-receptor docking study was evaluated for DS flavonol glycosides (Q 3-rut, I 3-glu, Q 3-rha 7-rha, K 3-glu-glu 7-rha and Q 3-rha-glu 7-rha) against influenza virus targets such as HA: Hemagglutinin (3LZG, H1N1, A/California/04/2009), NA: Neuraminidase (4WA4, H3N8, A/harbor seal/Massachusetts/1/2011), PA: Polymerase Acidic Protein (4AWM, pH1N1, 2009), PB2: Polymerase Basic Protein (4NCE, H3N2, Seasonal Flu), M: Matrix protein, Proton Channel (1EA3, H1N1, A/Puerto Rico/8/1934), M2: Proton Channel (6BKK, H3N2, A/Udorn/1972) protein using AutoDock Vina program (Version 1.1.2, available at http://vina.scripps.edu) (Enkhtaivan et al., 2017a). The crystal structures of the viral proteins were collected from Protein Data Bank (PDB, http://www.rcsb.org/pdb). Subsequently, polar hydrogen and Kollman charges were added to the NA receptor protein by AutoDockTools (Version 1.5.6, http://mgltools.scripps.edu) program. Moreover, water molecules were removed from receptor. The pockets of receptors were built by AutoDockTools grin box with default 0.375 Å spacing. All additional settings of AutoDock Vina program defaulted and best 9 binding position were extracted. The calculated 2D ligand interaction (5 Å distance) and 3D crystal structures were illustrated through Maestro (Version 11.5.010, Schrodinger LLC) (Enkhtaivan et al., 2017b).

2.15. Statistical analysis and correlation study

Analytical and biological replicates were used for the analysis and the data were analyzed by using Microsoft Excel and IBM SPSS Statistics 20 software (SPSS Inc., Chicago, IL, USA). Metabolite comparison study was analyzed by Statistica 7.1 (Statsoft, USA). The individual metabolites areas of LC-MS/MS data were analyzed by MS workstation MS Data Review (Version 8.1.2; Bruker) and then $\log_{10}$ values were used for calculation of correlation study. Correlation between metabolites and biological activities analyzed using Pearson's correlation coefficient test by SPSS software. The correlation results were plotted in heat map using MeV software (version 4.9.0, http://tm4.org). Values of TPC and TFC for plant fractions were compared with standard compound and amount were calculated using Microsoft Office Excel program with statistical function: FORECAST. The all results were the mean ± SD of triplicates.

3. Result and discussion

3.1. TPC, TFC and metabolite identification using LC-MS/MS

Metabolite composition (phenolic and flavonoid contents) is the important factor for determination of biological activities such as anticancer, antiviral, anti-inflammation, and antimicrobial of plant extracts. Previous researchers showed that the methanol extract of DS contained various cyclic peptides and ethanol extract of DS contented several dianthramides, flavonoids and coumarins (Hsieh et al., 2005; Tong et al., 2012; Ding et al., 2013). The methanol extraction of DS was further purified by using ethyl acetate, butanol and remaining with distilled water. TPC and TFC of DS extracts was calculated and presented in Fig. 2. EA fraction had extremely high TPC among the other fractions with 105.2 mg/g concentration. Also, Bu fraction (80.6 mg/g) was better than DW fraction (31.1 mg/g). TFC of EA fraction was

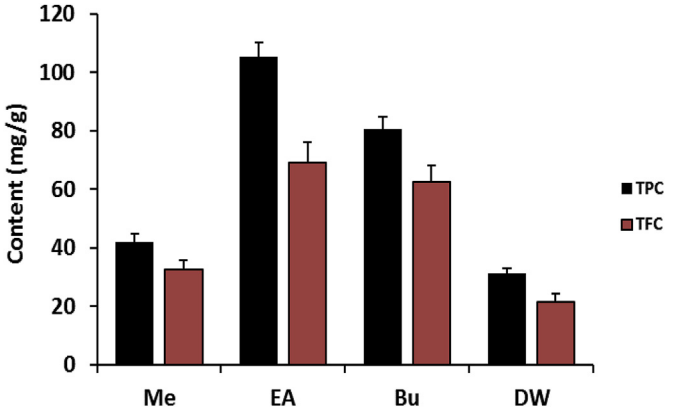

Fig. 2. Total phenolic and total flavonoid content of different extractions of *Dianthus superbus* L. The results are the mean ± SD of triplicates.

Table 1
List of the identified compounds using LC-MS/MS from *D. superbus* L.

NO	Compound name	RT (min)	UV(nm)	$[M+H]^+ m/z$	MS/MS	Molecular Formula	Area (*10^5mAU)			
							MeOH	EA	Bu	DW
1	Q 3-rut	7.2	276	611	465, 307, 303	$C_{27}H_{30}O_{16}$	3.47	3.59	5.21	0.71
2	I 3-glu	7.6	289	479	317, 162	$C_{22}H_{22}O_{12}$	2.87	1.27	3.45	0.55
3	Q 3-rha 7-rha	7.9	303	595	449, 303, 146	$C_{27}H_{29}O_{15}$	2.80	0.41	6.51	0
4	K 3-glu-glu 7-rha	5.9	228	757	611, 449, 287, 162, 146	$C_{33}H_{40}O_{20}$	2.81	0	1.44	6.99
5	Q 3-rha-glu 7-rha	6.1	235	772	611, 449, 303, 162, 146	$C_{33}H_{40}O_{21}$	4.07	0	4.84	0
6	Dianthin C	13.9	535	677	579, 514	$C_{36}H_{48}N_6O_7$	0.12	0.94	0	0
7	Dianthin D	9.3	354	712	684, 615, 599, 114	$C_{36}H_{53}N_7O_8$	1.09	6.91	1.20	0
8	Dianthin H	8.6	327	637	597, 468, 355, 254	$C_{31}H_{46}N_6O_7$	0.86	3.34	0	0
9	Dianthin F	7.5	286	548	401, 302, 155	$C_{30}H_{37}N_5O_5$	0.50	2.91	0	0
10	Tyrocidine B	10.1	386	1309	1195, 1096, 916, 805, 638, 472, 377	$C_{66}H_{87}N_{13}O_{13}$	0.12	0.16	0	0

Compounds were identified by LC-MS/MS and compared with reference compounds (I, isorhamnetin; K, kaempferol; Q, quercetin; glu, glucoside; rut, rutinoside; rha, rhamnoside).

recorded as highest having 69.1 mg/g concentration, which was high with TPC. Flavonoid content of Bu and DW fractions were 62.5 mg/g and 21.3 mg/g, respectively. Enkhtaivan et al., 2015 mentioned that TFC and TPC are positively correlated with antiviral and antioxidant activity against influenza virus infection *in vitro*. We investigated that, ethyl acetate as solvent is ideal for extraction and analysis of TFC and TPC, while butanol as best solvent for extraction and analysis of TFC in DS. Additionally, EA fraction mainly absorbing cyclic peptides and flavonoids with less than 3 sugar group. In the other hand, Bu fraction has been absorbed mainly in flavonoids and flavonoids with more sugar groups.

The metabolic distribution of DS extracts using EA, Bu and DW as extraction solvent were identified by LC-MS/MS analysis and presented in Table 1. Identification of metabolites of DS based on their UV spectra, released positive ions $[M + H]^+$ and reference data (Hsieh et al., 2004; Cuyckens and Claeys, 2004; Kachlicki et al., 2008; Tong et al., 2012). The 5 different cyclic peptides and 5 different flavonol glycosides were extracted and studied in EA, Bu and DW extracts of DS. Cyclic peptide content was higher in EA extraction than Bu; on the contrary, Bu extraction contained higher flavonol glycosides and lesser cyclic peptides. Cyclic peptides such as dianthin C, dianthin D, dianthin F, dianthin H, and tyrocidine B and few flavonol glucosides as Q 3-rut, I 3-glu and Q 3-rha 7-rha were detected in EA extraction.Q 3-rut, I 3-glu, Q 3-rha 7-rha, K 3-glu-glu 7-rha, Q 3-rha-glu 7-rha and dianthin F was observed in Bu extraction. In case of DW extraction, Q 3-rut, I 3-glu and K 3-glu-glu 7-rha were identified (Table 1). The reason for the polarity of different solvents used for extraction is because the cyclic peptides and flavonol glycosides selectively separates and distributed distinctively in specific extractions. Low polarity solvent EA extraction could lead to the collection of less amounts of mono and di glycosides of flavonoid but rich with cyclic peptides. Whereas, the polar solvent Bu was rich in mono, di and tri glycosides of flavonoids and only one cyclic peptide was identified. Also, DW extraction detected less amount of mono and tri glycosides of flavonoids but no cyclic peptides were detected. Considering the variation of metabolite contents in different extractions, biological activities and their correlations were studied.

3.2. ABTS and DPPH radical scavenging activity

Oxidative damage to human cells may cause heart diseases, stroke, diabetes, cancer and other disorders (Yu et al., 2007). Different extractions of DS were compared for their antioxidant potential by ABTS and DPPH radical scavenging activity assay and the IC_{50} value was calculated (Fig. 3) and estimated antioxidant effect for isolated compounds for DS were presented in Table 4. ABTS activity of MeOH, EA and Bu extraction were 5.2 μg/mL, 4.99 μg/mL and 5.02 μg/mL of IC_{50}, respectively. DPPH activity of MeOH, EA and Bu extraction were 54.3 μg/mL, 47.1 μg/mL and 42.4 μg/mL. In both ABTS and DPPH

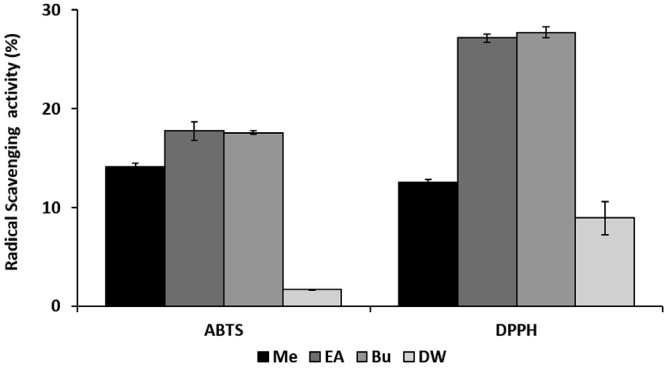

Fig. 3. ABTS (A) and DPPH (B) radical potential of different extractions of *Dianthus superbus* L at 1 μg/mL concentration. The results are the mean ± SD of triplicates.

radical scavenging activity of DW was found to be low (27.5 μg/mL and 177.4 μg/mL of IC_{50} values). ABTS radical scavenging activity of EA extraction was showed highest among the other extractions. However, in case of DPPH radical scavenging activity, Bu extraction was stronger than EA and DW extractions. However, including the correlation study, many metabolites distinctly correlated with DPPH and ABTS radical scavenging activity.

3.3. Anticancer activity and correlation of DS extractions

Anticancer activity of various DS extraction is presented in Table 2. The extracted cyclic peptides and TPC of EA extraction induced dramatic anticancer activity with all cancer cell lines used in this study (Table 4). EA extraction showed highest effectiveness against cancers *in vitro* with HeLa, SKOV, Caski and NCL-H1299 cell lines at 9.5 μg/mL, 9.6 μg/mL, 13.8 μg/mL and 69.9 μg/mL of IC_{50} values, respectively. The result showed that EA extraction was more effective against cancer cells

Table 2
Cytotoxic activity of different extractions of *D. superbus* L on cancer cell lines.

Extracts	Cancer cell lines (IC_{50}, μg/mL)			
	Hela	Skov-3	Caski	NCL
MeOH	120.2 ± 33.6	220.6 ± 24.1	209.6 ± 83.8	122.5 ± 11.7
EA	9.5 ± 0.8	9.5 ± 1.2	13.8 ± 4.7	69.9 ± 4.2
Bu	81.9 ± 13.5	68.4 ± 31.8	272.5 ± 61.3	70.0 ± 6.1
DW	127.3 ± 32.8	2897.6 ± 736.8	356.8 ± 88.2	106.1 ± 4.2
Berberine	8.0 ± 0.9	58.5 ± 0.9	42.7 ± 4.2	5.4 ± 0.1

IC_{50}: Inhibition concentration of 50% (μg/mL). The results are the mean ± SD of triplicates.

than Berberine, which is known as an anticancer agent. Our result confirmed that previous studies illustrated cyclic peptides from DS are toxic against cancer cell lines (Hsieh et al., 2004; Tong et al., 2012). In our observation, EA extraction content of cyclic peptides was higher than other extractions. Additionally, TPC of EA was highest among the others followed by Bu, MeOH and DW. SKOV cell is one of a most drug-resistant ovarian cancer cell and Gonera et al., (2014) reported that SKOV cell is not sensitive to anticancer drugs and toxins such as Adriamycin, diphtheria toxin and *cis*-platinum.

However it was interesting to observe that the EA and Bu extractions showed fascinating toxicity against SKOV cell at even less concentration such as 9.6 μg/mL and 68.4 μg/mL of IC_{50} values respectively, while Berberine was 58.5 μg/mL. Caski cell is a cervical cancer cell line that was used in this study was reported to contain human papillomavirus type 16 and 18 genomes (Yee et al., 1985). In present study EA extraction showed the highest activity against Caski cell line with 13.8 μg/mL IC_{50}. EA extraction appears to be a possible candidate inhibitor against human papillomavirus. The NCL-H1299 cell line used in this experiment is a human lung cancer cell, which is known to contain partial genes for deletion or decline of the p53 protein. This p53 protein helps to provide inhibition against influenza virus infections (Turpin et al., 2005). It shows the patient has lung cancer, which has more risk to get an influenza virus infection than normal people. Continually anti-influenza activities of DS extracts were tested. Correlation study results are presented in Fig. 5, cyclic peptides were highly correlated with anticancer activity compared to flavonol glycosides with no significant correlation.

3.4. Antiviral activity against influenza virus A and B viruses

Antiviral activity of DS extractions against influenza A and B viruses and cytotoxicity was evaluated at different concentrations (0.1 μg/mL to 100 μg/mL). SRB assay was used for determining the antiviral activity of various extractions of DS (Fig. 4 and Table 3). All extractions showed different activity against influenza virus. Among extractions, EA and Bu extractions showed similar excellent inhibition activity against influenza A virus with 4.45 μg/mL and 4.97 μg/mL IC_{50} values followed by MeOH (28.67 μg/mL) and DW (85.62 μg/mL). In case of influenza B virus, Bu was observed strongest with3.9 μg/mL of IC_{50} value and TI was 25.4. EA extraction showed 12.44 μg/mL of IC_{50} against influenza B virus. Bu, MeOH and DW extractions did not show any toxicity in non-infected MDCK cells, but EA extraction was detected to exhibit toxicity (CC_{50} was 75.91 μg/mL). However, TI value of EA was 17.1 and 6.1 against influenza A and B viruses, respectively. Cyclic peptides content was identified higher in EA than MeOH and Bu extractions. Reversely, Bu extraction revealed less cyclic peptides and great flavonol glycosides. Our result confirmed that Choi et al., presented quercetin-3-rhamnoside has strong inhibition activity against influenza A virus (Choi et al., 2009). Following the correlation study (Fig. 5), antiviral activity against influenza A and B viruses was extremely high correlated with both TFC and flavonol glycosides. The high content of flavonol glycosides in Bu extraction induced strong anti-influenza activity in both A and B viruses. Comparing the TI and a wide range of inhibition against influenza viruses of Bu extraction, it appears to be a potential candidate for new anti-influenza drug development (see Table 4).

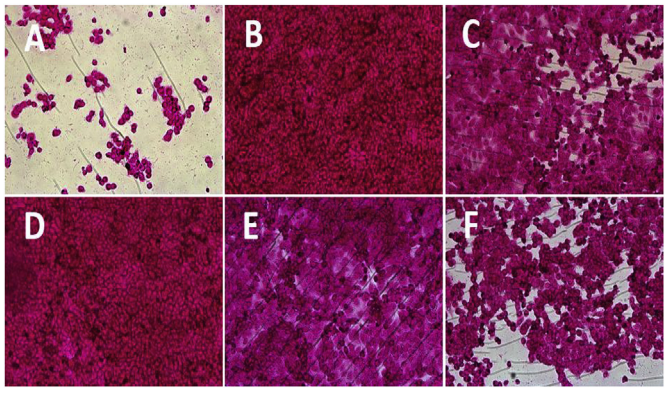

Fig. 4. The effect of different extracts of *Dianthus superbus* L on Influenza A/PR/8/34-induced CPE in the concentration of 10 μg/mL. (A) Infected cells only; (B) Non-infected MDCK cells; (C) Infected cells treated with MeOH extract; (D) Infected cells treated with EA extract; (E) Infected cells treated with Bu extract; (F) Infected cells treated with DW extract.

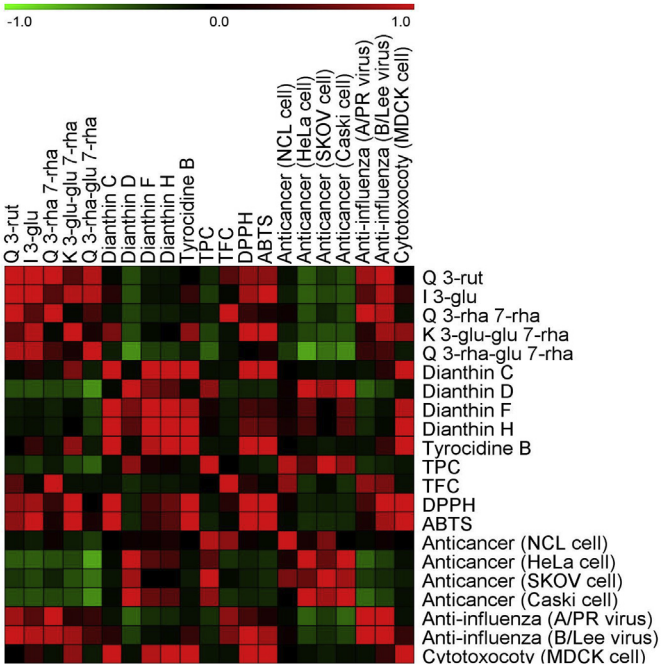

Fig. 5. The correlation between metabolite and biological activities of *D. superbus* L.

Table 3
Anti-influenza activity and cytotoxicity of different extractions of *D. superbus* L.

Extractions	CC_{50}	Influenza A/PR		Influenza B/LEE	
		IC_{50}	TI	IC_{50}	TI
MeOH	> 100	28.67 ± 18.1	3.5	31.25 ± 12.2	3.2
EA	75.91	4.45 ± 0.2	17.1	12.44 ± 1.8	6.1
Bu	> 100	4.97 ± 0.6	20.1	3.9 ± 0.5	25.4
DW	> 100	85.62 ± 17.09	1.2	196.1 ± 20.7	0.5

CC_{50}: Cytotoxic concentration of 50% (μg/mL), IC_{50}: Inhibition concentration of 50% (μg/mL), TI: Therapeutic index. The results are the mean ± SD of triplicates.

3.5. Neuraminidase (NA) activity of influenza virus

Influenza NA protein is a one of membrane glycoprotein, which has a role on release of new viral particle from host cells. Currently, well-known influenza drug Oseltamivir it inhibits viral infection through blocking of NA (Gansukh et al., 2016; Choi et al., 2009). However, some H1N1 isolates have recorded as resistant to Oseltamivir in 2007–2008 influenza seasons (Choi et al., 2009). Interestingly, the compounds Q 3-rut, I 3-glu, Q 3-rha 7-rha, K 3-glu-glu 7-rha, and Q 3-rha-glu 7-rha showed competitive docking result on NA protein and the binding affinity range between −7.4 kcal/mol to −8.4 kcal/mol (Table 5). Moreover, in same active site, Oseltamivir had been docked and binding value was −6.1 kcal/mol (Enkhtaivan et al., 2017).

Table 4
Estimated antioxidant, antiviral, anticancer activity and cytotoxicity of detected compounds from *Dianthus superbus* L calculated by correlation study.

NO	Compounds	Cytotoxicity	Anticancer				Anti-Influenza		Antioxidant	
		MDCK	HeLa	Skov-3	Caski	NCL	A/PR	B/LEE	ABTS	DPPH
1	Q 3-rut	82.5	79.0	49.0	63.2	54.0	95.3	95.8	93.5	75.4
2	I 3-glu	86.6	73.0	48.2	56.1	52.5	91.3	92.0	95.4	63.3
3	Q 3-rha 7-rha	77.1	85.8	52.7	71.2	56.3	96.8	97.2	89.3	86.2
4	K 3-glu-glu 7-rha	91.6	73.3	55.8	53.2	54.0	88.1	89.2	97.2	51.7
5	Q 3-rha-glu 7-rha	70.3	59.7	25.8	41.4	33.0	89.4	89.0	84.6	59.5
6	Dianthin C	100.0	84.5	81.2	75.7	81.2	76.7	79.4	96.8	62.0
7	Dianthin D	83.4	86.4	98.1	95.2	99.7	47.4	51.5	71.3	72.9
8	Dianthin H	97.7	86.3	90.6	85.4	92.4	65.4	68.9	89.9	66.6
9	Dianthin F	98.5	86.9	89.9	84.3	90.8	68.1	71.4	91.6	66.2
10	Tyrocidine B	99.8	83.8	79.3	73.8	79.0	78.1	80.6	97.4	61.0

Values calculated by Pearson's correlation coefficient and expressed as % inhibition. I: isorhamnetin; K: kaempferol; Q: quercetin; glu: glucoside; rut: rutinoside; rha: rhamnoside.

Table 5
Anti-influenza target approach through molecular docking (Affinity kcal/mol).

NO	Compound name	Surface Proteins		Polymerase		Matrix Proteins	
		NA	HA	PA	PB2	M1	M2
1	Q 3-rut	−8.4	−6.7	−9.8	−9.4	−6.1	−4.2
2	I 3-glu	−7.4	−6.5	−8.2	−7.9	−6.0	−3.7
3	Q 3-rha 7-rha	−8.6	−6.2	−9.7	−8.9	−5.6	−4.5
4	K 3-glu-glu 7-rha	−8.5	−6.5	−8.6	−8.8	−6.4	−4.7
5	Q 3-rha-glu 7-rha	−8.8	−6.8	−8.9	−9.2	−6.2	−4.6

HA: Hemagglutinin (3LZG), NA: Neuraminidase (4WA4), PA: Polymerase Acidic Protein (4AWM), PB2: Polymerase Basic Protein (4NCE), M1: Matrix protein, Proton Channel (1EA3), M2: Proton Channel (6BKK). I: isorhamnetin; K: kaempferol; Q: quercetin; glu: glucoside; rut: rutinoside; rha: rhamnoside.

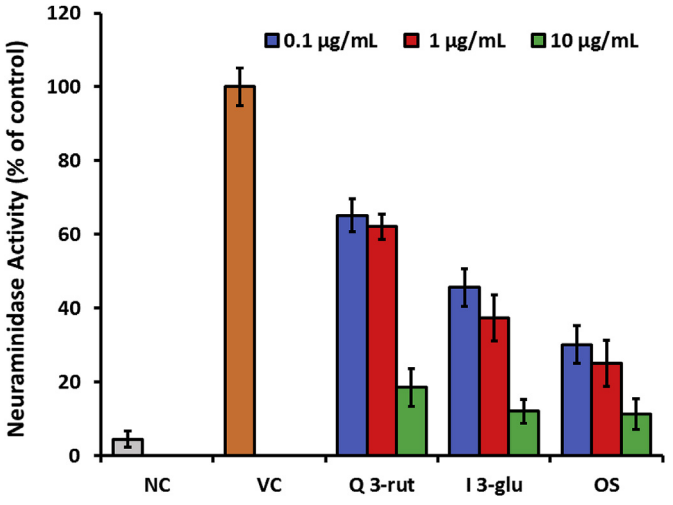

Fig. 6. The assay of the Neuraminidase activity of flavonoids. NC: Negative control, VC: Virus control, Q: Quercetin, rut: rutinoside, I: isorhamnetin, glu: glucoside, OS: Oseltamivir (Standard). The error bars are indicating the mean of standard deviation (± S.D) of triplicate.

Subsequently, for proving the computed result, we carried out NA inhibition assay with Q 3-rut and I 3-glu compared with standard NA inhibitor as Oseltamivir (Fig. 6). Q 3-rut and I 3-glu showed higher NA inhibition activity as dose dependent manner. Moreover, the Q 3-rut, I 3-glu and Oseltamivir were reduced NA activity up to 18.5%, 12.1% and 11.3% at 10 µg/M, respectively. Additionally, Q 3-rut and I 3-glu flavonol glycosides showed plentiful of interactions with hydrogen bonds with positive and negative charges between receptors and ligand.

3.6. Molecular docking study

The results for *in vitro* study showed significant anti-influenza activity for *D. superbus* L bioactive compounds including Q-3-rut, I-3-glu, Q-3-rha 7-rha, K-3-glu-glu 7-rha and Q-3-rha-glu 7-rha, which was further, studied using in silico molecular docking study and found that the results were highly correlated to anti-influenza activity. For probing of anti-influenza activity, six different potential target of influenza (HA, NA, PA, PB2, M1 and M2), a well-known anti-influenza drug target proteins were assayed using molecular docking methods. Molecular docking result showing that tested flavonol glycosides are with significant and higher binding activities towards influenza polymerase proteins, mainly PA and PB2 membrane glycoprotein as NA (Fig. 7). Previously, it was reported that various quercetin derivatives are highly potent towards inhibiting influenza PB2 protein in influenza A virus (Enkhtaivan et al., 2017). The influenza virus polymerase has a special cap-binding mechanism using host 7 methylated guanosine 3 phosphate as mRNA to start viral gene replication. Moreover, this PB2 structure is identical along on most of the different strains of influenza viruses (Enkhtaivan et al. 2017a, 2017b).

4. Conclusions

In this study we utilised the *Dianthus superbus L* and extracted various bioactive compounds using differential polarity of solvents and extracts were analyzed LC-MS to identify the metabolite content. DS has rich contents of metabolites such as cyclic peptides and flavonol glycosides. The cyclic peptides composition was higher in EA than Bu extractions but not detected in DW. The results showed that the polar solvent like Bu provides a better separation and more yields for flavonol glycosides, while EA used as ideal solvent for the extraction of cyclic peptides.

The results of the cytotoxicity study suggest that EA extraction showed strongest anticancer activities in various cancer cells because of the high content of cyclic peptides. Moreover, the Bu extraction showed highest therapeutic activity against influenza A and B viruses due to presence of high concentration of flavonol glycosides. The Q 3-rut and I 3-glu showed higher NA inhibition activity as dose dependent manner for influenza virus. The results showed that the flavonol glycosides highly correlated with antiviral activity, where as cyclic peptides highly correlated with anticancer activities using molecular docking and in silico studies. From obtained results we concluded that the *D. superbus L* deserves more attention as a folk medicine and further exploited as a pharmaceutical herbal product. Further, the *D. superbus* considered as a source for valuable phytochemicals for developing novel drug leads against influenza virus and cancer treatment. Further studies are needed for isolation and identification of individual compounds from the crude extracts with in vivo studies.

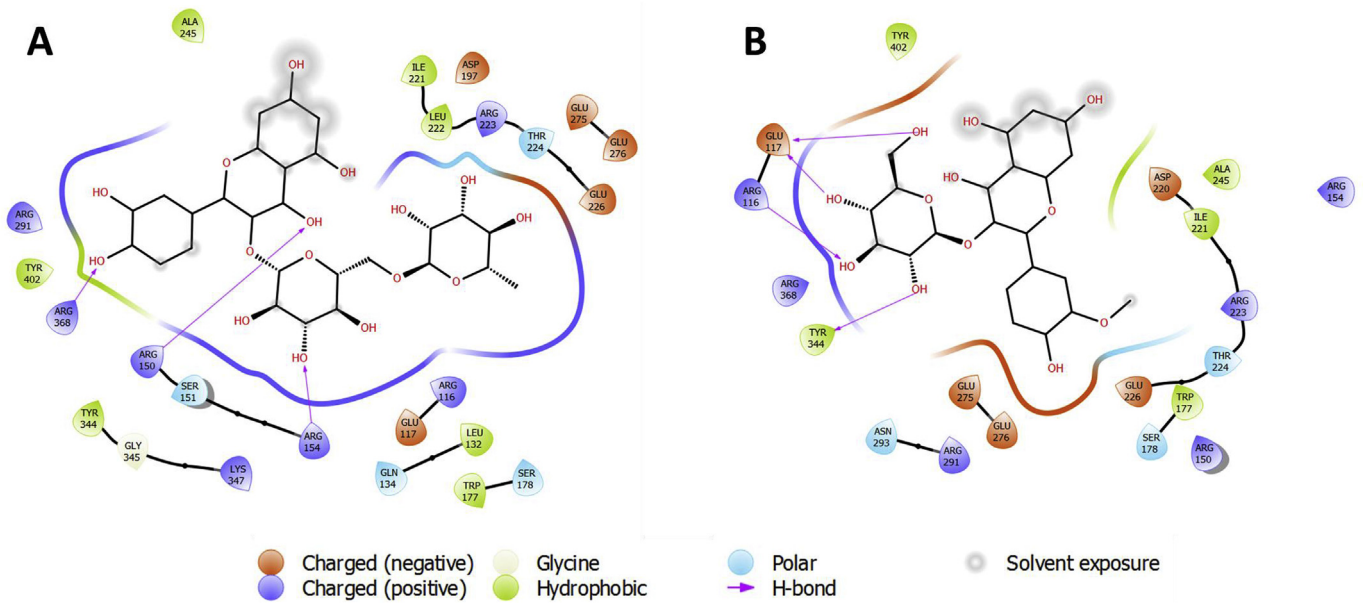

Fig. 7. Overall binding interactions of Q 3-rut (A) and I 3-glu (B) calculated in 5 Å distances from the influenza NA subunit (4WA4). Q: Quercetin, rut: rutinoside, I: isorhamnetin, glu: glucoside.

Conflicts of interest

The authors declare that they have no competing interest.

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Transparency document

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