DPPH, ABTS free radical scavenging, antibacterial and phytochemical evaluation of crude methanolic extract and subsequent fractions of *Chenopodium botrys* aerial parts

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Abstract: We investigated antioxidant, antibacterial potentials and secondary metabolites of *Chenopodium botrys* aerial parts to rationalize its effectiveness in free radicals induced disorders and infectious diseases. Antioxidant activity of plant extracts were investigated using DPPH and ABTS free radicals scavenging assays. Antibacterial potential was studied using well diffusion method. Phytochemical analysis was performed for the presence of secondary metabolites. In DPPH assay chloroform fraction (CHF), ethyl acetate fraction (EAF) and *n*-hexane fraction (NHF) were most active causing average inhibition of 65.9, 59.2 and 55.9% at concentration of 1mg/ml with IC_{50} values of 140, 30 and 590 µgml respectively. EAF, CHF and aqueous fraction (AQF) revealed highest scavenging effect against ABTS free radicals causing 85.46, 82.73 and 68.80% inhibition with of IC_{50} of 75, 94 and 530 µg/ml respectively. In antibacterial assay, CHF was found most effective against *S. aureus* presenting an inhibitory zone of 19 mm whereas; EAF, CHF and NHF were most active against *K. pneumoneae* with inhibitory zones of 27.1 mm, 25.4 and 18.7 mm respectively. *C. botrys* was tested positive for flavonoids, anthraquinones, saponins and tannins. Current findings revealed that that *C. botrys* is rich source of natural antioxidant and antibacterial bioactive compounds and may be further investigated.

Keywords: Antioxidant, antibacterial, DPPH, ABTS, *Chenopodium botrys.*

INTRODUCTION

Men have always used natural resources of healing substances to treat different human diseases. Efforts to cure the diseases by means of traditional medicine have been made in all parts of the world. At present, ethno-botanical and ethno-pharmacological experiences of the people are used in the treatment of wide range of diseases including cancer, AIDS, alzheimer's disease, alcoholism, microbial infections and early aging (Heinrich and Bremner 2006; Hameed, Ashraf *et al*. 2011). In developing countries medicinal plants provide a real alternative for primary health care system. According to an estimate, between 35,000 and 70,000 plant species are used in folk medicine worldwide as raw drugs, but their active constituents in pure form are still unexplored (Ali and Qaiser 2009).

The negative effects of oxidative stress to human tissues and cells caused by reactive oxygen species arising from aging and disease pathogenesis are well documented. Though the human body has inherent anti-oxidative mechanisms to counteract the damaging effects of free radicals, there is often a need to use dietary and/or medicinal antioxidant supplements, particularly during instances of disease attack and as protective agents. A lack of balance between free radicals such as singlet oxygen, superoxide anion radical, hydroxyl radical and hydrogen peroxide, and the natural detoxification capacity of the body in favour of the oxidant molecules causes oxidative stress leading to cellular and DNA damage as well as oxidation of low-density lipoproteins (Atmani, Chaher *et al*. 2009). Oxidative stress disorders caused by the actions of free radicals are associated with many acute and chronic diseases such as inflammation and neurodegenerative conditions including Alzheimer's disease (Hoozemans, Veerhuis *et al*. 2006). Owing to growing concerns about the toxicity and side effects of many synthetic therapeutic agents, there has been a renewed interest globally, in the search for antioxidants from natural sources, particularly medicinal plants (Ayaz, Junaid *et al*. 2014).

Medicinal plants are a potential sources of antibacterial agents. The emergence of multiple drug resistant pathogens and unpleasant side effects associated with the use of modern antibiotics have compelled the researchers to explore safe and effective remedies from natural resources. (Yasunaka, Abe *et al*. 2005). Moreover, a specific activity of specific specie of plant owe to the presence of certain secondary metabolites (Shah, Sadiq *et al*. 2014). These secondary metabolites have been reported to be responsible for various pharmacological activities. Various extraction techniques are employed to extract maximum bioactive compounds from plants. Maceration is one of these techniques, which is time consuming but effective procedure to get maximum secondary metabolites in large quantity (Singh 2008).

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C. botrys belongs to family *Chenopodiaceae,* which has been used in traditional medicine as anti-spasmodic, anti-asthmatic, and anthelmintic and as spice (Singh and Kachroo 1976). Essential oil isolated from *C. botrys* have been revealed to possess significant fungicidal and antibacterial activity (Maksimović, Đorđević *et al*. 2005). As no antioxidant, antimicrobial and phytochemical analysis studies has been reported on *C. botrys*, so the current study has been designed to investigate the antioxidant, antimicrobial potentials and phytochemistry of crude methanolic extract and its various fractions of *C. botrys.*

MATERIAL AND METHODS

Chemical and drugs

DPPH (CAS 1898-66-4 Sigma Aldrich CHEMIE GmbH USA), ABTS (CAS 30931-67-0 Sigma Aldrich USA), Ascorbic acid (GSK) and ceftriaoxne (Sami Pharmaceuticals, Pakistan) were used in the study. For phytochemical analysis and extraction all ingredients and solvents used were of pure analytical grade.

Collection of plant material

The aerial parts of *C. botrys* were collected from the locality of Lower Dir, Khyber Pakhtunkhwa, Pakistan in July 2013.The plant was identified and authenticated by plant taxonomist Prof. Dr. Jehandar Shah, Shaheed Benazir Bhutto University, Sheringal (Dir Upper) KPK, Pakistan. A specimen voucher (CB-1036) has been submitted to the herbarium of the same University for future reference.

Extraction and fractionation

C. botrys powder (2.2kg) was soaked in 80% of methanol (3 x 250ml) and filtered using muslin cloth. The filtrates were mixed and evaporated under reduced pressure at 45◦C using rotary evaporator (Heidolph Laborota 4000, Schwabach, Germany). This resulted in 435g crude methanolic greenish extract (19%). A portion of 400g of this was subjected to fractionation using n-hexane (3 x 250ml), chloroform (3 x 250ml), ethyl acetate (3 x 250ml) and water. The different fractions obtained were sealed and stored at 20◦C until needed for antioxidant and antibacterial and phytochemical evaluation (Kamal, Ullah *et al*. 2015; Zeb, Sadiq *et al*. 2015).

Phytochemical screening

For identification of secondary metabolites including alkaloids, glycosides, saponins, flavonoids and tannins, phytochemical investigations were carried out. For this purpose some chemical tests were performed on crude methanolic extract (CME) and on the powdered specimen of *C. botrys* using standard procedures as reported previously (Zeb, Sadiq *et al*. 2014). The tests performed are discussed as follows;

Alkaloids

For the detection of alkaloids the extract was treated with 2% HCl and then filtered. The filtrate was treat with Mayer's reagent and observed the presence of turbidity or yellow precipitate (Khan, Ullah *et al*. 2011).

Flavonoids

Aqueous filtrate of plant extract was mixed with 5 ml of ammonia solution. Concentrated H2SO4 was added to it. The presence of yellow color indicated the presence of flavonoids, which disappeared upon standing for a while (Dastagir, Hussain *et al*. 2012).

Tannins

Dried powder of plant having weight of 0.5 g was allowed to boil in 20 ml of water and then filtered. A few drop of ferric chloride (0.1%) was added to it and the brownish green or bluish black color was observed (Dastagir, Hussain *et al*. 2012).

Glycosides

For the detection of glycosides 2ml of glacial acetic acid was taken in a test tube and one drop of ferric chloride solution was added to it. About 5 ml of plant extract was added to it and after that 1 ml of concentrated sulphuric acid was also added to the test tube. The presence of glycoside will be indicated by the formation of a brown ring at the interface (Dastagir, Hussain *et al*. 2012).

Saponins

Powdered plant sample 0.2g was allowed to boil in 2 ml of distilled water and then filtered. About 1 ml of the filterate was mixed with distilled water and vigorously shaken to form stable froth. About 3 drops of olive oil was mixed with the froth and vigorously shaken which led to the formation of emulsion (Dastagir, Hussain *et al*. 2012)

Anthraquinone

For the detection of anthraquinone, a minute quantity of powdered plant sample was soaked in ether and a pink, red or violet color was detected (Dastagir, Hussain *et al*. 2012).

Antioxidant activities

DPPH free radical scavenging assay

The antioxidant potential of crude extract and subsequent fractions was determined using DPPH (2,2-diphenyl-1-picrylhydrazyl) (Sigma Aldrich USA). Test samples (2ml) of different concentrations (250-1000µg/ml) were taken in test tubes and 2 ml DPPH (2%) was added. Ascorbic acid was used as a standard and all measurements were done in triplicates. After 30 minutes, absorbance was determined at 517nm using UV spectrophotometer (Mazimba, Majinda *et al*. 2011).

Radical scavenging activity was calculated using the following formula;

Scavenging activity = [(blank absorbance-sample absorbance)/ (blank absorbance)] × 100.

ABTS free radical scavenging assay

ABTS (2, 2-Azinobis (3-ethylbenzothiazoline)-6-sulphonic acid) (Sigma Aldrich USA) assay was carried out according to the previously reported method (Gill, Brenwald *et al*. 1999). The assay is based on the capacity of antioxidants to scavenge ABTS radical cation causing a reduction in absorbance at 734 nm. The ABTS solution was prepared by mixing 7mM ABTS and 2.45 mM potassium persulphate (Riedel-de Haen Germany) and then incubated in the dark at room temperature for 16 h. Before the assay, the solution was diluted with methanol to give an absorbance of 0.706±0.001 at 734nm. Different concentrations (250-1000µg/ml) of CME and subsequent fractions were prepared in the methanol. Each concentration of tested samples was added 3ml of ABTS solution and measured absorbance after 1min for 6 minutes. Antioxidant activity measurements were carried out in triplicate. Ascorbic acid was taken as standard. The percent scavenging activity of the tested samples calculated using the formula,

Scavenging activity (%) = [(A – B) / A] x 100, where A is absorbance of ABTS and B is absorbance of ABTS and tested samples in combination.

Antibacterial assay

Collection and identification of bacteria

Bacterial strains including *Staphylococcus aureus*, *K. pneumoneae* and *P. aeruginosa* were used in the study. Bacterial strains were provided by microbiology department university of Malakand. These were identified by different biochemical tests and were preserved in freez-dried condition at 4°C in stab slant agar until later use (Barrow and Feltham 1993).

Standardization of bacterial suspension

Bacterial cultures were grown for 24 hours at 37°C and suspension with cell density of $1X10^8$ CFU/ml, were prepared using McFarland standard and were further diluted to a cell density of 1×10^6 CFU/ml using a UV visible spectrophotometer (Thermo electron corporation USA) at 625 nm. The standardization was maintained for the period of the study (Ayaz, Subhan *et al*., 2015).

Determination of bacterial susceptibilities

For the determination of antibacterial potential of CME and subsequent fractions, the samples of the plant material were prepared in a concentration of 10 mg/ml by dissolving 50 mg in 5 ml of methanol. The nutrient agar plates were prepared under laminar flow-hood and left for solidification. Then wells were formed by using sterilized cork borer. The plates were inoculated by the bacterial cultures using the sterilized swabs. The wells were filled with the plant extracts (100 µl in each well). For positive control ceftriaxone was added to the center of the nutrient agar plate. The Petri plates were incubated for 24 h at 37 °C. After incubation the Petri plates were checked for different zone of inhibition (mm) formed by the plant extracts. The experiment was performed in triplicate (Rahman and Rashid 2008).

Estimation of IC_{50} values

Median inhibitory values (IC_{50}) were calculated for enzyme inhibition and antioxidant activities using Microsoft Excel program.

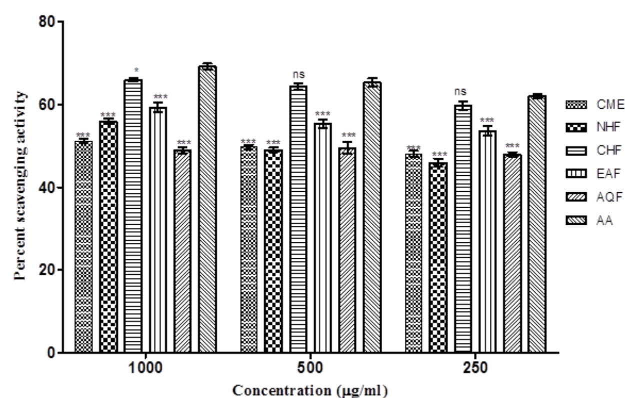

Fig. 1: Percent DPPH scavenging activity of various samples of *C. botrys.* The data noted were presented as mean ± SEM. Values significantly different as compare to positive control, *:P<0.05, ** :P<0.01, ***: P<0.001

STATISTICAL ANALYSIS

All experiments were performed in triplicate and values were expressed as were expressed as means ± S.E.M. One way ANOVA followed by multiple comparison Dunnett's test was used for the comparison of positive control with the test groups. The P values less than 0.05 were considered as statistically significant.

RESULTS

Phytochemical screening

CME of *C. botrys* was tested positive for the presence of saponins, tannins and flavonids while tested negative for alkaloids and glycosides as shown in table 1.

DPPH free radical scavenging effect

Fig. 1 shows the DPPH free radical scavenging effects of CME and subsequent fractions of *C. botrys.* Among the tested samples, CHF, EAF and NHF showed the highest DPPH scavenging activity with 65.9±0.41, 59.2±1.15 and 55.9±0.66% respectively at a concentration of 1mg/ml. Median inhibitory concentrations IC_{50} for different fractions were, CME (740), NHF (590), CHF (30), 140 and ascorbic acid (1460) µg/ml. All other tested samples showed moderate results as compared to the standard ascorbic acid. The order of percent DPPH scavenging activity all the samples was as CHF > EAF > NHF > CME > AQF.

Table 1: Results of phytochemical screening of *Chenopodium botrys*

Tests performed	Observations	Results
Alkaloids	Turbidity or colored precipitates formation	Negative
Flavonoids	A yellow color solution changed to colorless on acid addition	Positive
Tannins	A brownish-green or bluish-black color formation	Positive
Glycosides	Oxidation to anthraquinones	Negative
Saponins	1.stable froth formation 2.emulsion formation after olive oil	Positive
Anthraquinon	Red, violet or pink color formation in aqueous layer	Positive

Table 2: Antibacterial activity of various samples of *Chenopodium botrys*

Samples	*S. aureus*	*K. pneumoneae*	*P. aeruginosa*
Crude methanolic extract	11.2 mm ± 0.25	6.1 mm ± 0.31	15.2 mm ± 0.43
n-hexane	11.6 mm ± 0.65	5.3 mm ± 0.30	18.7 mm ± 0.31
Chloroform	19 mm ± 0.25	14.0 mm ± 0.35	25.4 mm ± 0.20
Ethyl acetate	14 mm ± 0.42	18.3 mm ± 0.30	27.1 mm ± 0.42
Aqueous	5.7 mm ± 0.64	10.0 mm ± 0.60	8.4 mm ± 0.40
Ceftriaxone	32.6 mm ± 0.57	30.0 mm ± 0.15	30.2 mm ± 0.25

The data noted were presented as mean ± SEM. Results are expressed as diameter of inhibitory zones (DIZ) in mm.

ABTS free radical scavenging effect

The ABTS free radical scavenging effects of all the tested samples of the plant are summarized in fig. 2. Among different fractions, EAF, CHF and AQF showed the prominent percent scavenging effect against ABTS free radical causing 85.46±0.53, 82.73±0.21 and 68.80±0 .57% inhibitions of free radicals at concentration of 1mg/ml displaying IC_{50} of 75, 94 and 530µg/ml respectively. All other fractions were effective in concentration dependent manner.

Table 3: IC50 for DPPH and ABTS results (µg/mL).

Fractions/ Samples	IC50 for DPPH (µg/mL)	IC50 for ABTS (µg/mL)
CME	740	840
NHF	590	590
CHF	30	94
EAF	140	75
AQF	1460	530
Ascorbic acid	22	8

Antibacterial effect

Investigating the antibacterial potentials of CME and subsequent fractions of *C. botrys,* it was found that CHF was most active against *S. aureus* with an inhibitory zone of 19±0.25mm. EAF and CHF showed strongest activity against *K. pneumoneae* with an inhibitory zone of 18.3± 0.30 and 140.35 mm respectively. Similarly EAF and CHF showed the strongest activity against *P. aeruginosa* with 27.10.42 and 25.40.20mm zones of inhibition respectively followed by NHF and CME with 18.70.31 and 15.20.43mm zones of inhibition respectively as shown in table 2.

DISCUSSION

Across the world, herbs have been used for the prophylaxis, treatment and cure of multiple disorders since long (Ullah, Subhan *et al*. 2015). The mere cause of its effectiveness is the presence of plethora of bioactive compounds (Kris-Etherton, Hecker *et al*. 2002). These bioactive compounds are grouped according to their chemical and physical properties. A specific group of compounds will have specific pharmacological properties and will be famous for those potentials (Vining 1990). For example flavonoids have been reported to possess strong antioxidant (Imran, Ullah *et al*. 2014; Ahmad, Ullah *et al*. 2015). Saponins have been reported to possess marked cytotoxic, anthelmintic and insecticidal potential (Ayaz, Junaid *et al*. 2014; Zeb, Sadiq *et al*. 2014; Ahmad, Ullah *et al*. 2015). In the current investigational study, it is evident from phytochemical screening that *C. botrys* possess almost all the major categories of bioactive compounds. So the pharmacological potential of this plant may be due to the presence these secondary metabolites.

The free radicals being responsible for multiple health anomalies have been investigated by numerous researchers to avoid their harmful effects. Most of the currently available radicals' scavengers are synthetic in nature and have been suspected to cause negative health effects. Hence, strong restrictions have been placed on their applications and there is a trend to replace them with naturally occurring antioxidants with high efficacy and safety levels. Additionally, these existing synthetic antioxidants are less soluble and show moderate antioxidant activity (Barlow 1990; Sadiq, Mahmood *et al*. 2015). The growing relevance of medicinal plants as possible sources for the discovery of novel antioxidant

molecules is often based on their long historical utilisation in folk medicine, especially in developing countries. In addition, the recognised health benefits of medicinal plants emanate from their prophylactic properties (Surveswaran, Cai *et al*. 2007). Investigating the CME and subsequent fractions of *C. botrys* for antioxidant potentials, it was found that CHF, EAF and AQF were most active against DPPH free radicals. Median inhibitory concentrations (IC_{50}) were also low for these fractions indicating that active compounds are concentrated in these fractions and can be subjected to isolation of pure compounds. While all other fractions showed moderate activity in concentration dependent manner. While for ABTS free radical scavenging, EAF, CHF and AQF were found with the highest percent inhibition effect and were most potent as indicated by their IC_{50} values. The other fractions also showed moderate percent scavenging activity.

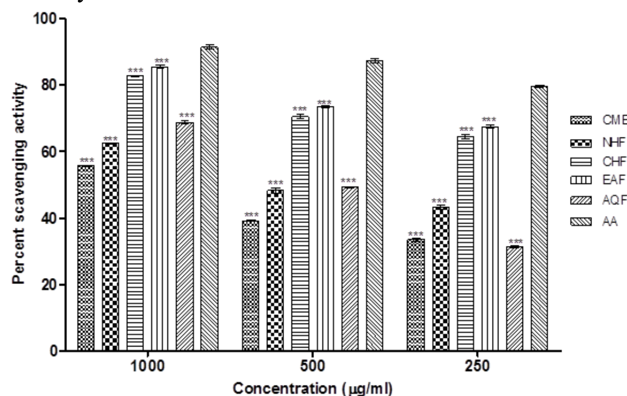

Fig. 2: Percent ABTS scavenging activity of various samples of *C.botrys.* The data noted were presented as mean ± SEM. Values significantly different as compare to positive control, *:P<0.05, ** :P<0.01, ***: P<0.001

Infectious diseases are the leading cause of death world-wide. Antibiotic resistance has become a global concern .The clinical efficacy of many existing antibiotics is being threatened by the emergence of multidrug-resistant pathogens and the unpleasant side effects associated with them (Westh, Zinn *et al*. 2004). Many infectious diseases have been known to be treated with herbal remedies throughout the history of mankind. Natural products, either as pure compounds or as standardized plant extracts, provide unlimited opportunities for new drug leads because of the unmatched availability of chemical diversity. There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases (Rojas, Bustamante *et al*. 2003). Therefore, researchers are increasingly turning their attention to folk medicine, looking for new leads to develop better drugs against microbial infections. While screening the CME and subsequent fractions of *C. botrys* for the antibacterial activity, all the samples showed activity towards all the bacterial strains used in this study. Among all fractions, EAF and CHF were most effective against *P. aeruginosa and K. pneumoneae* in comparison to the standard antibiotic used. Similarly CHF was most active against *S. aureus* followed by EAF and NHF as shown in table 2.

CONCLUSION

In a background of the ethnomedicinal uses and based on the results of this study, it may be concluded that *C. botrys* is rich in bioactive compounds which are responsible for their antioxidant and antibacterial potentials. This plant may be further investigated for the isolation and characterization of novel, cost-effective antioxidant and antibacterial compounds.

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