Phytochemical Profiling and Antiviral Potential of Elderberry (Sambucus Nigra) Extract against Herpes Simplex Virus and Human Papillomavirus: A Nutraceutical Approach

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ABSTRACT

Elderberry (*Sambucus nigra*) has been used for centuries to treat various ailments, including viral infections. However, the scientific evidence for its antiviral activity herpes simplex virus (HSV) and human papillomavirus (HPV) is scarce and inconsistent. This study aimed to evaluate the therapeutic potential of elderberry extract against HSV and HPV from a nutraceutical stand point by analyzing its phytochemical constituents. The volatile components of sambucol® was determined by high-performance liquid chromatography, the phytochemicals were identified by gas chromatography-mass spectrometry (GC-MS), and the proximate properties were measured. The GC-MS analysis of the black elderberry fruit extract revealed 58 unique compounds, the most abundant compound was 2-furanmethanol. The extract contained high amounts of vitamin C, B1, B6, B3, A and D3 vitamins, and phenolic compounds, anthocyanins, flavonoids, and phenolic acids. The extract also exhibited good nutritional value, with high levels of protein, fiber, and minerals. Black elderberry extract has robust chemical components with efficacy against viral infections and high potential for use in herpes simplex and human papillova virus management. However, further exploration of the activity of active chemical moieties identified should be carried out using *in vivo* and *in vitro* methods to elucidate this effect, mechanism and safety.

Keywords: Elderberry Extract, Phytochemicals, GC-MS Analysis. Antiviral Potential, Herpes Simplex Virus (HSV).

INTRODUCTION

Human papillomavirus (HPV) family *papillomaviridae* is icosahedral. The viral DNA is surrounded by a capsid which is made up of proteins L1 and L2 [1]. More than 200 variants of HPV have been identified based on DNA sequencing with 14 of these implicated in cancer [2]. HPV 16 and 18 have the most implicated cancer-causing variants identified [3]. Studies have indicated that HSV-2 infection could increase the expression of the HPV gene [4]. Another study has shown an association between hepatitis B infection and cervical cancer in women below the age of 50. HPV and HSV -2 infection are risk factors for HIV infection [5].

HSV-1 and HSV-2 belong to a family of enveloped viruses of the Herpesviridae family with only two of these specifically infect humans [6]. HSV-1 is commonly transmitted orally, while transmission of HSV-2 is mainly by sexual intercourse [7,8]. Infection with herpes simplex is usually asymptomatic but occasional intermittent symptoms may occur due to reactivation of latent infections [9]. Studies have shown that HSV-2 is a co-factor in HIV infection [10]. HSV-1 is known for causing

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blistering eruptions that primarily affect the mucous membranes in the mouth and genital areas [11]. HSV-2 infection also results in blistering lesions, along with general symptoms like itching, irritation, and skin abrasions. Additionally, HSV can lead to other conditions such as herpes stromal keratitis, encephalitis, and meningitis [12]. Despite the success recorded with screening and prophylactic measures for these viruses, challenges with vaccination (such as social stigma, and cost) and currently available prophylactic measures as well as medications used for the management of infections caused by herpes simplex and HPV virus has necessitated the use of other approaches for their management.

Extracts from fruit and flower of *Sambucus nigra* L, *(Adaxacede*) commonly called elderberry has been used as an antimicrobial and antiviral medication [13,14]. It is used as a diaphoretic, topical anti-inflammatory. diuretic, expectorant and laxative were well documented [15]. Its potency for inhibition of respiratory viruses has been reported [16]. Its efficacy in combating influenza and possibly COVID-19 has also been documented [17]. Recent studies have highlighted the anti-HIV activity of its extract, an effect it exerts by blocking the entry of the HIV virions into the host cells [18]. Another study has highlighted the efficacy of another species of Sambucus against the herpes simplex virus (HSV) [19]. The active moiety responsible for this effect is reported to be flavonoids and proanthocyanidins A-type contained in the extract [20]. Its activity against herpes simplex type 1 has also been documented [21]. This paper presents the results of studies on the analysis of vitamin composition, phytochemical constituents, and proximate properties of elderberry fruit extract Sambucus *nigra* (SA) highlighting its potential for use for prevention and inhibition of Herpes simplex virus and human papilloma virus.

METHODOLOGY

Materials

Sambucol® a marketed product of elderberry fruit (Sambucus *nigra*) Lot number N 077960101(USA) . Hydrochloric acid, ferric chloride, Mayer's reagent, chloroform, sulfuric acid chemicals were purchased from Merck. Vitamin standards A, B1, B3, B6, B9, C, D3, HPLC grade chemicals and reagents were purchased from Merck Specialty Pvt Ltd. Distilled water was obtained from the Central Research Laboratory LUTH. The Chemicals reagents were of analytical grade and no further purification was needed for their use.

Sambucol® (SA) Black elderberry contains cold-pressed black elderberry fruits preserved by the addition of potassium sorbate and citric acid. 10mL Sambucol® contains an equivalent of 3.8 g of cold-pressed elderberry extract.

QUALITATIVE PHYTOCHEMICAL SCREENING

Phytochemical screening was performed on the extract using standard procedures to identify the phytochemical constituents in the samples [22].

QUANTITATIVE PHYTOCHEMICAL SCREENING

Determination of tannin.

The technique described by Van-Burden&Robinson was used for quantitative tannin analysis [23].

Determination of alkaloids

Method employed by Akintelu, M. T., & Amoo, I. A. (2020) was used [24].

Determination of saponins

The technique provided by Obadoni & Ochuko (2002) was used to measure saponin quantitatively [25].

Determination of flavonoid

The flavonoid content of the ethanolic crude extract was determined using Aluminum chloride assay [26].

Determination of cardiac glycosides

8ml of the aqueous extract was measured and subjected to procedure described by Muhammad & Abubakar, 2016 [27].

Determination of phenol

0.5ml of the ethanolic crude extract was combined with 0.5ml of folin-ciocalteu reagent (1:1 diluted in distilled water) and incubated at 22°C for around 5 minutes. 2 mL of

20% sodium carbonate was added. After that, the mixture was incubated at the same temperature for 90 minutes, and the absorbance was measured at 650 nm [28, 29].

Determination of reducing sugar

1g of the ethanolic crude extract was macerated with 20 mL of distilled water. 1 mL of alkaline copper reagent was added to the aqueous extract, boiled for 5 minutes, cooled, then 1 mL phosphomolybdic acid, 2 mL distilled water was added. Absorbance was measured at 420 nm [30].

GC/MS FOR VOLATILE COMPONENTS OF BLACK ELDERBERRY EXTRACT

Agilent 7820A gas chromatograph attached to triple axis detector , 5975C inert mass spectrometer and electron-impact source was used. The stationary phase was HP-5 capillary column coated with 5% Phenyl Methyl Siloxane (30 m length x 0.32 mm diameter x 0.25 µm film thickness) (Agilent Technologies). The carrier gas Helium was set at a constant flow of 1.4871 mL/min, average velocity of 44.22 cm/sec and nominal pressure of 1.4902 psi. The mass spectrometer was set at 70eV, with ion source temperature of 230°C, transfer line temperature of 280°C and quadrupole temperature of 150°C. Acquisition of ion was via Scan mode (scanning from m/z 45 to 550 amu at 2.0s/scan rate [31].

DETERMINATION OF VITAMINS

Water soluble vitamins

5mg of Vitamins B1, B3, B6, B9, and Vitamin C were weighed separately and dissolved in 5ml of water to give concentration of 1000ug/ml. The mixed standard of the 5 vitamins was prepared by taking 1ml of each solution (5mL total volume of all vitamins) and making it up to 5mL with water to give 1 in 5 dilutions of each stock concentration and 200ug/mL of each vitamin standard in the mixed standard. The solvent used to dissolve Vitamin A, D3, and E was methanol. The solutions were filtered with a 0.22µm syringe filter and placed in the sonicator for 15 minutes.

Chromatographic condition.

The Mobile phase used for the assay of water-soluble vitamins was Methanol: hexane sulfonate buffer at a ratio of 24:76. The wavelength for detection was 245 nm, while the flow rate was 1 ml/min. The column used was Agilent zorbax SB- C8(4.6*150 mm 5 um). The Mobile phase solvents were filtered and sonicated for 15 minutes. For the fat-soluble vitamins, mobile phase was methanol: water 95:5 was used at a wavelength of 280nm and flow rate of 1ml/min.

Simultaneous characterization of vitamins

5mg of Vitamins B1, B3, B6, B9, and Vitamin C were weighed separately and each of the above was dissolved in 5ml of water to give a concentration of 1000 µg/mL. Mixed STD of the above five vitamins was prepared by taking 1 ml of each (total altogether is 5 ml) to give 1 in 5 dilution of each stock concentration and this gives 200 µg/ml of each standard in the mixed standard. Methanol was the solvent for the assay of Vitamin A and D3. The solutions were filtered with a 0.22 µm syringe filter and placed in the sonicator for 15 minutes. 500mg of vitamin D3(30000IU) was weighed and diluted with 50ml of methanol, filtered and sonicated to obtain a final solution containing 600 IU (15 µg/ml) of vitamin D3. A 30 µg/mL solution of vitamin A and Vitamin E was prepared and used for the analysis.

Preparation of black elderberry assay extract

Black elderberry extract 5mL was measured and diluted with 5ml of water. 1 in 5 dilutions of the solution in water was carried out. The solution was placed in the sonicator for 15 minutes. For the assay of fat-soluble vitamins, 5 ml of the extract was measured and 5 ml of methanol was added. The solution was placed in the sonicator for 15 minutes and a 1 in 5 dilution was prepared with methanol as solvent.

RESULTS AND DISCUSSION

QUALITATIVE AND QUANTITATIVE ANALYSIS

The phytochemical components of black elderberry extract revealed the presence of chemically active components such as reducing sugar, anthraquinones,

steroids, flavonoid, terpenoids, saponins, alkaloids, tannins and phenol but the absence of phlobatanin as documented on table 1. Standard procedures were used to identify the phytochemical constituents in the samples. Emulsion formation signified the presence of saponin, and formation of a yellow coloration highlighted the presence of flavonoids. Terpenoids, tannin, phenols, and steroids were also detected by the various colorations formed due to the addition of the assay reagents. The abundance of the phytochemicals was detected by the various quantitative techniques. Tannin had the highest concentration detected and saponin lowest. The phytochemical component of black elderberry fruit and conversely its extract is largely influenced by a number of factors largely categorized as intrinsic and extrinsic [32]. Analysis of the phytocomponents in extract and refined samples provides information on its potential pharmacological activity and use. A number of chemically active components including tannin (water soluble polyphenols) which was the most predominant with an average concentration of 91.41 ± 0.70mg/100g of the extract. Tannins are polyphenolic compounds found in many plants and are known for their antioxidant and astringent properties. Tannins are also known for their ability to bind and precipitate proteins and decrease the enzymatic activity of proteins with high affinity to the tannins due to the formation of enzyme complexes [33]. Tannins can interfere with different stages of viral infection, such as binding, penetration, replication, and spread. Tannins can also inhibit certain viral enzymes, such as DNA polymerase and topoisomerase II12. Some examples of tannins that have shown potent anti HSV-1 and-SARS-CoV-2 [34]. Phenols, flavonoids, reducing sugar and cardiac glycoside were also predominant in the extract, whereas the presence of alkaloids and saponins was detected in low concentrations. Anthraquinones, steroids and terpenoids were also identified in the extract. Previous studies have reported a high concentration of polyphenolics(tannins), flavonoids (anthocyanins), and procyanidins in the fruit extract [35]. Moreover, its antiviral activity has been alluded to the polyphenols and flavonoids [36, 37]. These polyphenols also mop up free radicals, protecting against oxidative and perioxidatve processes. Flavonoids (rutin, quercetin) and anthocyanins found in the extract also possess strong antiviral activity against DNA and RNA viruses including the Human immunodeficiency virus (HIV) [18] as well as HSV-2 [19].

Table 1: Quantitative analysis of active phytochemical components of black elderberry in mg/100g of fruit extract.

Phytocomponents	Qualitative analysis	Quantitative analysis mg/100g
Tannin	+	91.41 ± 0.71
Phenol	+	62.43 ± 0.502
Phlobatanin	-	Nd
Alkaloid	+	6.46± 0.10
Saponins	+	5.79± 0.02
Flavonoid	+	56.31± 0.40
Steroid	+	******
Anthraquinone	+	******
Terpenoid	+	******
Cardiac glycosides	+	31.74± 0.07
Reducing sugar	+	46.02 ± 0.19

Nd : not detected ******: not measured

GC/MS ASSAY OF VOLATILE COMPONENTS OF BLACK ELDERBERRY FRUIT EXTRACT

Numerous volatile compounds were identified in black elderberry fruit extract as illustrated in Figure 1 with furan methanol being the most abundant. Peaks consistent with labeled chemical components were identified using Mass Hunter\Library\NIST14.L. The GC-MS analysis of the black elderberry fruit extract revealed a total of 58 unique compounds. Among these, the most abundant compound was 2-furanmethanol, which comprised 11.53% of the extract. The least abundant compound in the extract was 1,3-Butadiene-1-carboxylic acid, which comprised only 0.37% of the extract. Other identified compounds with high abundance

include Furfural (5.43%), Sorbic acid (5.38%), 1-Piperidinepropanol, .alpha.-cycl (cycrimine) (5.22%), Propanamine (5.2%), 4-Methylcyclohexanol (2.77%), 2(5H)-Furanone (2.88%), Dodecane, 1,1-dimethoxy- (2.9%), 3-Heptanone (2.23%) and 4-Nitro-5-[(2-phenylhydrazin-1-ylidene)methyl]benzene-1,2-dicarbonitrile (1.32%). Previous reports of GCMS of elderberry extract has reported the presence of aldehydes (phenylacetaldehyde, benzaldehyde), fatty acid ester (ethyl linoleate), terpene alcohol (linalool) , aromatic phenolic (4-vinyl guaiacol) , ketones, and alcohols [21]. This is consistent with the result obtained from the assay where a number of fatty acids (Heptanoic acid 7-(butylthio) (0.74%), Hexyl ester decanoic acid (0.39%),Butyric acid, 4-phenyl-, 4-methyl-2-pentyl ester (1.87%), Fumaric acid, nonyl pentadecyl ester (1.94%), Butyric acid, 4-phenyl-, 4-methyl-2-pentyl ester (1.87%)), amines and peptides (2-Amino-1,3-propanediol (5.2%), 1-Piperidinepropanol, .alpha.-cycl (cycrimine) (5.22%), Propanamine (5.2%), 2-Ethyl-5-(2-fluorobenzylideneamino)-1,3,4-thiadiazole (2.88%), 2-methyl-2-pentyl-oxirane (0.49%)), alcohols (4-Methylcyclohexanol (2.77%), Isopropyl alcohol (2.9%), 2-ethyl-1- pentanol (0.39%), 2- butyl-1-octanol (0.39%), 2 methyly 3-Nonanol (1.86%)) and ketones (2-Cyclohexen-1-one (3.69%), 3-ethyl-4,5-dihydro-1,4-dimethyl-1H-pyrazole (0.51%), 3-Heptanone (2.23%), 3-Hexen-2-one, 3,4-dimethyl-, (E)- (0.97%), 3-Hexen-2-one, 3,4-dimethyl-, (E)-Cyclohexane-1,3-dione, 2-(2-hydroxyethylaminomethylene)-5,5-dimethyl (0.97%)). While the volatile compounds identified have no direct known link to its efficacy against herpes and HPV, compounds such as 2-furanmethanol and penicillamine have proven to have antimicrobial , anti-inflammatory and antioxidant properties which has implications in HIV, and STI prevention [38, 39,40]. The diverse array of compounds identified suggests synergism of multiple components of the extract as observed in a variety of herbal medications in order to exert the therapeutic effect.

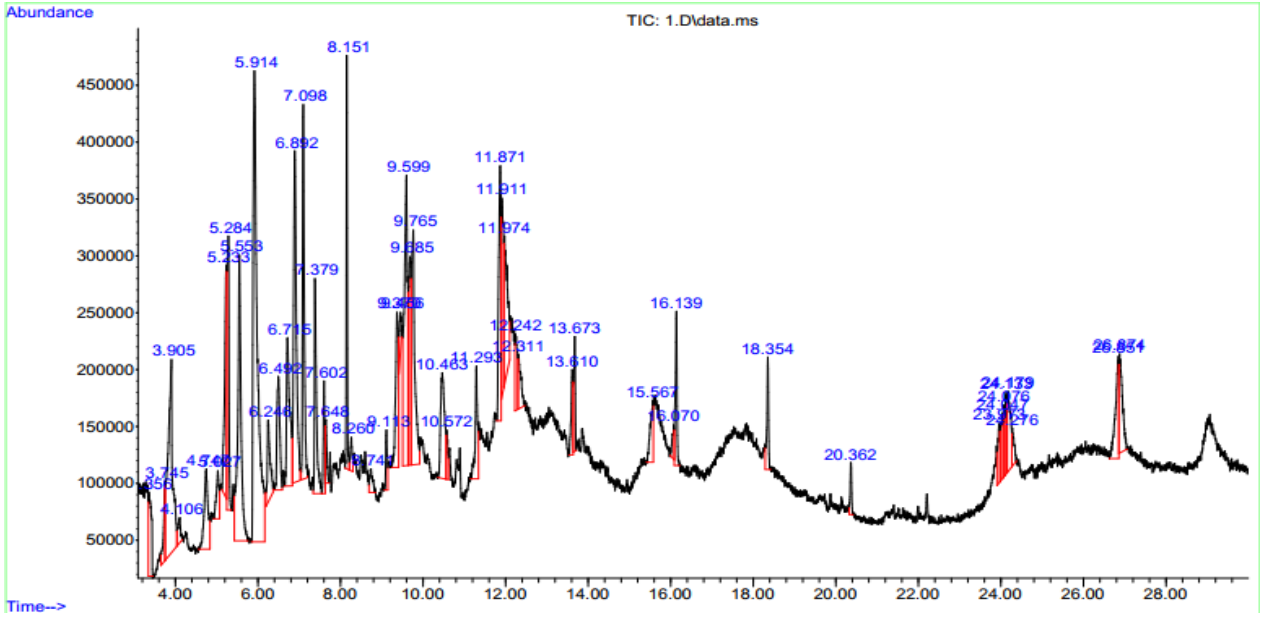

Fig 1: Analysis of volatile component of black elderberry fruit extract as analysed by gas chromatography/mass spectrometry (GCMS)

DETERMINATION OF VITAMINS IN BLACK ELDERBERRY EXTRACT

Analysis of water- and fat-soluble vitamins contained in the extract was assayed using HPLC. Peaks observed during the assay of the extract were consistent with those observed during the assay of vitamin standards as illustrated in Fig 2-3. The results of the vitamin assay revealed the presence of vitamin Bs and vitamin C, which are important for well-being, free radical eradication and immune support (Fig 2A). Significant levels of vitamin C were detected as illustrated by the sharp peak and area under the curve (% AUC 53.5972). This is consistent with what was described by Sidor and Gramza [41]. Niacin (Vitamin B3) Vitamin B6 and Vitamin B1 (Thiamine) were also detected with thiamine having the lowest percent area detected. Vitamin B3 (Niacin), known for its role in metabolism and DNA repair was detected in significant levels. Vitamins A and D were also detected as illustrated in Fig 3A. The high abundance of vitamin A (% Area 85.1131) crucial for mucosal barrier and immune function further supports the antiviral potential of the black elder fruit extract. Vitamin D is also well known for its immunomodulatory effects. These vitamins confirm the robust nutritional profile of the extract alluding to the multifaceted potential of the extract to prevent and eradicate viral infections (such as HPV and HSV) as well as promote wellbeing. While these vitamins were detected i*n vitro*, the bioavailability when applied *in vivo* is a critical factor for its efficacy.

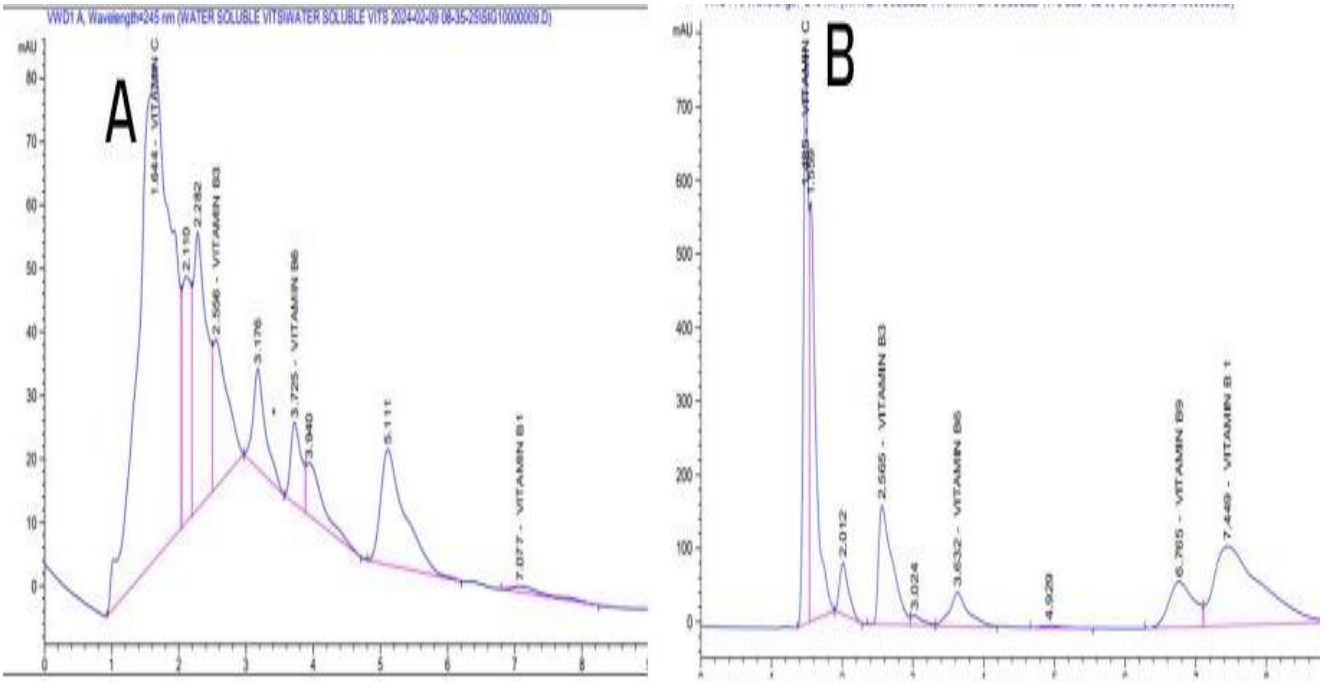

Fig 2: HPLC analysis of vitamins (A) water soluble vitamins detected in black elderberry extract (B) peaks detected during assay of water-soluble vitamins mixed standards

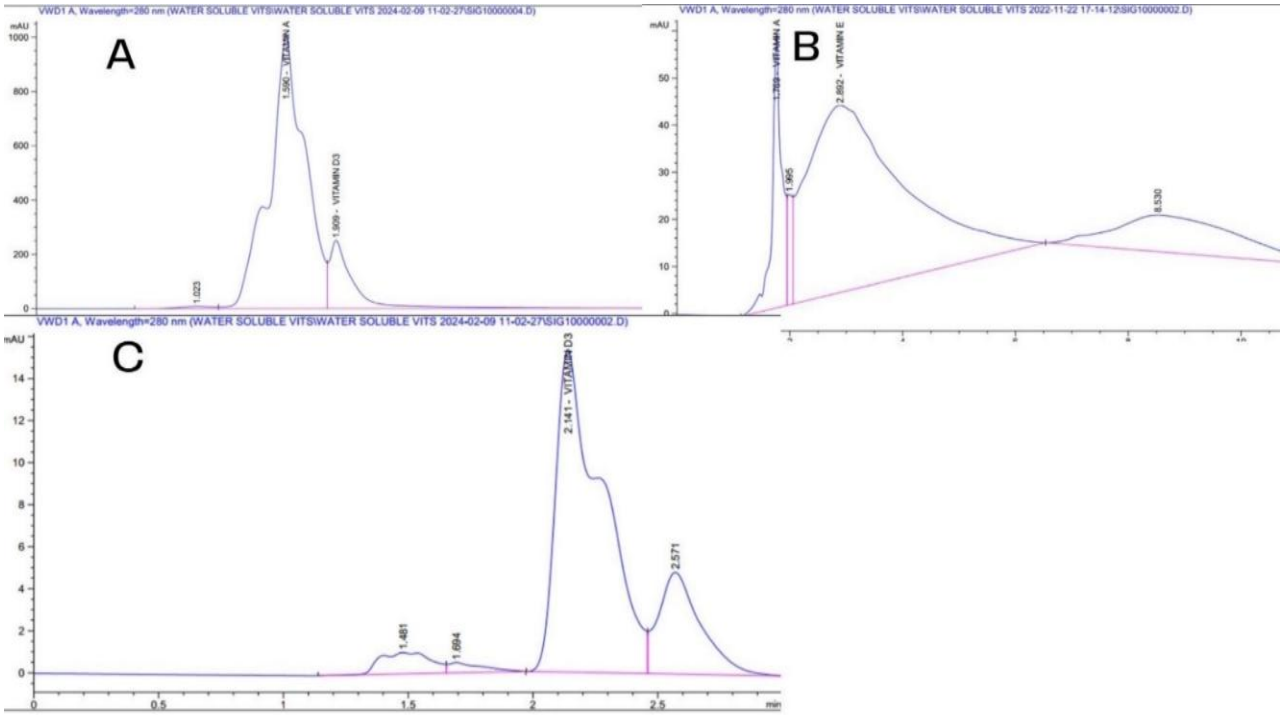

Fig 3: HPLC analysis of vitamin (A) Fat soluble vitamins detected in black elderberry extract (B) peaks detected during assay of fat-soluble vitamins mixed standards A and E (C) peaks detected during assay of fat-soluble vitamins standard E.

CONCLUSION

The analysis of the chemical components of black elderberry fruit extract demonstrates its robust nutritional and therapeutic (nutraceutical) potential for management of viral infections. The abundance of polyphenols, tannins and flavonoids as well as volatile components furan methanol and penicillamine has highlighted the potential of this extract for management of Herpes simplex and Human papilloma virus especially when used with other medications. The robust vitamin components supports its immense potential for preventing infections and promoting healing. Further exploration of the activity of active chemical moieties identified should be carried out using *in vivo* and *in vitro* methods to elucidate its activity against Herpes simplex and Human papilloma virus, its mechanism of action and safety profile.

List of abbreviations

HIV: Human immunodeficiency virus, STIs: sexually transmitted infections, GCMS: gas chromatography- mass spectroscopy, HPV: Human Papilloma Virus, HSV: Herpes Simplex Virus

DECLARATIONS

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and material

The datasets used during the current study are available from the corresponding author on request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

KOO conceived and executed the project and was a major contributor in writing the manuscript; POB and DO cowrote the manuscript, CPA and MOI supervised the project. All authors read and approved the final manuscript.

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التحليل الفيتوكيميائي والإمكانية المضادة للفيروسات لمستخلص التوت الأسود (سامبوكوس نيغرا) ضد فيروس الهربس البسيط وفيروس الورم الحليمي البشري

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ملخص

استخدم التوت الأسود (Sambucus nigra) منذ قرون لعلاج عديد من الأمراض، بما في ذلك الالتهابات الفيروسية. ومع ذلك، فإن الأدلة العلمية الداعمة لنشاطه المضاد للفيروسات، وخاصة ضد فيروس الهربس البسيط (HSV) وفيروس الورم الحليمي البشري(HPV) ، محدودة وغير متسقة. هدفت هذه الدراسة إلى تقييم الإمكانية العلاجية لمستخلص التوت الأسود ضد فيروسي HSV وHPV، من خلال تحليل مكوناته الفيتوكيميائية. تم تحديد محتوى الفيتامينات في ®Sambucol باستخدام كروماتوغرافيا السائل عالية الأداء(HPLC) ، كما تم التعرف على المركبات الفيتوكيميائية باستخدام كروماتوغرافيا الغاز – مطياف الكتلة(GC-MS) ، وقياس الخصائص التقريبية للمستخلص. أظهر تحليل GC-MSلمستخلص ثمار التوت الأسود وجود 58 مركبا كيميائيا فريدا، وكان أكثرها وفرة هو 2–فورانميثانول. كما احتوى المستخلص على نسب عالية من فيتامينات C، B1، B6، B3، A، وD3، إضافة إلى المركبات الفينولية، والأنثوسيانينات، والفلافونويدات، والأحماض الفينولية. وأظهر المستخلص أيضا قيمة غذائية جيدة، مع مستويات مرتفعة من البروتين والألياف والمعادن. وتشير النتائج إلى أن مستخلص التوت الأسود يمتلك إمكانية علاجية متعددة الجوانب ضد الالتهابات الفيروسية، نتيجة لقيمته الغذائية والعلاجية (الغذائية–الدوائية). وينصح بإجراء دراسات أكثر تعمقا حول النشاط الحيوي للمركبات الفعالة المحددة، لأن ذلك قد يؤدي إلى اكتشاف عوامل مضادة للفيروسات جديدة، خاصة للوقاية وعلاج الأمراض المنتقلة جنسيا.

الكلمات الدالة: مستخلص التوت الأسود، المركبات الفيتوكيميائية، تحليل GC-MS، الفعالية المضادة للفيروسات، فيروس الهربس البسيط .(HSV)

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