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Subcritical water extraction of withanosides and withanolides from ashwagandha (*Withania somnifera* L) and their biological activities

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ABSTRACT

Subcritical water extraction (SWE) applied to analyses the bioactives from ashwagandha *(W. somnifera)* at varying temperature (100–200 °C) and extraction time (10–30 min). The effect of temperature and time has been investigated in terms of extraction yield (EY), total phenolic content (TPC), cytotoxicity, antioxidant, and enzyme inhibitory activities. The withanosides and withanolides responsible for various biological effects were quantified using high performance liquid chromatography (HPLC). The HPLC analysis revealed Withanoside V, Withanoside IV, 12-Deoxywithastramonolide, Withanolide A, and Withaferin A as a principle bioactive compounds in SWE, with high in concentration compared to microwave-assisted extraction (MAE), Soxhlet extraction (SE) and maceration (MC). For SWE the highest EY (65.6%; 200 °C for 30 min), TPC (82.5 mg GAE/g DE), antioxidant activity (DPPH: 80.3%, FRAP: 60.5% and ABTS: 78.9), and potent enzyme inhibitory effects were observed. The SWE and Withaferin A showed significant reduction in cell viability of cervical cancer (HeLa) cells, with IC_{50} values 10 mg/ml and 8.5 μM/ml, respectively but no cytotoxic effect for normal cells (MDCK). Thus, SWE can provide effective extraction for ashwagandha withanosides and withanolides compared MAE, SE and MC to conventional methods, which could be used for extraction of pharmacologically active fractions with therapeutic applications.

1. Introduction

Natural bioactive compounds extracted from various medicinal plants received great attention and importance to explore and utilize towards prevention and treatment of various diseases throughout the world, having fewer side effects with impressive therapeutic action compared with the modern or synthetic medicines (Kesarwani and Gupta, 2013). Ashwagandha (*Withania somnifera L.* Dunal) is a medicinal herb that has been widely used in Ayurvedic and Chinese medicine for thousands of years. It belongs to the Solanaceae family, widely known as Indian ginseng, winter cherry and poison gooseberry (Ahmad and Dar, 2018). The plant possesses various medicinal properties including anti-inflammatory, anti-stress, anti-diabetics, anti-cancer, antioxidant, sleep-inducing, and drug withdrawal properties and considered as adaptogen, as it helps in human body to manage stress (Dar et al., 2015). Ashwagandha plant roots and leaves are widely used in preparation of various herbal drugs and constituent more than 200 formulations from Ayurveda, Siddha and Unani medicines, these formulations are having very effective treatment against various physiological disorders and diseases (Mirjalili et al., 2009). The major chemical constituents of ashwagandha are withanolides which showed various therapeutic and medicinal properties, including neuroprotective, hepatoprotective, anticancer, hypoglycemic, and antiarthritic effects (Singh et al., 2010; Chen et al., 2011).

The chemical composition of ashwagandha has been extensively studied and found several groups of bioactive chemical constituents such as flavonoids, tannin, alkaloids, sitoindosides, glycosides, withanicil, steroidal lactones, and alkaloids (Mir et al., 2012; Kalra and Kaushik, 2017). The various chromatographic and spectroscopic methods were used for analysis for ashwagandha and showed the presence of known withanosides, withanolides and steroidal lactones such withaferin A, withanoside IV or VI, withanolide D, dihydrowithanolide D, and withanolide sulfoxide (Trivedi et al., 2017). The extraction and identification of these chemical constituents, especially withanolides from ashwagandha using various conventional methods with aid of hazardous solvents like n-hexane, ethanol, chloroform, and methanol is

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a challenging task due to their chemical diversity, complex structure and low abundance (Mehrotra et al., 2011; Shahriar et al., 2013). However, most of these methods are energy-inefficient, time-consuming, hazardous to environment and human health due to excess utilization of organic solvents. Therefore, there is need to explode novel extraction technologies having low cost, short time consumption, easy for operation, and utilizing ecofriendly solvents (Lee et al., 2014; Zhang et al., 2018). Recently more attention has been increased for utilization of cheap, low time consuming and environmentally clean technologies. These technologies reduce the use organic solvents precluding its associated toxicity, with production of high quality and biologically active extracts. In this sense, the various clean and green methods such as supercritical fluid extraction (SFE) and sub critical water extraction (SWE) meet the requirements for extraction and biological analysis of herbal extracts compared to conventional techniques such as maceration, infusion, decoction, and Soxhlet extraction (Kumar et al., 2011; Dhanani et al., 2017).

Thus, this study is aimed to determine the effect and feasibility of SWE extraction of ashwagandha and its possible effect on concentration of bioactive compounds, phenolic content, antioxidant, and enzyme inhibitory activities, using optimized extraction conditions like temperature, pressure and time required for extraction, compared to those of conventional extraction (maceration, Soxhlet extraction, microwave-assisted extraction). The SWE method may provide rapid, ecofriendly, nontoxic, and efficient extraction of withanosides, withanolides and steroidal lactones from ashwagandha with significant antioxidant, anticancer and enzyme inhibitory effects.

2. Materials and methods

2.1. Plant sample

The dried ashwagandha *(W. somnifera)* plant sample (root and leaves) was purchased from Lamar Natural Pvt. Ltd., Mumbai, India in July 2018. Dried ashwagandha sample (1 kg) was minced and grinded with a mechanical grinder (Hanil Co. Seoul, South Korea) into a mesh size of 10–20 mm and stored at 4 °C, until further analysis.

2.2. Reagents and chemicals

The six reference standards, including, withaferin A, withanolide A, withanolide B, withanoside IV, withanoside V, and 12-deoxywithastramonolide was purchased from Phyto-Compounds Pvt. Ltd., Bangalore, India. 1,1-diphenyl-2-picrylhydrazyl (DPPH), Ferric chloride, 2,2′-Azinobis (3-ethyl- benzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Folin-Ciocalteu's phenol reagent, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), TPTZ (2,4,6-tripyridy-s-triazine), and gallic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade methanol, ethanol and distilled water were purchased from Duksan Chemicals Co. Ltd. (Seoul, South Korea).

2.3. Extraction procedure

2.3.1. Maceration (MC)

About 50 g of powdered ashwagandha plant sample was macerated overnight for 12 h in 500 ml of distilled water at 40 °C. The obtained extract was centrifuged at 3354 *g* for 10 min, supernatant was collected, filtered through muslin cloth and finally, the extract was lyophilized until a constant weight was obtained and further stored in amber colored bottle until further use (Nile et al., 2017).

2.3.2. Soxhlet extraction (SE)

50 g of powdered ashwagandha sample extracted with 500 ml of 80% ethanol for 5–10 h using Soxhlet extraction apparatus, at 60 °C. An extraction time of 7 h was taken as optimum by mass yield. Further, the obtained extract was filtered and concentrated to dryness with subsequent evaporation of alcoholic content using Rota vapour (temperature, 50 °C), further the obtained extract was lyophilized until a constant weight was obtained and stored at 4 °C until used for further studies (Nile et al., 2017).

2.3.3. Microwave-assisted extraction (MAE)

MAE was performed using microwave oven, accurate 50 g of powdered ashwagandha sample was extracted using 80% methanol (with 500 ml) for 10–40 min. On a mass yield basis, an extraction time of 20 min at 150 W microwave powers using 60 °C as optimum temperature was considered for extraction. The solution was later repeatedly extracted in triplicates, further the solution was filtered and concentrated to dryness using vacuum evaporator (temperature, 50 °C) and then subjected to lyophilization until a constant weight was obtained and kept in an amber bottle at −4 °C until further use (Karabegovic et al., 2014).

2.3.4. Subcritical water extraction (SWE)

The SWE was performed using house-made accelerated solvent extractor method described elsewhere (Cvetanović et al., 2017). The SWE unit equipped with a solvent controller and extraction done using Milli-Q water (Duksan Chemicals Co., Ltd. (Seoul, South Korea). The powdered ashwagandha plant sample (5 g) was placed in stainless-steel extraction cell containing filter paper in oven covered with heating system, the unit was equipped with temperature sensors, pressure control and all the parameters were monitored with digital displays. The extractions were carried out using temperature range 100–200 °C and extraction time 10–30 min interval, 10 MPa pressure was provided during extraction. The all extraction procedure was set and carried out as per the protocol described by Kumar et al. (2011). After complete extraction the obtained sample was collected in glass flask with the aid of nitrogen gas. The samples were collected in triplicates by repeating the procedure of extraction three times. The collected extracts were filtered, lyophilized (−80 °C, 48 h) and stored at 4 °C in dark until further experiments (Zhang et al., 2018).

2.3.5. Determination extraction yield (EY)

The dried ashwagandha sample was extracted using four extraction methods as described in section 2.3.1, 2.3.2, 2.3.3, and 2.3.4. The EY was calculated for each dried extract after complete evaporation of the solvents. The EY is the measure of the potency of solvent to extract components, which defined as mass ratio between amount of extract recovered (A1) with the initial amount of plant material (A0). The yield for each extraction was calculated using the formula, $EY = A1/A0 \times 100$ (Nile et al., 2017). For SWE we selected the optimized time (20 min) for further analysis, as we found maximum yield, high phenolic content and higher antioxidant activity at 20 min of extraction time (Table 1).

2.4. Determination of total phenolic content

The total phenolic content was determined using Folin–Ciocalteu colorimetric method as described by Nile and Park 2013. The results were calculated as mg gallic acid equivalents (GAE) mg GAE/100 g extract.

2.5. HPLC analysis

The HPLC system consisted of Agilent 1100 chromatograph (Agilent, CA, USA) using Discovery RP-C18 column (250 cm × 4.6 mm, 5 μm, Sigma-Aldrich Co. LLC), the system equipped with a DAD detector, solvent delivery system and auto-sampler. The column temperature kept at 30 °C and HPLC analysis done using mobile phase (acetonitrile (40%) and 0.1% acetic acid in water (60%)). Chromatographic separation was achieved with following gradient program: The isocratic elution mode with the flow rate of 1 ml/min, with gradient 5–45% B in 18 min; 45–80% B in 7 min; held at 80% B for

Table 1
The comparative results for TPC and antioxidant activities by ashwagandha using MC, SE, MAE, and SWE methods at different temperature and time intervals.

Extraction conditions	Solvent used	Extraction time	EY (%)	TPC (mg GAE/g)	Antioxidant activity (%)		
					DPPH	FRAP	ABTS
MC 40 °C	Water	12 h	20.8	32.5 ± 0.71^{i}	41.2 ± 1.24^{i}	20.3 ± 1.07^{i}	36.8 ± 1.15^{i}
SE 60 °C	Ethanol	7 h	25.7	42.2 ± 1.21^{h}	46.8 ± 2.11^{h}	25.8 ± 1.22^{h}	40.3 ± 2.01^{h}
MAE 60 °C	Methanol	20 min	30.2	50.6 ± 1.06^{g}	58.3 ± 1.98^{g}	31.9 ± 1.04^{g}	55.6 ± 1.42^{g}
SWE 100 °C	Milli-Q water	10 min	30.5	51.9 ± 1.20^{fb}	60.1 ± 1.32^{fb}	32.2 ± 1.01^{fb}	56.6 ± 1.44^{fb}
		20 min	35.8	56.4 ± 1.32^{f}	65.2 ± 2.13^{f}	35.6 ± 1.43^{f}	60.8 ± 1.61^{f}
		30 min	38.3	54.1 ± 1.09^{fa}	63.4 ± 1.11^{fa}	33.9 ± 1.61^{fa}	58.9 ± 1.35^{fa}
SWE 120 °C	Milli-Q water	10 min	38.5	57.3 ± 1.04^{eb}	66.8 ± 1.18^{eb}	38.6 ± 1.81^{eb}	63.8 ± 1.15^{eb}
		20 min	40.5	61.1 ± 1.28^{e}	70.1 ± 2.01^{e}	42.2 ± 2.11^{e}	68.1 ± 1.84^{e}
		30 min	42.9	58.9 ± 1.30^{ea}	68.4 ± 1.32^{ea}	40.1 ± 1.09^{ea}	65.3 ± 1.11^{ea}
SWE 140 °C	Milli-Q water	10 min	44.8	64.8 ± 1.03^{db}	71.9 ± 1.37^{db}	45.6 ± 1.16^{db}	65.9 ± 1.05^{db}
		20 min	45.2	70.3 ± 1.22^{d}	75.6 ± 1.45^{d}	50.8 ± 1.78^{d}	72.4 ± 2.05^{d}
		30 min	48.6	67.1 ± 1.13^{da}	73.4 ± 1.33^{da}	48.1 ± 1.03^{da}	68.2 ± 1.51^{da}
SWE 160 °C	Milli-Q water	10 min	50.9	75.3 ± 1.81^{ab}	76.7 ± 1.39^{ab}	55.3 ± 1.44^{ab}	74.1 ± 1.20^{ab}
		20 min	51.8	82.5 ± 1.08^{a}	80.3 ± 1.08^{a}	60.5 ± 2.03^{a}	78.9 ± 1.60^{a}
		30 min	55.1	79.8 ± 1.02^{aa}	77.9 ± 2.04^{aa}	57.8 ± 1.21^{aa}	75.9 ± 1.56^{aa}
SWE 180 °C	Milli-Q water	10 min	56.3	74.6 ± 1.52^{bb}	75.1 ± 1.10^{bb}	54.9 ± 1.07^{bb}	72.6 ± 1.34^{bb}
		20 min	58.2	80.8 ± 1.11^{b}	78.6 ± 2.03^{b}	57.6 ± 1.78^{b}	75.8 ± 1.09^{b}
		30 min	60.8	77.9 ± 1.31^{ba}	76.1 ± 1.67^{ba}	55.7 ± 1.33^{ba}	73.1 ± 1.81^{ba}
SWE 200 °C	Milli-Q water	10 min	61.9	72.8 ± 1.19^{ca}	70.4 ± 1.37^{cb}	50.8 ± 1.71^{cb}	69.8 ± 1.02^{cb}
		20 min	63.5	78.5 ± 1.31^{c}	76.9 ± 1.09^{c}	55.7 ± 2.01^{c}	73.6 ± 1.65^{c}
		30 min	65.6	73.2 ± 1.22^{cb}	72.1 ± 1.01^{ca}	52.3 ± 1.28^{ca}	71.3 ± 1.16^{ca}

Results expressed as mean ± standard deviation (SD) of three replicates. Different small letters in same column means significantly different values with $p < 0.05$, calculated by Duncan's multiple range tests. EY: Extraction yield; TPC: Total phenolic content; MC: Maceration; SE: Soxhlet extraction; MAE: Microwave-assisted extraction; SWE: subcritical water extraction.

3 min. Injection volume was 20 μL, pressure 3191 psi (220 bar). The solvents were degassed using vacuum. Sample 20 μg/ml in water: acetonitrile (80:20), samples filtered through a 0.45 μm membrane filter. The peaks were monitored at 230 nm using a UV–visible detector (UV–visible SPD −20 A). The obtained peaks in HPLC chromatogram were identified by comparing the retention time to that of standards (withanolide A, withaferin A, withanoside IV, withanoside V, withanolide B, and 12-deoxywithastramonolide) and their content in the extract was quantified based on area of the peak in HPLC chromatograph (Ganzeraa et al., 2003; Dhanani et al., 2017).

2.6. Antioxidant activities

The antioxidant activities of extracted sample of ashwagandha extracts and quantified bioactive compounds were determined using well known antioxidant assays, such as DPPH, FRAP and ABTS and the results were calculated using the formula; Antioxidant activity (%) = ((Ac − As)/Ac) × 100, Where Ac: absorbance of control and As: absorbance of the sample. (Nile et al., 2018 and Kim et al., 2019). The antioxidant activity of extracted compounds was calculated using trolox equivalents (Mocan et al., 2018).

2.7. Enzyme inhibitory effects

The enzyme inhibitory activities were studied against the enzymes which are mainly involved in various metabolic disorders, including α-amylase, and α-glucosidase tyrosinase, xanthine oxidase (XO), and cholinesterase (AchE: acetylcholinesterase and BchE: butyrylcholinesterase) comparing their results with those obtained with specific standard drugs like acarbose, kojic acid, allopurinol, and galantamine, respectively. The 1 mg/ml of each extract sample (SWE, MC, SE, and MAE) and extracted compounds (Withanoside V, Withanoside IV, 12-Deoxywithastramonolide, Withanolide A, and Withaferin A) were used for enzyme inhibitory activity and all experimental procedures were performed as per the methods described by Nile et al., 2018 and Grochowski et al., 2017.

2.8. In vitro cytotoxic effects

The cancer cells (HeLa) and normal cells (MDCK) were used for in vitro cytotoxic study. The cytotoxicity of SWE, MC, SE, and MAE extracts and quantified bioactive compounds were determined using sulforhodamine B (SRB) assay as described by Enkhtaivan et al., (2016). The absorbance was measured at 540 nm and 50% inhibitory concentration (IC_{50}) for cancer cells (HeLa) and cytotoxic concentration (CC_{50}) values for normal MDCK cells were calculated (Kim et al., 2019). Cell viability and proliferation for Hela cells were studied and measured as per the method previously described by Jiang et al., 2019.

2.9. Statistical analysis

The obtained results were statistically analyzed, and all values was expressed as mean with standard deviation (mean ± SD) of triplicates. The differences among different extraction methods and studied biological activities were analyzed using one-way analysis of variance (ANOVA) and confirmed with Duncan's multiple range test. The data were analyzed using Microsoft Excel and IBM SPSS Statistics 22.0 (SPSS, Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Extraction yields

The extraction yield (EY) for sub critical water extraction (SWE) at different temperatures (100–200 °C), optimized temperature (20 °C) and under high pressure (100 bar) compared to conventional methods like maceration (MC), Soxhlet extraction (SE) and microwave-assisted extraction (MAE) presented in Table 1. The results show that there is a significant increase in EY with increasing temperature, which was higher at 200 °C for 30 min, showing SWE (65.6%), however the EY at 160 °C for 20 min, showed high concentration of bioactive compounds with significant biological activities, compared to MC (20.8%), SE (25.7%) and MAE (30.2%), respectively. The results prove that the diffusion of water increased with increasing temperature by SWE, this indicates that the hot water help in easy releasing of the components

from ashwagandha plant matrix during the solid to liquid mass transfer mechanism (Lee et al., 2018).

The SWE method using hot water under high pressure is an emerging extraction technique recently used as an alternative method for replacement of conventional extraction methods like MC, SE and MAE. SWE is an eco-friendly efficient alternative method used for extraction of bioactive compounds which provides higher EY to from plant solid samples (Rodriguez-Meizoso et al., 2006). For SWE the optimal temperature for extraction of phytochemicals depends on the target compounds. The series of effects were observed for increased extraction temperature which includes solubility, desorption kinetics and improvement of the mass transfer. However, the effect of temperature in SWE mostly related to polarity change and weakening of hydrogen bonds. Sometimes the elevated temperatures may cause degradation of phytochemicals with servals paths (Plaza and Turner, 2015; Cvetanović et al., 2018). Thus, a concise study is important for maximize the yield of targeted components from final plant extracts.

3.2. Total phenolic content

Phenolics are important well known natural antioxidants from plant and stabilize free radical intermediates by donating an electron and hydrogen atom from their hydroxyl groups (Lee et al., 2018). The total phenolic content in SWE, MC, SE, and MAE extracts were determined spectrophotometrically by Folin–Ciocalteau method (Table 1), the highest total phenolic content in ashwagandha extracts was SWE (82.5), MC (32.5), SE (42.2), and MAE (50.6) mg GAE/g, respectively, which was obtained at 160 °C for 20 min for SWE. The results show that there is gradual increase in TPC with increased extraction temperature and time up to 160 °C for 20 min. This proves that the less energy is needed for breakdown of solute–matrix interactions and the solvent to solute interaction was enhanced with increased water temperature (Ho et al., 2007; Lee et al., 2018). However, there was a slight decrease in TPC of ashwagandha extracts with further temperature range that is extraction over 160 °C and 20 min in SWE. This suggests that the elevated temperatures may cause degradation of phytochemicals with servals paths in plant sample (Plaza and Turner, 2015; Cvetanović et al., 2018). The obtained results show the high concentration of TPC compared to the previous study, in which the effect of extraction methods on yield and phytochemical constituents of ashwagandha were studied (Dhanani et al., 2017).

3.3. RP-HPLC analysis of marker compounds

The phytochemical analysis for Ashwagandha extracts was determined by simple gradient elution-based RP-HPLC method and the chromatogram of extracted compounds has been shown in Fig. 1. Six marker compounds (Withanoside IV, Withanoside V, Withaferin A, 12-Deoxywithastramonolide, Withanolide A, and Withanolide B) were analysed in ashwagandha, which were extracted by SWE, MC, SE, and MAE methods. The structural details of these withanoside, withanolide and steroidal lactone lactones, presented in Fig. 2 and the concentrations of each compound in ashwagandha with respect to different extractions methods were presented in Table 2. In all extracts withanoside V was found in high amount compared to other withanoside, withanolide and steroidal lactone. However, the results for total withanolide content was higher in the SWE (150.7) as compared to MAE (119.88) ≥ SE (106.47) ≥ MC (85.15), μg/g, respectively. Dhanani et al., 2017 also studied extraction of withanoside, withanolide and steroidal lactone from ashwagandha using ultrasound assisted solvent extraction (UASE) and microwave assisted solvent extraction (MASE), found higher concentrations of these compounds compared to water–-ethanol and water conventional extraction methods.

3.4. Antioxidant activity

In this study the antioxidant activity of ashwagandha extracts were determined using DPPH, FRAP and ABTS antioxidant assays and the resulted values were presented in Table 1. The extracts obtained by SWE (160 °C for 20 min), showed relatively higher antioxidant capacity to those extracted with MC (40 °C for 12 h), SE (60 °C for 7 h) and MAE (60 °C for 20 min), respectively. However, the ashwagandha extracts by SWE at different temperature and time intervals presented significant statistical difference among the antioxidant values. These differences might be explained by the less or more extraction time (10–30 min) and temperature (100–200 °C) conditions applied for extraction of ashwagandha. The SWE extracts showed remarkably increased antioxidant activity with extraction temperature and time (Table 1). The highest values DPPH (80.36%), ABTS (78.98%) and FRAP (60.53%), respectively, observed at 160 °C for 20 min, compared the conventional extraction methods; MC (40 °C for 12 h), SE (60 °C for 7 h) and MAE (60 °C for 20 min), showing antioxidant values by DPPH (41.25%), ABTS (36.84%) and FRAP (20.29%) for MC, DPPH (46.80%), ABTS (40.33%) and FRAP (25.83%) for SE and DPPH (58.35%), ABTS (55.67%) and FRAP (31.91%) for MAE, respectively.

The high antioxidant activity for ashwagandha may be due to presence of various bioactive phytochemicals including flavonoids, phenolics, withanosides, withanolides, and steroidal lactones distributed among leaves, bark, stem, and roots (Siriwardane et al., 2013). In general, there is significant correlation between the antioxidant activity and the type/amount bioactive compounds present in plant extracts, such as phenolics, flavonoids, carotenoids, lactones, and other pigments like proanthocyanins etc. (Skerget et al., 2005). On the other hand, it was reported that the results of antioxidant activity and concentration of bioactive compounds such as phenolics and flavonoids were greatly influenced by different temperatures selectivity in SWE (Lachos-Perez et al., 2018). High temperature condition mainly contributed towards effective extraction of bioactive compounds and increased percent antioxidant capacities by SWE compared to conventional extraction methods. However, the results with temperatures higher than 160 °C were not significant, as the main antioxidant constituents start degrading in SWE (Ko et al., 2014), this study also agrees with these findings. Previous studies found that there was great variation and significant difference in ashwagandha bioactive compound composition and biological activities of extracts, extracted and prepared using different extraction techniques (Dhanani et al., 2017).

3.5. Enzyme inhibitory effects

In recent years there is an increased prevalence of some chronic diseases worldwide including skin problems, hyperuricemia and gout, Alzheimer's disease, and diabetes mellitus. These diseases are identified as global health problems among many people; occurred due to the activity of various metabolic enzymes responsible for metabolism in human body (Mocan et al., 2018; Grochowski et al., 2017). Thus, inhibition of the catalytic functions of these enzymes is a key finding for management these diseases. In this context, the target enzymes which are mainly involved in metabolic pathways and alleviates the symptoms must be targeted including; tyrosinase responsible for skin disorders and browning of food products, xanthine oxidase involved in hyperuricemia or gouty arthritis, cholinesterase's develop Alzheimer's disease, amylase and glucosidase markers in diabetes mellitus. Many drugs are available in market as inhibitors of these enzymes including, kojic acid, galantamine, allopurinol, acarbose, respectively (Baltas et al., 2016; Zengin et al., 2018; Nile et al., 2018). However, these synthetic drugs exhibit several side effects and disorders including; nausea, diarrhea, headache, gastrointestinal problems, skin rashes, and allergies reactions (Etxeberria et al., 2012; Murray et al., 2013; Nile et al., 2018). Thus, effective remedy using natural drugs from medicinal plants is an important new line of research to control these diseases without any

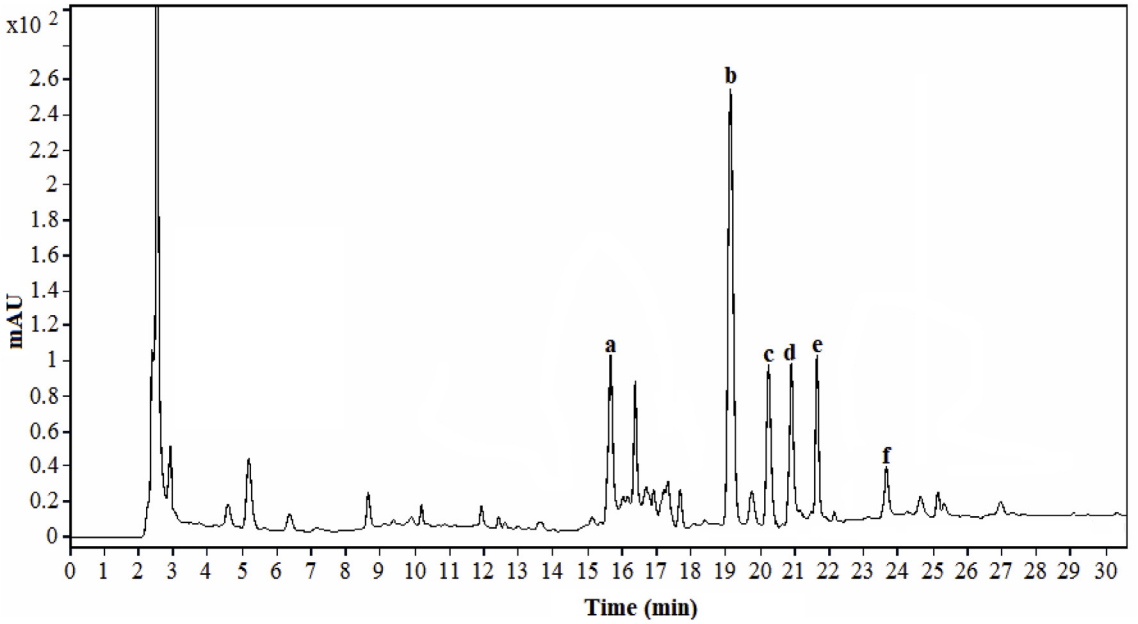

Fig. 1. HPLC profile of *W. somnifera* with SWE (a: Withanoside IV, b: Withanoside V, c: Withaferin A, d: 12-Deoxywithastramonolide, e: Withanolide A, and f: Withanolide B).

side effects.

In this research the inhibitory effects of different ashwagandha extracts were investigated using in vitro enzyme inhibition assays and the resulted values were presented in Table 3. The SWE extracts revealed potent inhibitory activity at 500 μg/ml (160 °C for 20 min), compared to MC, SE and MAE. The results show that all the ashwagandha extracts had potent inhibitory effects towards xanthine oxidase, AchE, BchE, α-amylase, and α-glucosidase, and tyrosinase (Table 3). Previous studies showed that this plant is widely utilised to in treatment of a variety of infectious diseases, also treat the tremors and inflammation especially rheumatoid arthritis, diabetes, osteoarthritis, and gout (Ahmad and Dar, 2018). Thus, these findings suggest that the SWE extract optimized at 160 °C for 20 min could be having high concentrations of principle bioactive compounds which mainly responsible for these enzyme inhibitory effects, which could be used as natural inhibitors or pharmacologically-active fractions derived from ashwagandha. However, further in vivo studies and clinical trials are important for future drug developments as commercial inhibitors from ashwagandha.

3.6. In vitro cytotoxic effects

The compounds derived from natural products as well as their man-made counterparts have achieved greater prominence and preference in the area of cancer therapy and hold good potential as anticancer agents on account of their safety, potency and efficacy (Ahmad et al., 2017). The cancer cells HeLa (cervical cancer) and MDCK (normal cells) were used to determine the cytotoxic effect of SWE, MC, SE, and MAE extracts (Table 4). It was found that the ashwagandha extracts are more

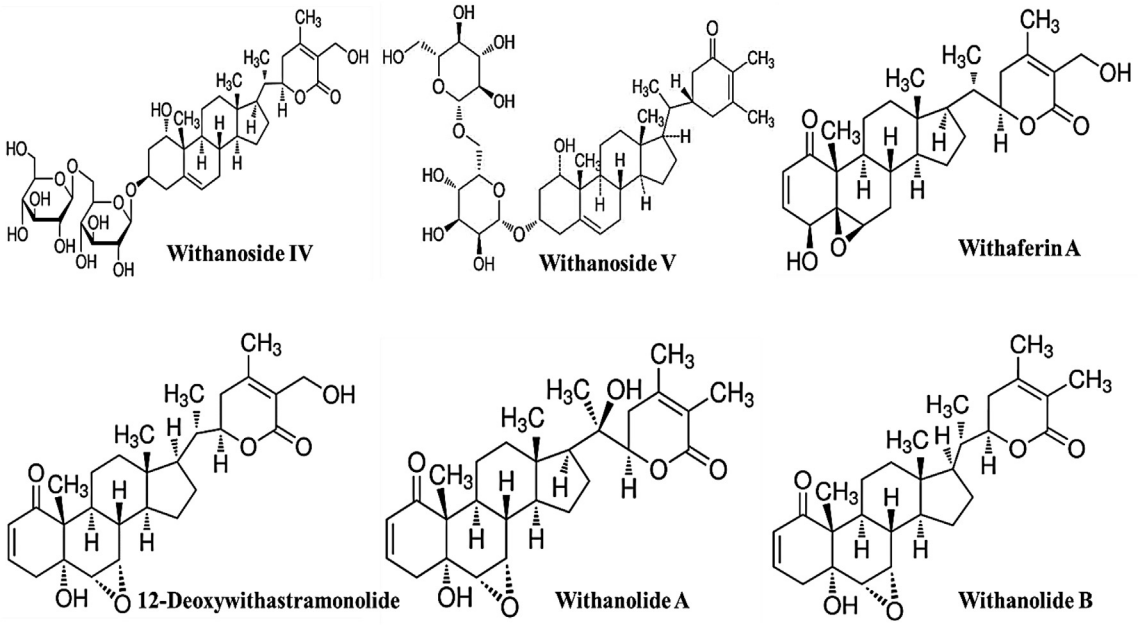

Fig. 2. Chemical structures of phytochemicals quantified in *W. somnifera* extract.

Table 2
Chromatographic parameters and concentration of six reference analytes in ashwagandha with different extraction methods.

Peaks	Bioactive compounds	R_t (min)	Concentration (μg/g) DW			
			SWE 160 °C 20 min	MC 40 °C 12 h	SE 60 °C 7 h	MAE 60 °C 20 min
1 (a)	Withanoside IV	15.8	22.10 ± 0.25	10.05 ± 0.13	15.20 ± 0.46	17.08 ± 0.30
2 (b)	Withanoside V	19.2	37.08 ± 1.09	25.14 ± 0.85	28.42 ± 1.18	31.11 ± 1.01
3 (c)	Withaferin A	20.5	30.14 ± 1.17	19.56 ± 1.21	21.87 ± 1.14	24.18 ± 1.08
4 (d)	12- DOWAM	21.0	33.06 ± 1.24	20.12 ± 1.04	23.75 ± 1.47	27.20 ± 1.11
5 (e)	Withanolide A	21.8	20.14 ± 1.41	8.25 ± 0.65	14.07 ± 1.09	15.21 ± 0.48
6 (f)	Withanolide B	23.8	8.18 ± 0.22	2.03 ± 0.19	3.16 ± 0.43	5.10 ± 0.14
Total			150.7	85.15	106.47	119.88

DW: Dry weight. Values are expressed as the mean ± SD (standard deviation). Values (a-f) represents elution of compounds in HPLC chromatograph and values (1–6) represents peak numbers in HPLC chromatograph for respective compound. 12-DOWAM: 12-Deoxywithastramonolide. MC: Maceration; SE: Soxhlet extraction; MAE: Microwave-assisted extraction; SWE, subcritical water extraction.

effective against HeLa cancer cells and it inhibited cell growth up to 78% (SWE), 88% Withaferin A, compared to Berberine (standard) 90% (Fig. 3). The study shows that the SWE is most effective extract causing potent cytotoxicity for Hela cancerous cells with IC_{50} values 10 mg/ml, while the withaferin A showed significant reduction in cell viability for cervical cancer (HeLa) cells with IC_{50} 8.5 μM/ml, respectively. However, it was also found that these extracts not having any cytotoxic effects for MDCK normal cells but may be having cytotoxic effects at higher concentrations (> 70.8 mg/ml, CC_{50}) (Table 4). The previous study reported that the cytotoxic effects of methanolic and ethanolic extracts of ashwagandha and found that the methanolic extract was more effective towards cytotoxicity showing IC_{50} of 40 and 30 mg/ml in 10% and 50% DMSO, respectively (Srivastava et al., 2016). Previous phytochemical studies revealed that Withaferin A is widely distributed in leaves, bark, stems and roots of ashwagandha and it was found that this compound is more effective ingredient showing potent anticancer activity and cytotoxicity towards cancerous cells (Siriwardane et al., 2013; Srivastava et al., 2016). Thus, SWE of ashwagandha accounting for the higher anticancer activity (lower IC_{50}) towards Hela cancerous cells will be utilised for further pharmacological studies for development of natural anticancer agent.

Table 4
Comparative cytotoxic effect of ashwagandha extracts (mg/ml) with MC, SE, MAE, and SWE methods.

Extraction conditions	Optimized extraction time	IC_{50}	CC_{50}
		Hela	MDCK
MC 40 °C	12 h	30.6 ± 1.2^{i}	65.8 ± 1.4^{i}
SE 60 °C	7 h	25.8 ± 1.1^{h}	68.2 ± 1.5^{h}
MAE 60 °C	20 min	21.8 ± 1.7^{g}	70.8 ± 2.1^{g}
SWE 100 °C	20 min	18.2 ± 1.3^{f}	72.6 ± 1.4^{f}
SWE 120 °C	20 min	15.9 ± 1.3^{e}	73.9 ± 1.1^{e}
SWE 140 °C	20 min	13.5 ± 1.4^{d}	75.1 ± 1.3^{d}
SWE 160 °C	20 min	10.0 ± 0.8^{a}	78.2 ± 1.2^{a}
SWE 180 °C	20 min	12.2 ± 0.9^{b}	76.9 ± 1.7^{b}
SWE 200 °C	20 min	11.8 ± 0.8^{c}	75.6 ± 1.3^{c}

Values represent the mean ± standard deviation (SD) of triplicate determinations. Different superscript letters indicate statistically significant differences among the various extracts ($p < 0.05$). IC_{50}: 50% inhibitory concentration for cancer cells (HeLa) and CC_{50}: 50% cytotoxic concentration for normal MDCK cells. MC: Maceration; SE: Soxhlet extraction; MAE: Microwave-assisted extraction; SWE, subcritical water extraction.

3.7. Biological activities of extracted compounds

Phytochemical investigation of ashwagandha resulted in the extraction of six withanoside, withanolide and steroidal lactone compounds (Withanoside IV, Withanoside V, Withaferin A, 12-Deoxywithastramonolide, Withanolide A, and Withanolide B), which were extracted by SWE, MC, SE, and MAE methods (Fig. 2). Among all the compounds the Withaferin A showed highest antioxidant (Fig. 4a), cancer cell cytotoxicity (Fig. 4b) and enzyme inhibitory activity (Fig. 5) compared to all other compounds. The HeLa cell inhibition pattern by Withaferin A comparing with standard drug Berberine were showed in Fig. 3. The previous research on ashwagandha also showed that the Withaferin A, acts as potent inhibitor of angiogenesis with promising anticancer activity (Jayaprakasam et al., 2003; Yang et al., 2007). The withanolides from ashwagandha are considered to have several pharmacological and biological activities with varying concentration from milligram to micrograms range. Previous reports suggested that the withanoside, withanolide and steroidal lactone compounds has promising anticancer drug activity due to due to its apoptotic, cytotoxic

Table 3
Comparison of enzyme inhibitory effects for ashwagandha extracts using MC, SE, MAE, and SWE methods.

Extraction conditions	Time	Tyrosinase inhibition (mg KAE/g extract)	XO inhibition (mg AP/g extract)	AChE Inhibition (mg GT/g extract)	BChE Inhibition (mg GT/g extract)	α-amylase inhibition (mg AE/g extract)	α-glucosidase inhibition (mg AE/g extract)
MC 40 °C	12 h	7.12 ± 0.44^{i}	0.98 ± 0.03^{i}	3.12 ± 0.04^{i}	2.06 ± 0.02^{i}	6.82 ± 0.18^{i}	4.56 ± 0.88^{i}
SE 60 °C	7 h	10.85 ± 0.31^{h}	1.85 ± 0.02^{h}	4.98 ± 0.07^{h}	2.95 ± 0.05^{h}	11.12 ± 0.15^{h}	8.98 ± 0.77^{h}
MAE 60 °C	20 min	15.19 ± 0.88^{g}	3.45 ± 0.05^{g}	6.12 ± 0.06^{g}	3.70 ± 0.08^{g}	18.02 ± 0.77^{g}	12.85 ± 0.89^{g}
SWE 100 °C	20 min	18.90 ± 1.06^{f}	3.89 ± 0.06^{f}	7.02 ± 0.10^{f}	5.45 ± 0.02^{f}	21.13 ± 1.01^{f}	18.22 ± 1.02^{f}
SWE 120 °C	20 min	20.41 ± 1.09^{e}	4.76 ± 0.04^{e}	10.85 ± 0.07^{e}	7.10 ± 0.07^{e}	25.12 ± 1.14^{e}	21.78 ± 1.07^{e}
SWE 140 °C	20 min	24.18 ± 1.11^{d}	5.18 ± 0.03^{d}	13.18 ± 0.06^{d}	9.98 ± 0.03^{d}	33.01 ± 1.03^{d}	28.51 ± 1.39^{d}
SWE 160 °C	20 min	30.11 ± 1.41^{a}	6.82 ± 0.02^{a}	16.56 ± 0.02^{a}	12.71 ± 0.08^{a}	40.12 ± 1.20^{a}	33.02 ± 1.11^{a}
SWE 180 °C	20 min	28.63 ± 1.31^{b}	6.06 ± 0.05^{b}	15.62 ± 0.03^{b}	10.45 ± 0.02^{b}	38.02 ± 1.41^{b}	30.19 ± 1.33^{b}
SWE 200 °C	20 min	27.11 ± 1.01^{c}	5.92 ± 0.07^{c}	14.01 ± 0.09^{c}	9.04 ± 0.06^{c}	37.81 ± 1.33^{c}	29.87 ± 1.02^{c}

Results expressed as mean ± standard deviation (SD) of three replicates. Different small letters mean significantly differences with $p < 0.05$, calculated by Duncan's multiple range tests. KAE: kojic acid equivalents, AP: allopurinol equivalents, GT: galanthamine equivalents, AE: acarbose equivalents. MC: Maceration; SE: Soxhlet extraction; MAE: Microwave-assisted extraction; SWE, subcritical water extraction.

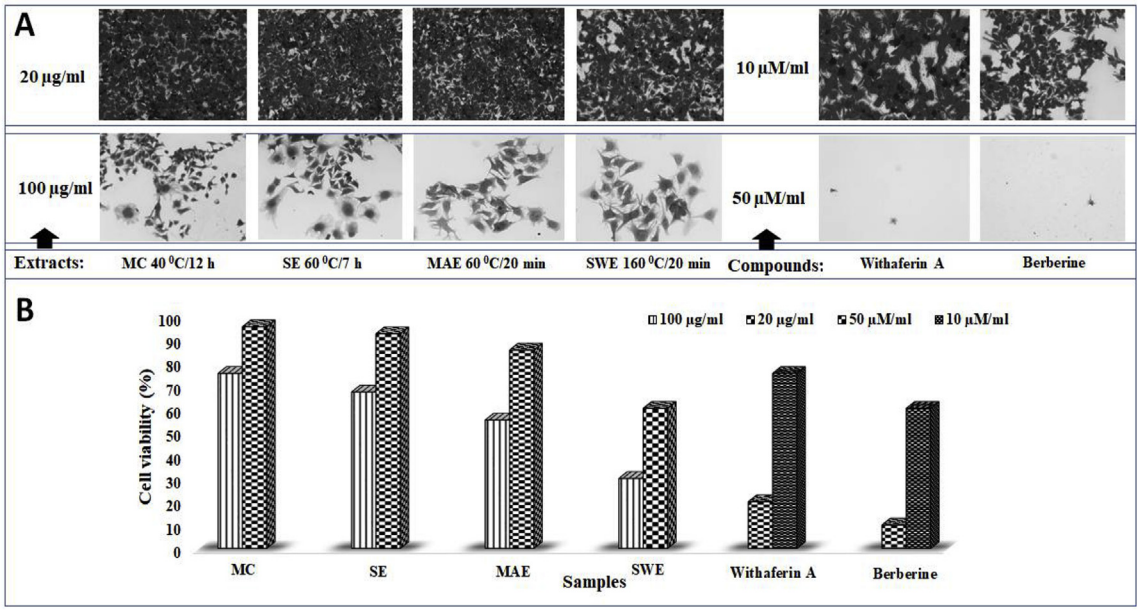

Fig. 3. The cytotoxic effect of *W. somnifera* extracted at different temperature and time conditions, withaferin A and berberine (Standard drug) on HeLa cell lines (A: Microscopic observation and B: Cell viability of HeLa cells).

antimitotic and anti-angiogenesis properties (Ahmad et al., 2017; Ahmad and Dar, 2018). Thus, this result shows that the extracted withanoside, withanolide and steroidal lactone compounds from ashwagandha by using SWE optimized at 160 °C for 20 min could be used as promising anticancer, antigout and antidiabetic compounds and the SWE method provided information for maximum yield for these compounds compared to other extraction methods like MC, SE and MAE.

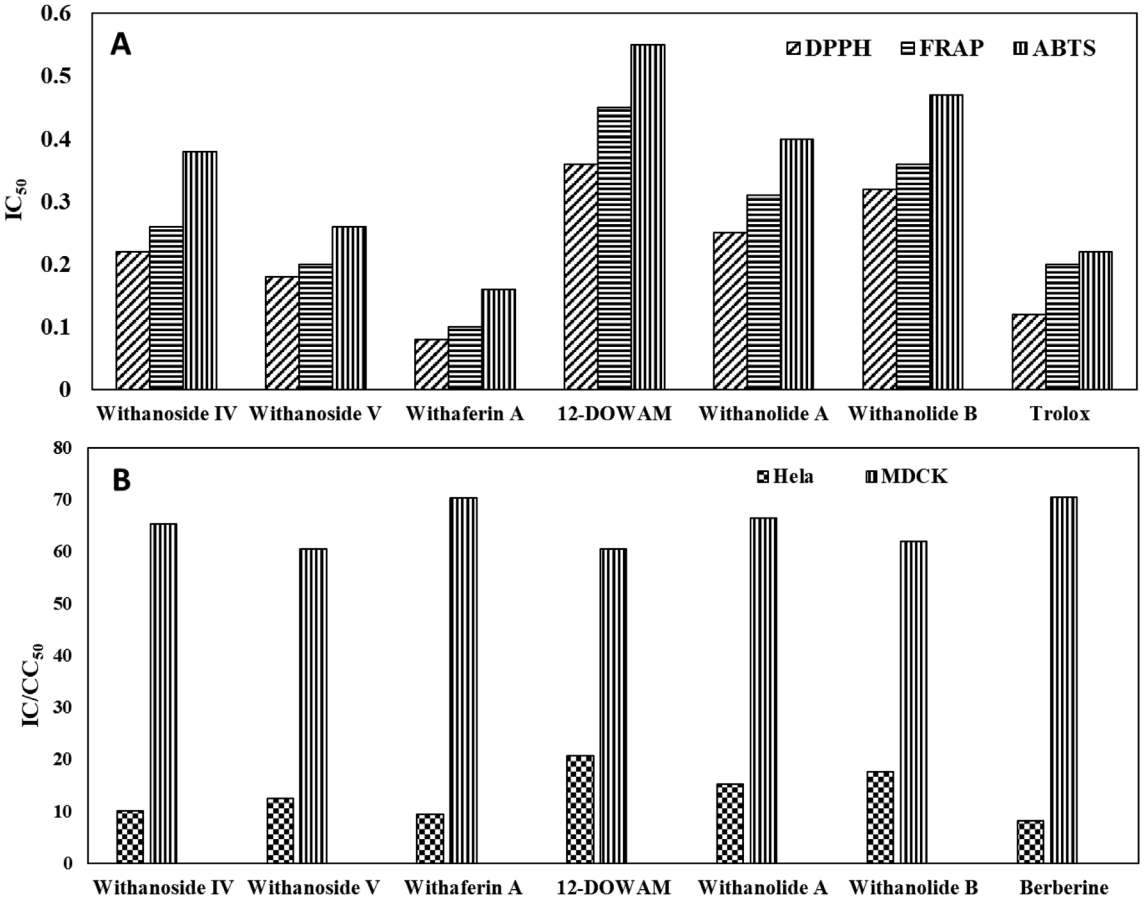

Fig. 4. Biological activity of extracted compounds; A: Antioxidant activity (IC_{50} mg/ml) and B: Cytotoxicity effect (IC/CC_{50} µM/ml)) by extracted bioactive compounds from ashwagandha using SWE (160 °C at 20 min). Values expressed as for all assays. 12-DOWAM: 12-Deoxywithastramonolide.

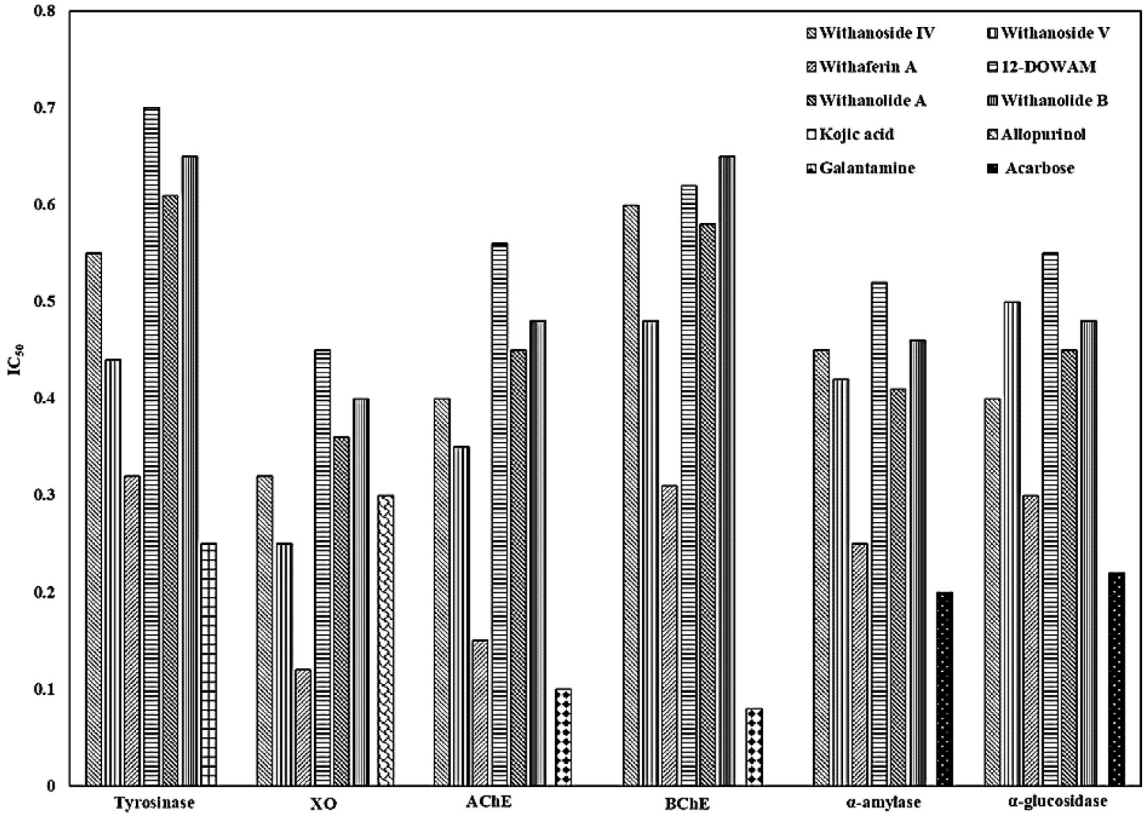

Fig. 5. IC_{50} (mg/ml) values for enzyme inhibitory effects for extracted bioactive compounds from ashwagandha using SWE (160 °C at 20 min). 12-DOWAM: 12-Deoxywithastramonolide.

4. Conclusions

The results show that the ashwagandha, is an important medicinal plant which has been used in Ayurvedic and indigenous medicine for treatment of various diseases. The plant extract using SWE showed high concentration of withanoside, withanolide and steroidal lactone compounds with promising biological activities. This indicate that the SWE at 160 °C for 20 min could be used as new method for extraction of bioactive compounds from ashwagandha, instead maceration (MC), Soxhlet extraction (SE) and microwave-assisted extraction (MAE) traditional methods of extractions, which were more time consuming and required hazardous solvents. The SWE method can be an eco-friendly viable and alternative method used for extraction of bioactive compounds from ashwagandha compared to MC, SE and MAE.

However, further studies are required to control and predict the analyses and standardization of different parameters in SWE using mathematical models. Such green extraction methods help in production of improved yield and high-quality bioactive compounds with possible health benefices, which also help to save energy and time. So, this study could help in production and utilization of pharmacologically-active fractions from ashwagandha.

Conflicts of interest

The authors declare that they have no competing interest.

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