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PHARMACOLOGICAL STUDIES OF ISOLATED COMPOUNDS FROM *ADHATODA VASICA AND CALOTROPIS PROCERA* AS AN ANTIOXIDANT AND ANTIMICROBIAL BIOACTIVE SOURCES

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Abstract

Adhatoda vasica and *Calotropis procera* species are worldwide available but poorly investigated as an important resource of bioactive compounds and antimicrobial activities for controlling of many infectious diseases. This article explores the activities of isolated compounds using advanced technology in context of bacterial diseases and suppression of infectious diseases through stictic acid and usnic acid content isolated first time from these species. The estimation of different compounds was carried out in leaves samples of both plants while various compounds like usnic acid, stictic acid and p-hydroxy-benzoic contents isolated through high performance liquid chromatography. The total antioxidant activities of 2, 2'-azino-bis (3- ethylbenzothiazoline-6-sulphonic acid (ABTS) and some active compounds evaluated via different extraction methods such as methanol, ether, acetone and crude extraction with the quantity of these compounds. The Broth microdilution method was performed to determine the minimum inhibitory concentration (MIC) for both species. The obtained data for each parameter was interpreted by applying Complete Randomized Design (CRD) along with factorial arrangements. Mean comparison was performed using LSD test at 5% probability level. The efficiency of extraction of the solvents for the usnic acid, stictic acid and p-hydroxy-benzoic contents found to be methanol > ether > acetone > crude extraction. The MIC values of all extracted compounds showed maximum level of bacterial control. The MIC of methanol extracts of *Adhatoda vasica* is E-coli E1 (516) µg/mL while *Calotropis procera* plant's extract showed higher antimicrobial activity for E-coli E1 511 µg/mL. All bacterial strain showed maximum resistance especially the kidney failure strain E. coli O157:H7. It was concluded that isolation of usnic acid, stictic acid first time from these two species recommended that both species can act as the best resources of antioxidant and antimicrobial agents in controlling of various bacterial diseases.

Key words: Usnic acid, Bacterial diseases, Antioxidant and antimicrobial agents.

Introduction

Adhatoda vasica belong to family *Acanthaceae* that commonly known as Malabar Nut plants Anndy *et al.*, (2003). *Calotropis procera* is a species of flowering plant which belongs to the family *Asclepiadaceae lima*). It is widely distributed in Asia and subcontinents as well as in West Africa and other parts of the tropics Irvine (Asahina & Shibata, 1995). Antioxidants and bioactive compounds were widely distributed in plants which have been reported to excess multiple biological effect, including antioxidant, free radicals scavenging abilities, anti-inflammatory, anti-carcinogenic Miller (1996). *Adathoda vasica* is a commonly consuming herb in "Ayurveda" which is 5000 years old traditional medications and many experimentations and observations suggest that the medicinal plants identified as the source of numerous medicines for the control of different infections in the human body Cakir *et al*., (2004). Parotta, (2001) reported that the secretions from the root bark of *Calotropis procera* used traditionally for the treatment of skin diseases, enlargements of abdominal viscera and intestinal worms, bacterial infections control in the human body Contreras *et al.,* (2015). In recent year, various studies reported that usnic acid, p-coumaric displays a wide range of biological activities including antioxidant, anti-inflammatory, anti-microbial and anti-carcinogenic activities, improvement of vision, and induction of apoptosis and neuroprotective effects on the human bodies Gulluce *et al*., (2006). Many disease of E-coil caused illness, either diarrhea or illness outside of the intestinal tract and failure of kidney in the human bodies (Irvine, 1961). Herbal species have potential for the preparation of the natural products that used in medicine, food, fodder, perfume, spice, dyes for different purposes throughout the world (James *et al*., 2009). Brand, (2012) reported that *Adhatoda vasica and Calotropis procera* have several potential activities, such as anti-mycobacterial, antitumor, antiprotozoal, anti-mutagenic, anti-allergenic, analgesic, antipyretic, anti-proliferative. Herbal species produce number of antioxidants Gulluce *et al*., (2006). They have properties because they act as hydrogen donors and singlet oxygen quenchers and, therefore, have redox capacity (Khan *et al.,* 2012). Herbal species from different parts of world have been investigated in term of their enormous potential activity of antioxidants activity (Cakir *et al*., 2004). Recent studies by Contreras *et al.,* (2015) showed that some metabolites found in these herbal species, have very good anti-bacterial potential. Lima *et al*., (2104) reported that *Adhatoda vasica and Calotropis procera* are effective against Gram positive and Gram negative bacteria Cocchietto. Many industries used usnic acid as a pure substance in toothpaste, creams, mouthwash, sunscreens, powder, extract and tincture, as these have potential for drugs as an active ingredient for antibacterial properties (Mazza, 2007). Usnic acid and stictic acid are potent agents for controlling cell stress, cellular senescence, and cell cycle and control the pathways of the protein p53 which causes cancer (Miller, 1996). The current study reported the usnic acid, stictic acid first time from *Adhatoda vasica* and *Calotropis procera* through high

performance liquid chromatography-diode array detection (HPLC-DAD). Furthermore; solvent extraction method was also employed for determination of various bioactive compounds and their total antioxidants activity was tested via standard methods.

Materials and Methods

Materials for experimental analysis: Trolox [(±)-6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid], an HPLC grade methanol, ethanol and usnic acid purchased from Sigma-Aldrich (Steinheim, Germany). Stictic acid purchased from ChromaDex (California, USA). Analytical-grade acetone, dimethyl sulfoxide (DMSO), tetrahydrofuran and orthophosphoric acid (89%) purchased from Merck (Merck, Darmstadt, Germany).

Collection of herbal species and experimental site: Leaves of *Adhatoda vasica* and *Calotropis procera* freshly plucked from Khanpur valley, Haripur Pakistan. The mature leaves collected from valley. The leaves were chosen of correct species and uniformity on the basis of its bright green color, having no pigmentation and blemishes on leaves. These species shifted into Horticulture laboratory, The University of Haripur for further analytical process. The repository reference number of these species is 47070771.

Preparation of plant sample: The leaves samples were washed thoroughly with tap water and crushed. The samples were then placed in the conventional oven at 73°C for 48 hours. After the drying of the samples, the dried samples ground with a domestic electric grinder and the powdered samples then stored in air tight glass jar and kept away from the direct sunlight for further processing.

Plant Extracts used during the experiment

- Leaves of *Calotropis procera*
- Leaves of *Adhatoda vasica*

Pure sample preparation and extracts: The plant extract prepared by taking 1 g of powdered dry sample and 10 ml of distilled water added to it. The sample then mixed with the help of mechanical shaker and filtered through filter paper. The plant sample then transferred to the separate bottle and the pure solution was kept in the refrigerator as described by Odugbemi, (2006).

HPLC analysis for bioactive compounds: The organic solvent extracts (5 mL) evaporated to dryness. Finally, residues dissolved in 2 mL of dimethyl sulfoxide. The dimethyl sulfoxide extracts used for the HPLC analysis. Chromatographic separations were carried out using an X Bridge C18 (3.5 µm, 4.6 x 250 mm) column from waters. The mobile phase consisted of 0.25% orthophosphoric acid and 1.50% tetrahydrofuran in water (solvent A) and methanol (solvent B). The gradient conditions are as follows; 0-15 min 30-70% A, 15-30 min 70-100% A, 30-35 min 100% A, 35-36 min 100-30% A, and 36-50 min 30% A with a total run time of 50 min. The column was equilibrated for 10 min prior to each analysis.

Antioxidant activity assay: The total antioxidant activities of both species determined with the 2, 2'-azino-bis (3- ethylbenzothiazoline-6-sulphonic acid (ABTS) method as described by Sarıburun *et al.,* (2010). The absorbance measured by spectrophotometry (Varian Cary 50, Australia) at 734 nm against a blank after 6 min. The results expressed as mg of trolox equivalent (TE) per 100 g of dried weight.

Bacterial strains: The different bacterial strains that used in this study were Staphylococcus aureus 1, 2 and E. coli number are 1 to 5 isolates. E. coli O157:H7 for special bacterial strain for kidney failure.

Determination of minimum inhibitory concentration (MIC): Broth microdilution testing was performed to determine the MICs of the herbal species according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2003). The bacterial cultures prepared in Mueller-Hinton Broth (MHB) at 37ºC for 16-20 h. Herbal species extracted with methanol; then methanolic extracts prepared in phosphate buffered saline (v/v) after buffer preparation then volume make up to mark. The optical densities (ODs) of the cultures measured at a wavelength of 595 nm (Bio-Rad, iMark).

Statistical analysis: The data was analyzed under Complete Randomized Design (CRD) along with factorial arrangements. Mean comparison performed using LSD test at 5% probability level. The developed methods are confirmed and aureate the measurement of all bioactive compounds, confirmed through standards digital library. The data of HPLC-DAD was measured by the help of Chrom Gate v 3.31 Knauer software was used.

Results and Discussion

Herbal species' leaves, flowers, and barks showed a rich source of bioactive compounds which can used as a reserve for antioxidant and antimicrobial agents (Mishra *et al*., 2011). The current study showed that the *Adhatoda vasica* and *Calotropis procera* species may replace many existing species which are used as antioxidant or antimicrobial agents.

Identification of p-coumaric and p-hydroxy-benzoic acids in *Adhatoda vasica* and *Calotropis procera*: The concentrations of p-coumaric and p-hydroxybenzoic acids in the four different extracts of *Adhatoda vasica* and *Calotropis procera* determined by HPLC-DAD (Table 1). Identification of these compounds achieved by comparison of their retention time values with the standard substance purchased from Chroma Dex and Sigma-Aldrich. The higher values of p-coumaric 10.81±0.48 recorded in the methanolic extract. While lower concentrations were observed in crude extraction (0.91±0.01). The highest concentration of p-coumaric in *Calotropis procera* (11.81±0.49) observed in methanol extraction followed by the similar trend in crude extraction (Table 1). P-hydroxy-benzoic was higher in methanol extract (12.81±0.41) in leaves of *Adhatoda vasica*. Lower values were noted in crude extraction

methods as compared with others methods of extraction. P-coumaric concentrations that obtained by the HPLC method indicate that the order of solvent efficiency is methanol > Ether > ethanol > Cured extraction, followed by similar order of solvents extractions for p-hydroxy-benzoic acids. The best solvent extraction of these two compounds was methanol because these are soluble and extracted in methanol as reported in the literature (Parotta, 2001). The results of current investigation were in accordance of previous studies which was reported by several Scientist like (Skaltsa *et al.*, 2014; Khan *et al*., 2012; Wong *et al*., 2006; Cakir *et al*., 2004). These results showed that maximum quantity of these bioactive compounds are present in leaves extracts as reported by Asahina & Shibata, (1995).

Identification of stictic and usnic acids: The identification of stictic and usnic acids from different extraction methods presented in the (Table 2). Results showed that *Adhatoda vasica* and *Calotropis procera* contained higher stictic acid and usnic acids. The higher stictic acid (13.80±0.42) in *Adhatoda vasica* was recorded in methanol extraction, while lower extraction was recorded in the crude extraction (11.81±0.42) followed by similar trends in *Calotropis procera* in comparison of others solvents extraction methods such as ether, acetone and crude extraction. The higher soluble method of extraction found to be methanol while ether as second best method of extraction whereas crude extraction proved to be lowers one. Furthermore, with our best of knowledge these herbal species showed maximum contents of bioactive compound like stictic and usnic acids which were first time reported during this investigated via diverse extraction methods followed by quantitative analysis of these compounds through advanced technology. Pal & Rahaman, (2015) reported that different plant species have variation in the P-coumaric content due to the genetic potential and spatial differences. Stictic acid is an aromatic organic compound, and used as a source of many medicines and byproduct used in daily life Perry *et al*., (1999). The stictic acid concentration in four solvent extract was reported first time in this study. Usnic acid is found in several lichens and herbal species, is especially plentiful in the genera like Cladonia, Usnea, Alectoria, Lecanora, Ramalina and Evernia Cocchietto (Mazza, 2007; Qureshi *et al*., 2006).

Antioxidants activity of *Adhatoda vasica* and *Calotropis procera*: The antioxidants activities of both herbal species presented in (Table 3). The highest antioxidants activity was noted in ether extraction for *Adhatoda vasica* (14.71±0.01 mg TE100 g-1) and the methanolic extract (12.81±0.40 mg TE100 g^{-1}. *Calotropis procera* contained higher contents of TA (13.81±0.41 mg TE100 g^{-1}) in methanol extraction while *Adhatoda vasica* contained (11.92±0.02 mg TE100 g^{-1}) when compared with other extractions methods including ether, acetone and crude extraction but reduced in crude extraction with lower antioxidant activity in same extraction (10.92±0.01 mg TE100 g^{-1}). Results recommended that ether found to be best extraction method for antioxidants activity as compared to any other methods of extraction.

Table 1. Identification of p-coumaric and p-hydroxy-benzoic compounds with different extraction methods.

Species	Extraction method	*p*-coumaric	*p*-hydroxy-benzoic
Adhatoda vasica	Methanol	10.81 ± 0.48	12.81 ± 0.41
	Ethanol	1.11 ± 0.17	2.11 ± 0.16
	Ether	9.72 ± 0.06	8.72 ± 0.05
	Crude extraction	0.91 ± 0.01	0.96 ± 0.02
Calotropis procera	Methanol	11.81 ± 0.49	12.82 ± 0.42
	Ethanol	2.11 ± 0.15	1.11 ± 0.17
	Ether	10.72 ± 0.02	9.72 ± 0.05
	Crude extraction	1.91 ± 0.02	0.91 ± 0.01

Mean of two determinations ± SD of species

Table 2. The amounts of stictic and usnic acids extracted from *Adhatoda vasica* and *Calotropis procera species* using different solvents Methods (milligrams per gram).

Species	Extraction method	Stictic acid	Usnic acid
Adhatoda vasica	Methanol	13.80 ± 0.42	11.81 ± 0.42
	Ethanol	2.11 ± 0.15	3.11 ± 0.19
	Ether	11.72 ± 0.05	9.72 ± 0.06
	Crude Extraction	0.81 ± 0.01	1.96 ± 0.01
Calotropis procera	Methanol	10.81 ± 0.49	11.82 ± 0.42
	Ethanol	1.11 ± 0.15	2.11 ± 0.17
	Ether	11.71 ± 0.01	10.72 ± 0.05
	Crude Extraction	1.92 ± 0.03	2.91 ± 0.01

Mean of two determinations ± SD of species

Table 3. Total antioxidants activity (ABTS) of *Adhatoda vasica* and *Calotropis procera* (mg TE100g^{-1}) by different extraction methods.

Methods of extractions	*Adhatoda vasica* (mg TE100g^{-1})	*Calotropis procera* (mg TE100g^{-1})
Methanol	12.81 ± 0.40	13.81 ± 0.41
Ethanol	11.11 ± 0.11	12.11 ± 0.12
Ether	14.71 ± 0.01	11.71 ± 0.02
Crude extraction	11.92 ± 0.02	10.92 ± 0.01

The values are the mean of two species extracts
TE = Trolox equivalent
± SD of species

Table 4. Validation parameters and recovery of stictic, usnic acids and p-hydroxy-benzoic in different extract methods.

Validation parameters	Stictic acid	Usnic acid	*p*-hydroxy-benzoic
LOD (mg L^{-1})	0.90	0.99	1.22
LOQ (mg L^{-1})	2.12	3.11	3.01
Recovery (%)			
Methanol	0.99	0.99	0.99
Ethanol	97.31 ± 2.12	92.33 ± 2.13	93.31 ± 2.12
Ether	91.21 ± 1.71	90.21 ± 1.72	90.22 ± 1.70
Crude	96.97 ± 1.77	95.96 ± 1.76	91.96 ± 1.36
Extraction	90.34 ± 1.55	89.34 ± 1.52	81.33 ± 1.31

LOD limits of detection, *LOQ* limits of quantification

Table 5. Minimum inhibitory concentration of two herbal species methanolic extracts for different bacterial strains.

Isolates ID	MIC (µg/mL)	
	Adhatoda vasica	*Calotropis procera*
E. coli E1	516	511
E. coli E2	230	232
E. coli E3	200	201
E. coli E4	240	242
E. coli E5	330	331
E. coli O157: H7	270	260
S. aureus 1	333	334
S. aureus 2	320	320

Validation of the analytical methods: The usnic acid and stictic acid obtained by HPLC. The linearity of HPLC- DAD method investigated for anti-oxidative range 10-12 (mg TE100g^{-1}), stictic acid 0.90 and usnic acid 0.99 mg/L at nine concentrations levels (Table 4). The ranged of LOD for stictic acid 0.90 and usnic acid 0.99 mg/L while p-hydroxy-benzoic is 1.22. Whereas the LOQ ranged for stictic acid 2.12 and usnic acid is 3.11 mg/L while p-hydroxy-benzoic is 3.01 mg/L (Table 4). The higher extraction efficiency of stictic acid in methanol extract and lower values of stictic acid were obtained in crude extraction. In contents higher values of usnic acid observed in ether while lower contents noted in crude extraction. Higher values of p-hydroxy-benzoic was observed in methanol extracts.

Antimicrobial activity of *Adhatoda vasica* and *Calotropis procera*: The antimicrobial activities of both species were studied by the broth of microdilution methods to test for susceptibility of these extracts while results presented in the (Table 5). The MIC of methanolic extract of *Adhatoda vasica* for E-coli E1 (516) µg/mL while *Calotropis procera* plants extract showed higher antimicrobial activity from E-coli E1 511 µg/mL. Maximum antimicrobial activity from *S. aureus* 1 showed higher MIC 334 µg/mL in *Calotropis procera* while *Adhatoda vasica* extract showed 330µg/mL. All bacterial strain showed maximum susceptibility for control of antimicrobial activity. Lower activity was recorded by E-coli E3 bacterial strain. In fact these extracts have potential role for control of bacteria (Table 5). Higher values O157:H7 bacterial strain showed maximum in MIC from *Adhatoda vasica* leaves extracts as compare to *Calotropis procera* extracts. Both extracts showed inhibition and decline in the bacterial strains. Today the world faced many infectious diseases which associated with the health problems. Medicinal chemists are always in search for new antimicrobial agents to control of different diseases (Pragasam & Rasool, 2013). 4-Hydroxy benzoic acid has vast range of antimicrobial agents for positive and negative bacteria (Lima *et al*., 2013).The biological activity of these species proves that they contain vast range of diverse bioactive compounds reflecting potential control of dissimilar bacteria in human (Roy *et al*., 2005).

Conclusion

The study revealed that *Adhatoda vasica* and *Calotropis procer* are the rich resources of different metabolites as a new source of active compounds for different drug formulation which were poorly studied yet. In conclusion, our findings indicate these two species may be used as a best resource of antioxidant and antimicrobial agents for control of different bacterial diseases. According to estimation of these compounds it is proposed that these

two species must be taken under investigation for the biological activities for various diseases.

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