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Four bioactive compounds isolated from the stem of *Anethum sowa* L. and their bioactivities

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Abstract

Anethum sowa L., a well-known herb in folk medicine, has a greater medicinal significance due to its diversified activities such as antioxidant, antimicrobial and antispasmodic activity. Very few bioactive compounds have been reported from this species. Our study focused on the isolation, structure elucidation and bioactivity assay of the compounds. In the present work, 6-hydroxy-1, 3-dimethoxy-7-methyl-xanthen-9-one **(**AS-1) from dichloromethane extract and scopoletin **(**AS-2**),** 1, 3, 4-trimethoxy-xanthen-9 -one **(**AS-3**)**, graveolone **(**AS-4) from ethyl acetate extract of stem of *A. sowa* were isolated from the stem as well as the plant for the first time. All the characterizations and chemical structures of the compounds were determined by extensive modern spectroscopic techniques such as ultraviolet (UV), infrared (IR), mass, Nuclear Magnetic Resonance (NMR) spectrophotometer. Moreover, the cytotoxic, antimicrobial and antioxidant activity of AS-2, AS-3 and AS-4 were assessed. AS-2 exhibited significant activity against *Salmonella typhi* while mild antifungal activity against *Aspergillus niger*. Furthermore AS-3 revealed significant antifungal activity against *Sacharomyces cerevacae* as well as antibacterial activity against *Salmonella typhi*. Besides AS-4 exhibited moderate antibacterial activity against *Bacillus megaterium.* In addition AS-2, AS-3 and AS-4 presented mild cytotoxic with respect to positive control (Vincristine sulphate) while AS-3 exhibited moderate antioxidant activity as compared to positive control (Ascorbic acid).

Keywords

Anethum sowa , Apiaceae, brine shrimp lethality bioassay, disc diffusion, free radical scavenging.

Introduction

From primordial time, plants are significantly used for medicinal purposes. Many recent studies have shown that medicinal plants such as *Cannabis sativa* L.*, Calotropis procera* (Aiton) W.T.Aiton*, Chenopodium quinoa* Willd and *Chenopodium murale* L., contain a variety of biological active compounds (1-4). The chemical constituents derived from medicinal plants were frequently contemplated as more functional due to less toxicity, natural and appetizing effect, long shelf-life and relatively lower undesirable side-effects (5). For that reason, many civilizations used the different parts of medicinal plant for cooking, scenting agent in regular life and as an efficacious therapeutic aid for various diseases. On the contrary, the medications like the crude form of

the plants revealed a number of perilous side effects owing to the presence of some harmful ingredients along with the active constituents in older ages. The researchers are deeply altering their concentration and effort to natural products to develop effective drugs against deadly diseases as well as infections (6). Natural bioactive compounds from plants are known for their versatile biological activities such as antifungal, antibacterial, anticancer, antioxidant, anti-inflammatory etc. (7-10).

The common name of *Anethum sowa* L. belonging to Apiaceae family, locally known as *Shulfa* in Bangla, is Dill. It is an annual or a biennial aromatic herb having a smooth plant surface which is completely devoid of hair or pubescence. The genus *Anethum* comprises 37 species. Among them, four species, for instance, *Anethum graveolens* L., *Anethum sowa* L., *Anethum foeniculoides* and *Anethum theurkauffii* were available. At present, only two species such as *Anethum graveolens* L. and *Anethum sowa* L. are being found (11). It is extensively planted in the northern part of Bangladesh and all over India, Pakistan and Myanmar. It is frequently grown with the weed and even in the canal escapes of irrigated agricultural area in cold season (12). The ripe dried seed of *A. sowa* is longer. Dill-apiol and essential oil derived from seed have insecticidal, ovicidal and synergistic activity against flour beetles (13, 14, 15). Moreover essential oils have antioxidant and antimicrobial activities (12). Ethanol extract of the stem has antioxidant, antifungal and insecticidal activities (16, 17, 18). As condiment and tea, dill weed is used in fresh and dried condition. The aromatic herb is generally consumed for essence and process of different types of perishable foods like pickles, salads, sauces and soups. In culinary purposes, fresh or dried leaves have a favorable use in the preservation and cooking of fish and meat, sandwiches, fish sauces and sour vinegar. An essential oil obtained from dill oil is used as flavoring in food industry. To aromatize detergents and soaps, it is used in the perfume industry as well as a substitute for caraway oil (19, 20).

In addition, the isolation of propiophenone biphenyl derivative constituents (21), piperine and β-sitosterol glucoside (22), dillapional (23), limonene and carvone (24) from *A. sowa* are reported from earlier phytochemical investigations. Although some works on *A. sowa* have been explored till now, insufficient information is revealed regarding the phytochemical and pharmacological aspects of the stem of *A. sowa* grown in Bangladesh. This is why the stem of *A. sowa* found in Bangladesh has been selected for extensive phytochemical and biological studies in the present research work. In this present study, we have reported one compound isolated from dichloromethane extract and three compounds from the ethyl acetate extract of stem of *A. sowa* as well as the cytotoxic, antimicrobial and antioxidant activity of scopoletin **(**AS-2**),** 1, 3, 4-trimethoxy-xanthen-9-one **(**AS-3**)** and graveolone **(**AS-4).

Materials and Methods

Collection of plant material

Fresh stem of *A. sowa* were collected from Keranigonj, Dhaka, Bangladesh. The identification of the plant was approved by Bangladesh National Herbarium Centre, Dhaka with a voucher specimen (DACB Accession Number-31,282) recorded at Bangladesh National Herbarium Centre, Dhaka.

Instrument and Operating condition

Electro-thermal melting point apparatus (Stuart Scientific SMP3, UK) and OptiMelt (MPA100), Stanford Research Systems, US were used to record melting points of the isolated compounds. SHIMADZU FTIR spectrophotometer (model IRAffinity-1) was employed to measure Infrared spectra as KBr disc. Under ultraviolet light at 254 nm for fluorescence quenching spots and at 366 nm for fluorescent spots, TLC chromatograms were detected. To record ^{1}H NMR and ^{13}C NMR spectra in deuterated solvent ($CDCl_3$), BrukerAvance spectrometer equipped with 400 MHz was extensively used. To measure the chemical shifts (δ), the residual proton of the solvent was used as an internal standard and these were measured in ppm relative to $CDCl_3$ (δ_H 7.25, δ_C 77.2) and coupling constants (J) are given in Hz. Gas Chromatograph Mass spectrometer (model GCMS-QP2010 Ultra) was used to scan Electron Impact Mass Spectrometry (EI-MS). The IR, UV, MS, 1D and 2D, ^{1}H and ^{13}C NMR spectroscopic techniques were extensively exploited to elucidate the structures of the isolated compounds. UV-Visible Spectrophotometer (Shimadzu, Japan) was utilized to record the UV absorbance. Solvents were evaporated with the help of vacuum rotary evaporator (BUCHI, Rotavapor R-210 Switzerland). Analytical graded chemicals and solvents obtained from commercial sources E. Merck (Germany), BDH (England) and Sigma Aldrich (Germany) were used.

Preparation of the extracts

The stem of the herb was separated, chopped into small pieces and dried in air under the shed (25-30°C) in the absence of sunlight. The dust free air-dried material was ground into powder in a grinding machine (100 mesh).Under the cold extraction process, the powdered material of stem (1.5 kg) was successively soaked by n-hexane, dichloromethane, ethyl acetate and methanol with three times soaking of each solvent at room temperature where they were soaked for 2 days. As a result, sticky mass collected from n-hexane extract (7.2 g), dichloromethane extract (5.8 g), ethyl acetate extract (8.7 g) and methanol extract (15.9 g) was obtained from the filtrate of corresponding solvent subjected to evaporation by rotary evaporator under reduced pressure.

Isolation of the compounds

Firstly, dichloromethane extract (5.0 g) of the stem of *A. sowa* was subjected to column chromatography eluted with *n*-hexane followed by mixtures of *n*-hexane-dichloromethane, dichloromethane, dichloromethane-ethyl acetate, ethyl acetate, ethyl acetate-methanol and finally 100% methanol with increasing polarity. Eight

fractions were formed from total 60 fractions as per TLC patterns for further purification. After the column fraction 5 (collection number 29-36) was concentrated, a yellow solid was precipitated out and separated from solution followed by washing with minimum amount of methanol to get AS-1 (9.1 mg) in a pure state.

On the contrary, ethyl acetate extract (8.0 g) of stem of *A. sowa* was undergone column chromatography eluted with *n*-hexane, *n*-hexane-ethyl acetate mixture, pure ethyl acetate and ethyl acetate-methanol mixture with increasing polarity for fractionation. Seven fractions were made from total 55 fractions according to their TLC behavior for further purification. Depending on the TLC behavior, the column fraction 3 (collection number 16-21) was dried by rotary evaporator and then subjected to PTLC (stationary phase: silica gel PF_{254}, mobile phase: toluene: ethyl acetate= 9:1, thickness of plates: 0.5mm). From the developed plates one yellow band (R_f= 0.40 in 10% ethyl acetate in toluene) was scrapped and extracted with ethyl acetate. Consequently, the yellow solid AS-2 (2.9 mg) was isolated in pure form after evaporation of solvent.

On the other hand, column fraction 5 (collection number 31-38) was passed through a medium sized column made with silica gel in 100% dichloromethane and eluted with dichloromethane-ethyl acetate solvent systems with increasing polarity. Depending on the TLC behavior, fraction number 12 and 13 among 40 fractions were dried and a yellow amorphous solid was found in pure form denoted as AS-3 (3.7 mg).

Afterwards collection number 14-18 of the same column showed single spot on the TLC plate in different solvent systems and dried using rotary evaporator to get light yellow solid which was designated as AS-4 (6.5 mg).

Bioassay of Isolated Compounds

Bioactivity of isolated compounds (AS-2, AS-3 and AS-4) from stem of *A. sowa* were carried out by determining the cytotoxic effect using brine shrimp lethality bioassay, antimicrobial activity by observing the zone of inhibition and antioxidant activity by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method.

Cytotoxic activity

The cytotoxic activity of AS-2, AS-3 and AS-4 was performed against *Artemia salina* (L.) by brine shrimp lethality bioassay method (25, 26). Isolated compounds were dissolved in DMSO under serial dilutions such as 150, 75, 37.5, 18.75, 9.375, 4.684, 2.344, 1.172, 0.586 and 0.292 µg/mL. For each experiment 10 shrimps were taken in a test tube filled with simulated brine water (5 mL). Then each of these test solutions was added to the test tube and incubated at room temperature for 24 h. A plot of the percentage of the shrimps against the logarithm of the sample concentrations is drawn for the determination of median lethal concentration (LC_{50}) of the test samples compared to vincristine sulphate (LC_{50}= 0.57) which was considered as positive control.

Antimicrobial activity

The antibacterial activity of the isolated compounds AS-2, AS-3 and AS-4 were evaluated at 400 µg/disc against some pathogenic bacteria and fungi listed in the Table 2 by disc diffusion method (27, 28) where Ciprofloxacin (30 µg/disc) was used as standard antibiotic disc.

Antioxidant Activity

In addition the free radical scavenging activity of AS-2**,** AS-3 and AS-4 was assayed spectrophotometrically by DPPH method (29, 30). Different concentrations (500, 250, 125, 62.5, 31.25, 15.625, 7.813, 3.906, 1.953, 0.977 µg/ml in methanol) of ascorbic acid solution (1 mL) as well as the solution (1 mL) of isolated compounds from stems was mixed separately with 3 mL of 0.4 mM DPPH solution. The mixture was retained in dark for 30 minutes to determine the absorbance at 517 nm by UV-visible spectrophotometer where ascorbic acid was used as positive control. For higher free radical scavenging activity, the lower absorbance of the reaction mixture is obtained. In DPPH assay, the scavenging activity of the sample depends on the degree of decolorization of DPPH from purple to yellow. The scavenging activity was calculated using the equation:

$$\text{Scavenging activity (\%)} = \frac{A-B}{A} \times 100$$

where A is the absorbance of positive control and B is the absorbance of positive control with the sample. IC_{50} (50% inhibitory concentration) value was calculated from the graph which was drawn by plotting the scavenging activity (%) against concentration.

Spectral Data of Isolated Compounds

Compound AS-1

Yellow solid; mp 178-179 ^{0}C;UV (CH_3OH) λmax 384 nm; IR (neat) υ3070 (=C-H), 2922, 2851, 1670 (>C=O), 1614, 1580 (C=C, aromatic), 1377, 1329, 1209,1161, 1136 (C-O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.85 (1H, s, H-5), 6.57 (1H, s , H-4), 6.38 (1H, s , H-2), 6.26 (1H, s , H-8), 3.95 (3H, s , -OC<u>H</u>$_3$at C-1), 3.90 (3H, s , -OC<u>H</u>$_3$at C-3), 2.48 (3H, s , -C<u>H</u>$_3$at C-7) ppm; ^{13}CNMR ($CDCl_3$) δ 182.8 (C-9), 166.6 (C-6), 161.4 (C-3), 159.0 (C-1) , 156.5 (C-12), 155.8 (C-11), 141.2 (C-13), 110.2 (C-8), 108.8 (C -7), 105.8 (C-5), 104.8 (C-10), 97.9 (C-4), 97.0 (C-2), 55.8 (-O<u>C</u>H$_3$) 55.4 (-O<u>C</u>H$_3$) 20.5(-<u>C</u>H$_3$); MS (m/z) 286 (M$^+$, base peak), 270, 257, 243, 228, 214, 199, 185,129, 115, 77, 69, 51.

Compound AS-2

Yellow solid; mp 203-205 ^{0}C;UV (CH_3OH) λmax 352, 301, 262, 232 nm; IR (neat) υ3335 (br. O-H), 3028 (ar. C-H), 2922, 2851, 1699 (>C=O), 1607, 1564,1510 (ar. C=C), 1288, 1261, 1138, 1016 (C-O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 7.58 (1H, d, J=9.6 Hz, H-4), 6.91 (1H, s, H-8), 6.83 (1H, s, H-5), 6.26 (1H, d, J=9.6 Hz , H-3), 6.13 (1H, s, -O<u>H</u>), 3.94 (3H, s, -OC<u>H</u>$_3$at C-6); ^{13}CNMR ($CDCl_3$) δ 161.4 (C-2), 150.2 (C-7), 149.6 (C-9), 144.0(C-6), 143.2 (C-4), 113.4 (C-3), 111.5(C-10), 107.4 (C-5), 103.2 (C-8), 56.4 (-O<u>C</u>H$_3$); MS (m/z) 192 (M$^+$, base peak), 177, 164, 149, 121, 79, 69, 65, 51.

Compound AS-3

Yellow amorphous solid; mp 175-176 ^{0}C;UV (CH_3OH) λ_{max} 409, 315 nm; IR (neat) υ>3000 (=C-H), 2936, 2827, 1639 (>C=O),1589, 1553, 1502, 1454 (C=C, aromatic), 1250, 1182,1138, 1105, 1057 (C-O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 8.44

(1H, d, J=8.0 Hz, H-8), 7.71 (1H, t , J=7.6, 8.0 Hz , H-6), 7.50 (1H, d, J=8.0 Hz , H-5), 7.29 (1H, t, J= 8.0& 7.6 Hz , H-7), 6.27 (1H, s , H-2), 4.01 (3H, s, -OC<u>H</u>$_3$ at C-1), 3.92 (3H, s, -OC<u>H</u>$_3$ at C-4) , 3.83 (3H, s, -OC<u>H</u>$_3$ at C-3); ^{13}CNMR ($CDCl_3$) δ 180.8 (C-9), 159.3 (C-1), 156.2 (C-11), 142.0 (C-12), 140.5 (C-3), 133.9 (C-6), 130.2(C-4), 126.6 (C-8), 121.5(C-7), 120.8(C-13), 114.5(C-5),105.8(C-10), 86.7 (C-2), 60.8 (-O<u>C</u>H$_3$ at C-4), 56.0 (-O<u>C</u>H$_3$ at C-1), 34.1(-O<u>C</u>H$_3$ at C-3); MS (m/z) 286 (M^+), 285, 270 (base peak), 242, 199, 170, 143, 128, 115,86, 77, 63, 51.

Compound AS-4

Light yellow solid; mp 178-179 ^{0}C;UV (CH_3OH) λ_{max} 345, 307, 253, 222 nm; IR (neat) υ>3000 (=C-H), 2928, 2868, 1730 (>C=O), 1684 (>C=O), 1626 (C=C, aromatic), 1395, 1296, 1211,1146,1101(C-O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 8.02 (1H, s, H-5), 7.66 (1H, d , J=9.6 Hz , H-4), 6.83 (1H, s, H-10), 6.29 (1H, d, J=9.6 Hz , H-3), 2.76 (2H, s, H-7), 1.48 (6H, s, 2 C<u>H</u>$_3$ at C-8); ^{13}CNMR ($CDCl_3$) δ 190.8 (C-6), 162.4 (C-2), 159.9 (C-11), 159.3 (C-14), 143.3 (C-4), 127.3 (C-5), 117.6 (C-13), 114.6 (C-3), 113.3 (C-12), 105.6 (C-10), 80.7(C-8), 48.6(C-7), 26.7(2 <u>C</u>H$_3$); MS (m/z) 244 (M^+), 229 (base peak), 201, 189, 160, 132, 104, 91, 76, 69.

Statistical analysis

Triplicate data of each compound in the assessment of the cytotoxic, antimicrobial and antioxidant activity were taken and average value was calculated.

Results and Discussion

Isolation of compounds from stem

Compound AS-1 (9.1 mg) was found as yellow solid and soluble in chloroform. The melting point of the compound was recorded as 178-179°C. It showed single spot on the TLC plate at R_f 0.39 in 10% ethyl acetate in toluene.

The mass spectrum of AS-1 showed a molecular ion peak at m/z 286 corresponding to a molecular formula $C_{16}H_{14}O_5$. The absorption band in the UV spectrum at λ_{max} 384 nm suggested the presence of conjugation and chromophoric groups in the molecule. The IR spectrum of the AS-1 exhibited absorption bands at 3070 cm^{-1} for aromatic C-H and 2922 and 2851 cm^{-1} due to saturated C-H stretching vibrations. The absorption band at 1670 cm^{-1} was found due to C=O stretching and bands at 1614 and 1580 cm^{-1} was obtained due to aromatic C=C stretching vibrations. The absorption bands at 1209, 1161 and 1136 cm^{-1} revealed the presence of C-O stretching vibrations in the molecule.

The ^{1}H NMR spectrum exhibited signals for highly characteristic four aromatic proton resonances at δ 6.38 (1H, s), 6.57 (1H, s), 6.85 (1H, s) and 6.26 (1H, s) which could be assigned to four protons: H-2, H-4, H-5 and H-8 respectively. The position of two protons attached to C-2 and C-4 was confirmed by the ^{1}H-^{1}H correlations indicated in the COSY spectrum and this was supported by the ^{1}H-^{13}C long range correlations in the HMBC spectrum. Two sharp singlets, each of three protons intensity, at δ 3.95 and 3.90 were ascribed to two methoxyl groups at C-1 and C-3 position. The presence of only methyl group in the molecule could be attributed by a singlet at δ 2.48.

The presence of 16 carbons in the molecule was clearly indicated by the 16 signals in the ^{13}C NMR spectrum. The analysis of ^{13}C NMR, DEPT-135 and DEPT-90 spectral data confirmed that the molecule contains three methyl (two methoxyl and one methyl), four methine and nine quaternary carbons. The ^{13}C NMR spectrum also showed two methoxyl carbons resonating at δ 55.8 (-OCH$_3$ at C-1) and 55.4 (-OCH$_3$ at C-3). The signal at δ 182.8 clearly showed the presence of carbonyl group at position 9 and the signal at δ 166.6 indicated the carbon (C-6) which is directly attached to the hydroxyl group. All the above data suggested that the compound AS-1 is a xanthone derivative containing two methoxyl, one hydroxyl and one methyl groups. The ^{1}H-^{1}H correlations in the COSY spectrum, the ^{1}H-^{13}C direct correlations in the HSQC spectrum and the ^{1}H-^{13}C long range correlations in the HMBC spectrum given in the Fig. 2 strongly supported the following structure of the compound AS-1 shown in the Fig. 1. Based on all spectroscopic data, literature values (31) and melting point of the compound, it was confirmed that the compound AS-1 is 6-Hydroxy-1, 3-dimethoxy-7-methyl-xanthen-9-one. Finally, the structure of the compound was confirmed by the fragment ions present in the mass spectrum.

Compound AS-2 (2.9 mg) was isolated as yellow solid and soluble in chloroform. The melting point of the

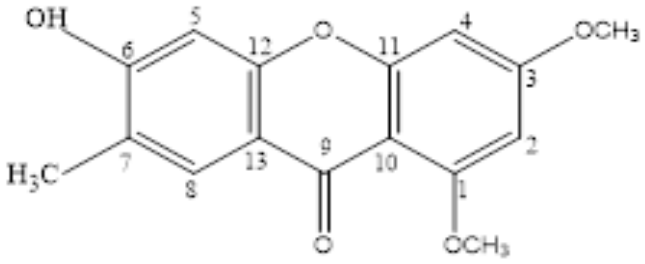

Fig. 1.Structure of 6-Hydroxy-1, 3-dimethoxy-7-methyl-xanthen-9-one (**AS-1**)

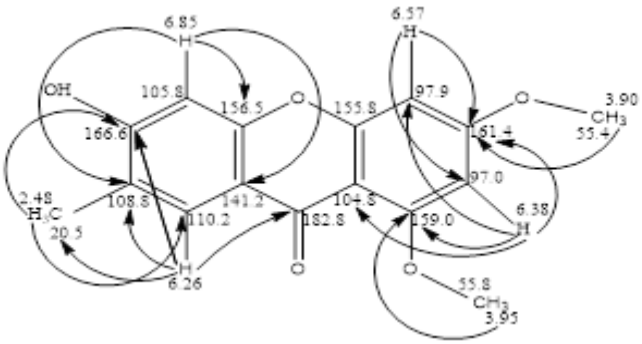

Fig. 2. HMBC spectrum of 6-Hydroxy-1, 3-dimethoxy-7-methyl-xanthen-9-one (**AS-1**)

compound was recorded as 203-205 °C. The compound showed single spot on the TLC plate with R_f value 0.36 in 10% ethyl acetate in toluene.

The mass spectrum of AS-2 showed a molecular ion peak at m/z 192 equivalent to a molecular formula $C_{10}H_8O_4$. The absorption bands in the UV spectrum at λ_{max} 232, 262, 301 and 352 nm suggested the presence of conjugation and chromophoric groups in the molecule. The IR spectrum of AS-2 showed absorption band at 3335 cm^{-1} due to the O-H stretching vibrations and absorption bands

at 2922 and 2851 cm^{-1} due to saturated C-H stretching vibrations. The absorption band at 1699 cm^{-1} was attained due to C=O stretching and bands at 1607, 1564,1501 cm^{-1} were found due to aromatic C=C stretching vibrations. The spectrum also showed absorption band at 3028 cm^{-1} due to aromatic C-H stretching vibrations. The absorption bands at 1288, 1138, 1016 cm^{-1} indicated the presence of C-O stretching vibrations in the molecule.

The ^{1}H NMR spectrum showed two doublets at d 6.26 (1H, d, J = 9.6 Hz) and 7.58 (1H, d, J = 9.6 Hz) characteristic to H-3 and H-4 protons respectively of the pyrone ring of coumarin skeleton. This was supported by ^{1}H-^{1}H correlations present in the COSY spectrum. The presence of two 1H singlets at d 6.83 and 6.91 were attributable to H-5 and H-8 respectively. In this spectrum a three-proton singlet at d 3.94 was assigned for a methoxyl group at C-6. The spectrum also showed a singlet at δ 6.13 which could be assigned to a hydroxyl proton at C-7.

The presence of 10 carbons in the molecule was clearly indicated by the 10 signals in the ^{13}C NMR spectrum. The analysis of ^{13}C NMR and DEPT spectral data confirmed that the molecule contains one methyl (OMe at C-6), four methine (C-3, 4,5 and 8) and five quaternary (C-2, 6, 7,9 and 10) carbons present in the structure. The signal for carbonyl carbon was appeared at δ 161.4 (C-2). Such a downfield appearance of C-2 is due to conjugation and esteric oxygen. The signal at δ 56.4 represents the carbon of methoxy group. All the above data suggested that the compound AS-2 is a coumarin derivative containing one methoxyl and one hydroxyl group. The ^{1}H-^{13}C direct correlations present in the HSQC spectrum and ^{1}H-^{13}C long range correlations in the HMBC presented in the Fig. 4 spectrum strongly supported the following structure of the compound AS-2 shown in the Fig. 3. Based on all spectroscopic data, literature values (32) and melting point of the compound AS-2, it was confirmed that the compound is Scopoletin. Finally, the structure of the compound was confirmed by the fragment ions present in the mass spectrum.

Compound AS-3 (3.7 mg) was isolated as yellow amorphous solid and soluble in chloroform. The melting point of the compound was recorded as 175-176 °C. A distinct yellow colored single spot was observed on the TLC plate with R_f value 0.38 in 10% ethyl acetate in toluene.

The mass spectrum of the compound showed a molecular ion peak at m/z 286 corresponding to a molecular formula $C_{16}H_{14}O_5$. The absorption bands in the UV spectrum at λ_{max} 315 and 409 nm suggested the presence of conjugation and chromophoric groups in the molecule. The IR spectrum of the compound showed absorption bands at >3000 and 2936 due to aromatic C-H and at 2827 cm^{-1} due to saturated C-H stretching vibrations. The absorption band at 1639 cm^{-1} was found due to C=O stretching and bands at 1589, 1553, 1502 and 1454 cm^{-1} were obtained due to aromatic C=C stretching vibrations. The absorption bands at 1250, 1182, 1138, 1105 and 1057 cm^{-1} indicated the presence of C-O stretching vibrations in the molecule.

The ^{1}H NMR spectrum exhibited signals for a highly characteristic ABCD spin system with four aromatic proton resonances at δ 7.50 (1H, d, J=8.0 Hz), 7.71 (1H, t , J=7.6, 8.0 Hz), 7.29 (1H, t, J= 8.0, 7.6 Hz) and 8.44 (1H, d, J=8.0 Hz), which could be assigned to four adjacent protons H-5, H-6, H-7 and H-8, respectively in the same ring. This was confirmed by ^{1}H-^{1}H correlations present in the COSY spectrum. The sharp singlet at δ 6.27 was attributable to the aromatic proton at C-2 of other ring. Three sharp singlets, each of three proton intensity, at δ 3.83, 3.92 and 4.01 were ascribed to three methoxyl groups at C-3, C-4 and C-1 position respectively.

The presence of 16 carbons in the molecule was clearly indicated by the 16 signals present in the ^{13}C NMR spectrum. The DEPT 135 spectrum indicated that out of the 16 carbons in AS-3, 8 are attached to protons. The analysis of ^{13}C NMR, DEPT-135 and DEPT-90 spectral data confirmed that the molecule contains three methyl (OMe at 1, 3 and 4), five methine (C-2, 5, 6, 7 and 8) and eight quaternary (C-1, 3, 4, 9, 10, 11, 12 and 13) carbons. The ^{13}C NMR spectrum also showed three methoxyl carbons resonating at δ 60.8 (-OCH_3 at C-4), 56.0 (-OCH_3 at C-1) and 34.1(-OCH_3 at C-3). The signal at δ 180.8 clearly showed for carbonyl carbon at C-9. All the above data suggested that the compound AS-3 is a xanthone molecule containing three methoxyl groups.The ^{1}H-^{13}C direct correlations present in the HSQC spectrum and the ^{1}H-^{13}C long range correlations presented in the HMBC spectrum shown in the Fig. 6 strongly supported the following structure of the compound AS-3 exhibited in the Fig. 5. Based on all spectroscopic data, literature values (33) and melting point of the compound, it was confirmed that the compound AS-3 is 1, 3, 4-Trimethoxy-xanthen-9-one. Finally, the structure of the compound was confirmed by the fragment ions present in the mass spectrum.

Compound AS-4 (6.5 mg) was isolated as light yellow solid and soluble in chloroform. The melting point

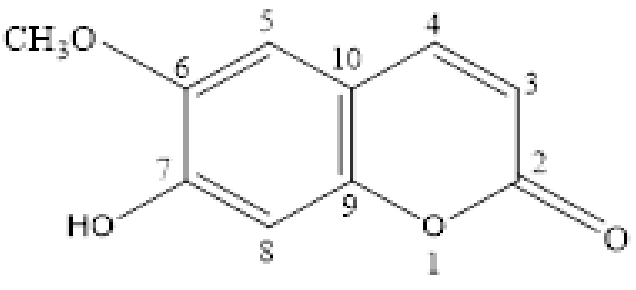

Fig. 3. Structure of scopoletin **(AS-2)**

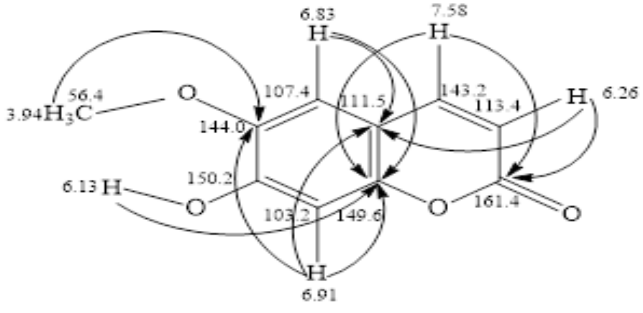

Fig. 4. HMBC spectrum of scopoletin (**AS-2**)

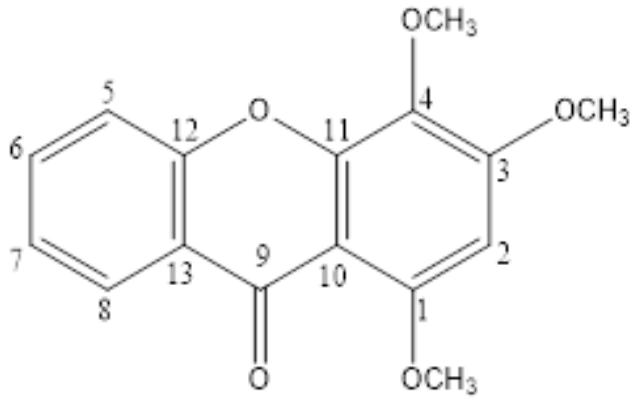

Fig. 5. Structure of 1, 3, 4-trimethoxy-xanthen-9-one (**AS-3**)

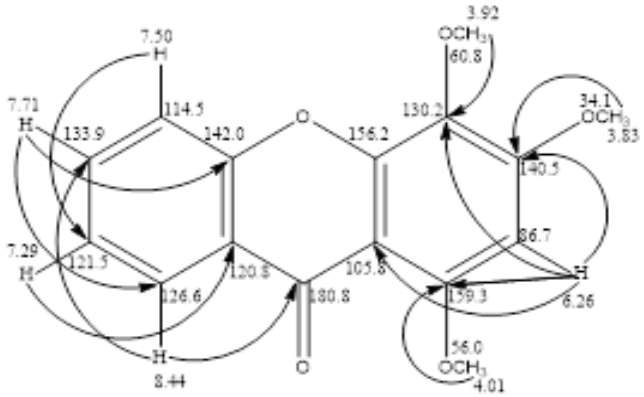

Fig. 6. HMBC spectrum of 1, 3, 4-trimethoxy-xanthen-9-one (**AS-3**)

of the compound was found at 178-179 °C. The compound showed single spot on the TLC plate with R_f value 0.34 in 10% ethyl acetate in toluene.

The mass spectrum of the compound showed a molecular ion peak at m/z 244 which is corresponding to a molecular formula $C_{14}H_{12}O_4$. The absorption bands in the UV spectrum at λ_{max} 222, 253, 307 and 345 nm suggested the presence of conjugation and chromophoric groups in the molecule. The IR spectrum of the compound showed absorption bands at >3000 and 2928, 2868 cm^{-1} due to aromatic C-H and saturated C-H stretching vibrations respectively. The absorption bands at 1730 and 1684 cm^{-1} were found due to C=O stretching and band at 1626 $cm^{-1}$was attained due to aromatic C=C stretching vibrations. The absorption bands at 1395, 1296, 1211, 1146 and 1101 cm^{-1} indicated the presence of C-O stretching vibrations in the molecule.

In the ^{1}H-NMR spectrum of AS-4, two singlets in the aliphatic region were found. The singlet at δ 1.48 corresponding to 6H which were safely assigned to two methyl groups attached to C-8. The another singlet having 2H appeared at δ 2.76 confirmed the presence of methylene protons at C-7 which was again supported by the negative signal at δ 48.6 in the DEPT-135 spectrum. The 1H doublets at δ 7.66 and 6.29 indicated the presence of two protons at C-4 and C-3respectively. The position of these protons was again confirmed by the ^{1}H-^{1}H correlations present in the COSY spectrum. The other two aromatic protons at C-5 and C-8 were easily assigned by two 1H singlets at δ 8.02 and 6.83 respectively.

The presence of 14 carbons in the molecule was clearly indicated by the 13 signals in the ^{13}C NMR spectrum. The signal at δ 26.7 represents two methyl carbons at C-8. The analysis of ^{13}C NMR, DEPT-135 and DEPT-90 spectral data confirmed that the molecule contains two methyl, one methylene (C-7), four methine (C-3, 4,5and 8) and seven quaternary (C-2,6, 8, 11, 12, 13 and 14) carbons. Two carbonyl carbons were appeared by the characteristic signals at δ 190.8 (C-6) and 162.4 (C-2) in the ^{13}C NMR spectrum. The signals at δ 159.9 (C-11) and 159.3 (C-14) indicated two aromatic carbons which were attached to oxygen. The aliphatic carbon at position-8 attached to oxygen could easily be assigned by the signal at δ 80.7.

All the above data analysis recommended that the compound AS-4 is a coumarin derivative known as Graveolone. The^{1}H-^{13}C direct correlations present in the HSQC spectrum and the ^{1}H-^{13}C long range correlations indicated in the HMBC spectrum depicted in the Fig. 8 strongly supported the following structure of the compound AS-4 shown in the Fig. 7. Based on all spectroscopic data, literature values (34) and melting point of the compound, it was confirmed that the compound AS-4 is Graveolone. Finally, the structure of the compound AS-4 was confirmed by the fragment ions present in the mass spectrum.

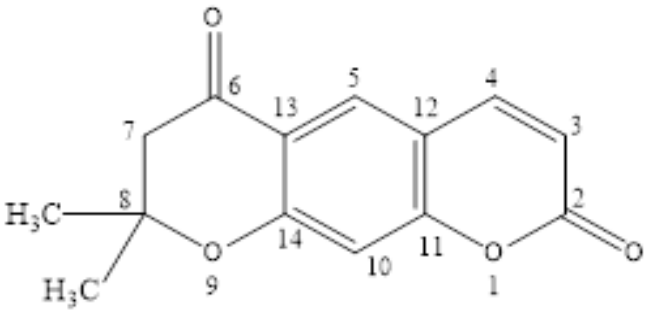

Fig. 7. Structure of graveolone **(AS-4)**

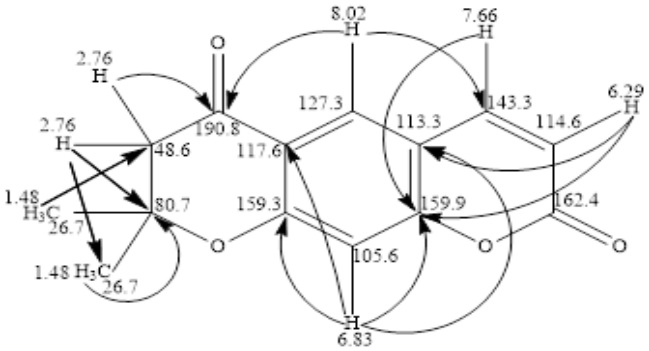

Fig. 8. HMBC spectrum of graveolone **(AS-4)**

Cytotoxicity, antimicrobial and antioxidant activity of AS -2, AS-3 and AS-4

Test samples showed different mortality rate at different concentrations. The mortality rate of brine shrimp was found to be increased with the increase of concentration of each sample. In the estimation of cytotoxic activity, the LC_{50} were found to be 12.59, 15.58, 13.30 and 0.45µg/mL for compound AS-2, AS-3 and AS-4 and VS respectively shown in the Table 1. Based on the value of LC_{50} it can be

predicted that AS-2**,** AS-3 and AS-4 showed mild cytotoxic effect on brine shrimp nauplii in comparison with vincristine sulphate used as positive control.

Table 1. Cytotoxicity of AS-2, AS-3 and AS-4

Sample	LC_{50} (ug/ml)
AS-2	12.59
AS-3	15.58
AS-4	13.30
VS	0.45

AS-2: Scopoletin, **AS-3:**1, 3, 4-trimethoxy-xanthen-9-one, **AS-4:** graveolone, **VS**: vincristine Sulphate.

In the evaluation of antimicrobial activity mentioned in the Table 2, Scopoletin (AS-2) exhibited significant activity at zone of inhibition of 27mm against *S. typhi* whereas mild antifungal activity against *Aspergillus niger* at zone of inhibition of 06mm. Moreover it has moderate activity against *Bacillus cereus* and *Escherichia coli* whereas was resistant to the rest of the tested bacteria and fungi. On the contrary, 1, 3, 4-Trimethoxy-xanthen-9-one (AS-3) showed significant antifungal activity against *Sacharomyces cerevacae* as well as significant antibacterial activity against *Salmonella Typhi*. Moreover AS-3 had mild antibacterial activity against *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus*. On the other hand, moderate antibacterial activity of Graveolone (AS-4) was found against *Bacillus megaterium* whereas mild activity was obtained against *Shigella boydii*, *Sarcina lutea* and *Escherichia coli*.

Table 2. Antimicrobial activity of AS-2, AS-3 and AS-4

Test bacteria and fungi	Diameter of zone of inhibition (mm)			Standard Ciprofloxacin (30 µ gm/disc)
	AS-2	**AS-3**	**AS-4**	
Gram Positive bacteria				
Bacillus cereus	16	NA	NA	40
Bacillus megaterium	NA	NA	24	45
Bacillus subtilis	NA	11	NA	35
Staphylococcus aureus	NA	13	NA	43
Sarcina lutea	NA	NA	14	39
Gram Negative bacteria				
Escherichia coli	17	11	14	38
Vibrio mimicus	NA	NA	NA	37
Shigella dysenteriae	NA	NA	NA	35
Pseudomonas aeruginosa	NA	NA	NA	42
Shigella boydii	NA	NA	16	38
Salmonella Paratyphi	NA	NA	NA	30
Salmonella Typhi	27	19	NA	35
Vibrio parahaemolyticus	NA	NA	NA	37
Fungi				
Sacharomyces cerevacae	NA	23	NA	38
Candida albicans	NA	NA	NA	38
Aspergillus niger	6	NA	NA	35

AS-2: scopoletin, **AS-3:**1, 3, 4-trimethoxy-xanthen-9-one, **AS-4:** graveolone, NA: No Activity

In the assessment of free radical scavenging activity given in the Table 3**,** it is clearly seen that AS-3 showed moderate antioxidant activity while AS-2 and AS-4 exhibit mild antioxidant activity as compared to positive control used as ascorbic acid. This is why it can be said that AS-2, AS-3 and AS-4 can reduce to the lowest possible amount of reactive oxygen species to prevent diseases.

Table 3. Antioxidant activity of AS-2, AS-3 and AS-4

Sample	IC_{50} (µg/mL)
AS-2	21.21
AS-3	14.45
AS-4	25.30
Ascorbic Acid	5.8

AS-2: scopoletin, **AS-3:**1, 3, 4-trimethoxy-xanthen-9-one, **AS-4:** graveolone

Conclusion

In the present study, four compounds were characterized from the stem of *A. sowa* for the first time. From the bioactivities of AS-2, AS-3 and AS-4**,** it comes to end that AS -2, AS-3 and AS-4 have mild to significant cytotoxic, antibacterial and antioxidant activity against different species.

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Authors contributions

MAM designed the study and performed the experiments while MASq prepared the manuscript and helped in data analysis. Finally KS supervised the experiments, corrected the manuscript and gave significant suggestions to upgrade the assessment.

Compliance with ethical standards

Conflict of interest:The authors have declared that there is no conflict of interest.

Ethical issues: None.

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