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Pharmacognostical, Antioxidant and Antimicrobial studies of aerial part of *Pulicaria crispa* (Family: Asteraceae)

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ABSTRACT

The study was designed to investigate the active phytochemicals present in the methanolic extract (ME) of *Pulicaria crispa* (*P. crispa*). The antimicrobial and antioxidant activities were also explored. These investigations were carried with an aim to use this plant in future. The morphological, microscopical and physicochemical investigation was carried out before the investigation of phytochemicals for authentication purpose. The antimicrobial activity was tested against Gram-positive, Gram-negative bacteria, *Aspergillus niger* and *Candida albicans*. Ampicillin was used as a standard for comparing the zone of inhibition. The in vitro 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and Ferric reducing ability (FRAP) models were used for the investigation of antioxidant activity. The results of the authenticated plant, phytochemical examination was shown the presence of biological active compounds. Marked antimicrobial and antioxidant activities were observed which may be due to the presence of tannins, phenols and flavonoids in the ME. The outcome of the present finding was suggested that it may be a good source of active antibacterial and antioxidant phytochemicals.

Keywords: Authentication, *Pulicaria crispa*, Phytochemical, Antimicrobial and Antioxidants.

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INTRODUCTION

The Plant *P. crispa*, family Asteracea, scattered all over the world. The family of *Pulicaria* species plant is generally divided into 12 sub-families and representing nearly 10% of all flowering plants [1]. The approximately 1,620 genera and 23,600 species are known in this family. The majority of Asteracea plants are herbaceous in nature [2]. Except, of the Antarctica continent, Astraceae family is distributed all over the world. *P. crispa* is used in traditional medicine for the treatment of various ailments [3]. The plant has been used for many years in conventional medicine for the cure of heart diseases and as gastroprotective, due to its antioxidative nature [4]. The oil of arial parts of *P. undulata* from Saudi Arabia was investigated by GC/MS and found it has rich in phenolic compounds and monoterpene hydrocarbons low in sesquiterpene [5]. Some of *Pulicaria* species have been used as galactagogues, antiepileptics, antimicrobial, antifungal and antioxidant due to presence of active phytochemicals [6,7]. This plant also used by the people of southern Egypt and Saudi Arabia to treat inflammation, insect repellent and as an herbal tea [8]. A phytochemical study of the *P. crispa* (Forssk.) shows the presence of sesquiterpenes [9]. The phytochemical investigation of the oils of some species showed the presence of monoterpenes, diterpenes and sesquiterpenes which showed antibacterial and antioxidant activities [10]. The use of antibiotics has been exercised since their innovation in the 20th century and due to the emergence of multi-drug resistant pathogens, it becomes a global challenge to the medical world to explore an active antibiotic against resistance [11]. It is now widely recognized that there is a need to develop

novel antibiotic agents to minimize the risk of antimicrobial resistance. The standardization of *P. crispacanth* is achieved by stepwise pharmacognostic studies. The detection and quality of the herbal medicine need to develop some standard parameters to ensure reproducibility for its safety and efficacy. The morphological and anatomical characters are a simple pharmacognostic method used for standardization of medicinal plant [12]. Commercially, phytochemical analysis of the medicinal plants is essential and has great importance in pharmaceutical companies for the manufacture of the new herbal drugs. Oxidative damage plays a key role in the progress of several human diseases. Generally, aerobic bacteria have redox enzymes that mediate oxidative phosphorylation with an oxygen molecule, produce reactive oxygen species (ROS) that play a significant pathological role in the various infections [13]. *P. crispacanth* wildgrown in Al-Kharj region, but till this time as per our knowledge not explored for their medicinal values. Hence, the present study was aimed to standardize pharmacognostical parameters such as macroscopic, microscopic and physicochemical evaluation. Later ME of *P. crispacanth* was further investigated for phytochemicals, antimicrobials and antioxidant activities.

MATERIAL AND METHODS

Plant

The *P. crispacanth*, aerial parts were collected from Alkharj-the central region of Saudi Arabia, in March 2015 and authenticated by Dr. Yousef Yaquob, College of Pharmacy, Department of Pharmacognosy, KSU, Al-Riyadh, Kingdom of Saudi Arabia. The voucher sample is reserved in College of Pharmacy Herbarium-Prince Sattam Bin Abdulaziz University for future reference. The plant materials were collected and proceeded to shade drying and milling to the fine powder before proceeding of further investigation.

Pharmacognostical studies

The macroscopic characters of plant leaves, stem, roots, flowers inflorescence and powder of dried aerial parts, were carried out and tabulated. The microscopy of leaf transverse sections (TS) was prepared by using chloral hydrate solution following the previous method [14]. The mounted slides were observed under the microscope (Inco-Ambala). The various physico-chemical parameters such as Ash content (total ash, water soluble ash, acid insoluble ash), moisture contents and the extractive values (hexane, chloroform, methanol and water) were carried out following the reported methods [15].

Methanolic extract (ME)

The *P. crispacanth* methanol extract was prepared by soaking 250 g dried powder samples in 2.5 L of methanol for 24 h in a percolator. The extract was filtered and evaporate under reduced pressure using rotary evaporators, recycle of collecting solvents in plant sample until the colour of sample become nearly colorless.

Phytochemical study

The ME was subjected to qualitative examination for the detection of various phytochemical constituents using reported procedures [16-17]. The qualitative identification of different phytochemicals was performed by employing Mayer's, Wagners and Dragendorff's reagent (alkaloids), Modified Borntrager's and Keller-Killiani (glycosides), Foam test (saponins), Salkowski's and Liebermann Burchard's Test (steroids and triterpenoids), Stain Test (fat and oil), ferric chloride (phenols and tannins), Lead acetate test (flavonoids) and Ninhydrin and Biuret (proteins).

Antimicrobial Studies:

The antimicrobial activity of ME was tested against different Gram positive and Gram negative bacteria and yeast and mold strains. The microorganisms were obtained from Department of Pharmaceutical microbiology, Prince Sattam Bin Abdulaziz University, Alkharj, Kingdom of Saudi Arabia.

Antimicrobial evaluation

Staphylococcus aureus (ATCC 29213), *Staphylococcus aureus* (clinical isolate), *Bacillus subtilis* (ATCC 10400) as Gram positive bacteria and *Escherichia coli* (ATCC 10536), *Klebsiella pneumoniae* (ATCC 13882), *Proteus vulgaris* (clinical isolate) as Gram negative bacteria were used for the antibacterial study. *Aspergillus niger* (ATCC 16404) and *Candida albicans* (NCYC 1363) were used for the anti-fungal study. These microbes were selected on the basis of pharmacological and clinical relevance [18]. The bacterial strains were developed in Mueller-Hinton agar (Oxoid, Basingstoke, England) plates at 37°C, and the fungus strains were subculture in PDA (Potato dextrose agar) media at 25°C and both the stock cultures were maintained at 4°C. The antibacterial and anti-fungal studies were investigated by the agar disk diffusion method [19].

The ME was fully dissolved in dimethyl sulfoxide (DMSO) and sterilized by filtration using a sintered glass filter, and stored at 4°C. The test sample (ME, 100 µg/mL) and the standard drug (Ampicillin, 10 µg/mL) were prepared in double distilled water (DDW). The sterile agar plates (Mueller-Hinton) were seeded with bacterial strains (10⁸ cfu) and allowed to reside at 37°C for 3 h. After solidification of agar, 5 mm holes were made with previously sterile borer. The test strains (0.25 mL) were inoculated in

the sterile media separately. The zones of growth inhibition were measured after 24 h of incubation at 37°C for bacteria and 48 h at 28°C for fungus [20]. The value less than 8 mm was considered as inactive against microorganisms.

Antioxidant evaluation

DPPH radical scavenging assay

The DPPH radical scavenging assay of ME was carried out according to previous method [21]. At 517 nm, DPPH radicals showed a strong absorption (Abs), and with reduction by an antioxidant compound indicated by colour changed from purple to yellow. A part (1 mL) from each of the different concentrations (10-1000 µg/mL) of ME or ascorbic acid (standard) was added to 1 mL of DPPH (1 mmol/L in methanol). It is incubated in a dark for 30 min, then there action mixtures were vortex and incubate in a dark for approximately 30 min. The absorbance was measured against control (DPPH having 1 mL of methanol without extract). Percentage radical scavenging activity of DPPH was calculated using the following formula:

Scavenging activity (%)

$$= [(control\ absorbance - sample\ absorbance) / (control\ absorbance)] \times 100.$$

IC₅₀ value was the effective concentration that could scavenge 50% of the DPPH radicals.

Ferric-reducing power assay

The reducing ability of the ME was determined according to the procedure [22]. 1 mL of the ME or standard (100 µg/mL) with different concentrations (250-2000 µg/mL) was mixed with 2.5 mL of phosphate buffer and 2.5 mL of potassium ferricyanide. The mixture was incubated at 50 °C for 20 min, then cooled. After that 2.5 mL Trichloroacetic acid (TCA), was added to the mixture and centrifuged at 3000 rpm for 10 min. The supernatant was mixed with FeCl₃ and distilled water. The absorbance was measured at 700 nm using UV spectrophotometer.

Statistical analysis:

All the experiment were taken in triplet. Some of data were presented as mean values ±SD and data was analysis of variance (ANOVA) using GraphPad Prism 6.

RESULTS

Pharmacognostical standardization

The macroscopic studies of the *P. crisper* were performed and tabulated (Table. 1). The results were shown that the plant contains green, oval-lanceolate leaves; stem branched from base with fibrous taproot. The flowers yellowish or white in colour with involucral bracts equally with ray and disc florete. The microscopic characters showed covering trichomes pollen grain with tuber cell, trichomes, spiral vessels and fibers (Fig.1). The section of leaf showed upper epidermis with cutical, palisade mesophyll, spongy mesophyll contain vascular bundles, lower epidermis (Fig. 2). The physico-chemical parameters were shown as, total ash (1.31%), water soluble ash (0.37%) and acid soluble ash (0.92%), moisture contents (1.95%) and the extractive contents was highest in distilled water (15.4%) (Table 2). The ME of *P. crisper* was subjected to the preliminary phytochemical studies and results were shown the presence of alkaloids, phenols, flavonoids and tannins (Table 3).

Antimicrobial Studies

The zone of inhibition of antibacterial studies was shown in Fig-3. The antibacterial activity of the ME was carried out and showed on *K.pneumoniae* (28 mm), *B. subtilis* (22 mm) and *E.coli* (21 mm) which are comparatively less, potent than Ampicillin. No considerable (≥ 8) action on the three strains *Staphylococcus aureus* (ATCC 29213), *Staphylococcus aureus* (clinical isolate), *Proteus vulgaris* (clinical isolate) were seen. An antifungal activity was carried out and mention in Fig-4, and it was found that ME solution has comparatively less inhibitory effect on *Aspergillus niger* (18 mm), when compared to the standard (23 mm).

Antioxidant evaluation

In DPPH assay, ME demonstrated a significant antioxidant potential in a concentration dependent manner (Fig.4). The higher percentage inhibition of ascorbic acid account, the better antioxidant potential, compare to ME. ME at concentration 10. 50. 100, 500 and 1000 µg/mL showed scavenging activities of 19.57, 41.40, 76.78, 81.23 and 79.84%, respectively, while ascorbic acid scavenging activities showed 48.65, 71.90, 84.62, 92.56 and 96.51%, respectively.

In present Ferric-reducing power assay (Fe^{3+} to Fe^{2+}) increased with the concentration of the extract. The reducing power of ascorbic acid (Standard) was found to be higher than ME. ME at concentration of 250. 500. 1000, and 2000 µg/mL showed average reducing activities of 0.555, 0.897, 1.744 and 2.618 respectively, while ascorbic acid average reducing activities showed 1.598. 1.878, 2.768 and 2.967 respectively (Fig. 5).

Table:1: *P. crispa* macroscopic study

S.N	Structure	Colour	Types
1	Leaf	Yellow-Green	oval-lanceolate
2	Stem	Yellow-	Branched from base
3	Root	Brownis	Fibrous Taproot
4	Flower	White & Yellowish	Involucral bracts (Ray floret=Disc floret)
5	Powder	Yellowish Brown	Coarse
6	Taste	NA	Astringent
7	Flavor	NA	Aromatic

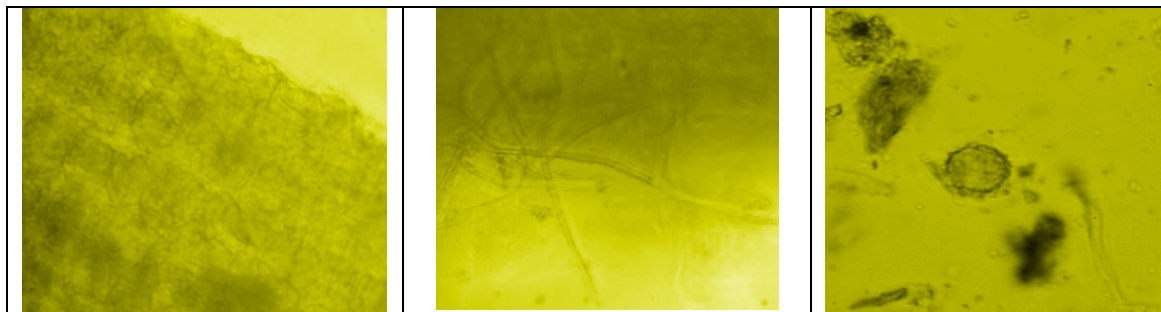
Table 2: *P. crispa*Physiochemical evaluations

Parameter	Result (Percentage)		Colour
Ash value	Total Ash (%w/w)	1.31	-
	Acid insoluble ash (%w/w)	0.92	
	Water soluble ash (%w/w)	0.37	
Percentage Moisture content	Moisture content	1.95	-
Extractive value	Hexane	1.5	White
	Chloroform	3.5	Light green
	Methanol	6.8	Green
	Distilled water	15.4	Dark yellow

Table:3: *P. crispap*preliminary phytochemical study

Phytoconstituent	Test	Result
Alkaloids	Mayer's Test	+
	Wagner's Test	++
	Dragendroff's test	+
Carbohydrates	Molisch's test	++
	Benedict's test	+
	Fehling's test	++
Glycosides	Modified Borntrager's Test	-
	Keller-Killiani test	-
Saponins	Foam Test	++
Test for steroids and Triterpenoids:	Salkowski's Test	++
	Liebermann Burchard's Test	++
Fats & oils	Stain Test	++
Phenols & Tannins	Ferric Chloride Test	++
Flavonoids	Lead acetate Test	++
Proteins & Aminoacids	Ninhydrin Test	+
	Biuret Test	+

+ = present; ++ = strongly present; - = absent.



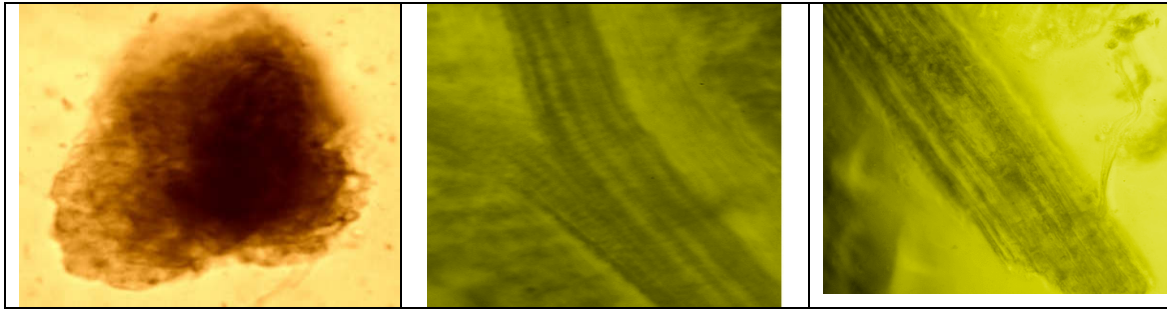


Fig. 1: Powder microscopy study (×40); 1=Epidermal cell, 2=epidermis containing covering trichomes, 3=Pollen grains, 4=Tannin containing cells, 5= spiral vessels, 6=Fibers

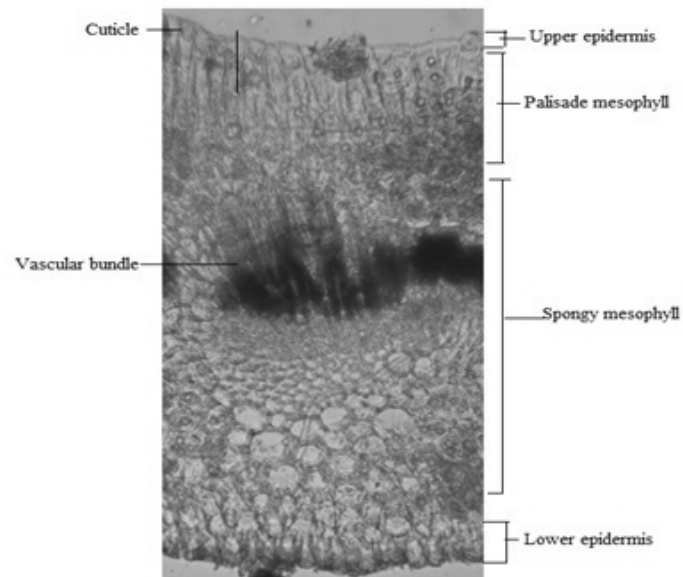


Fig. 2: *P. crispa* Leaf (T. S.) at 20X : Upper epidermis with cuticle, Palisade mesophyll, Spongy mesophyll with vascular bundle and Lower epidermis.

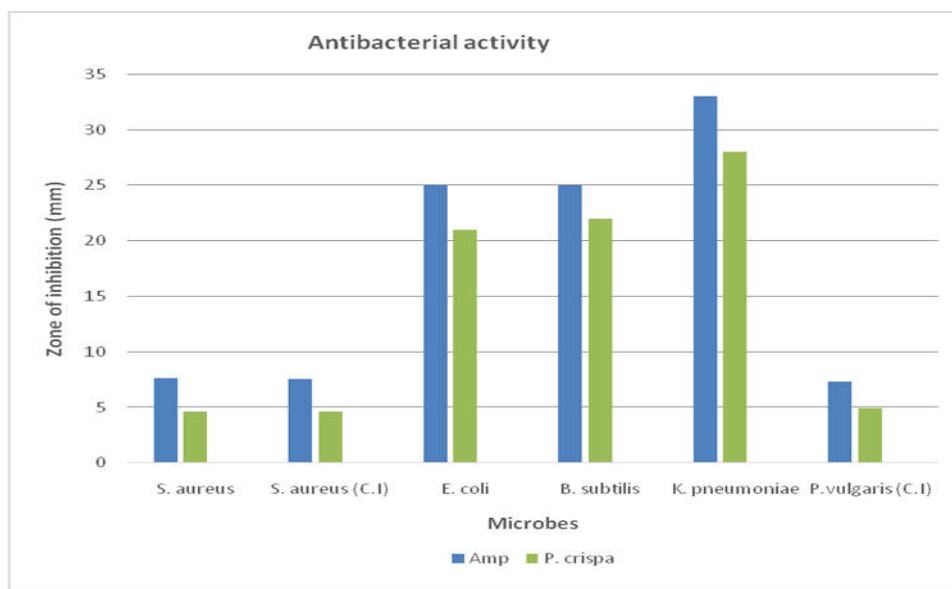
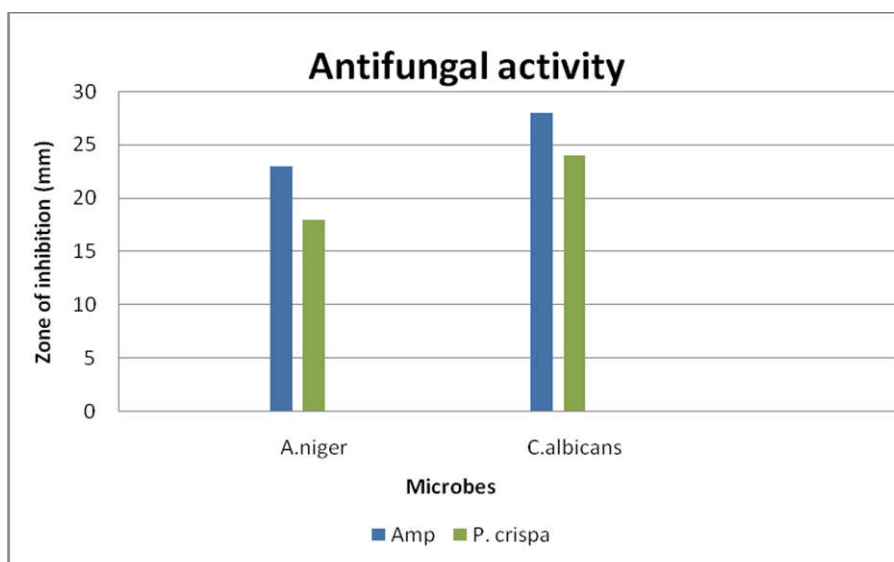
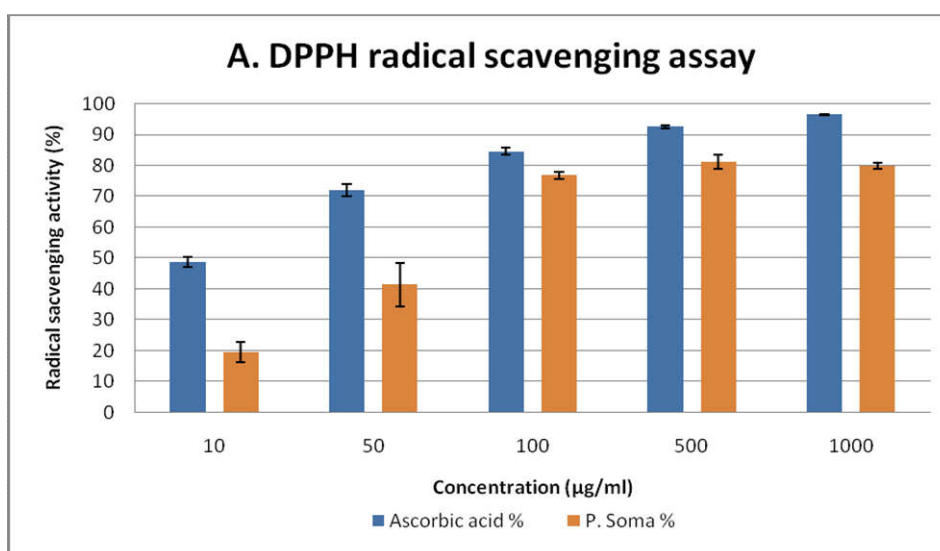
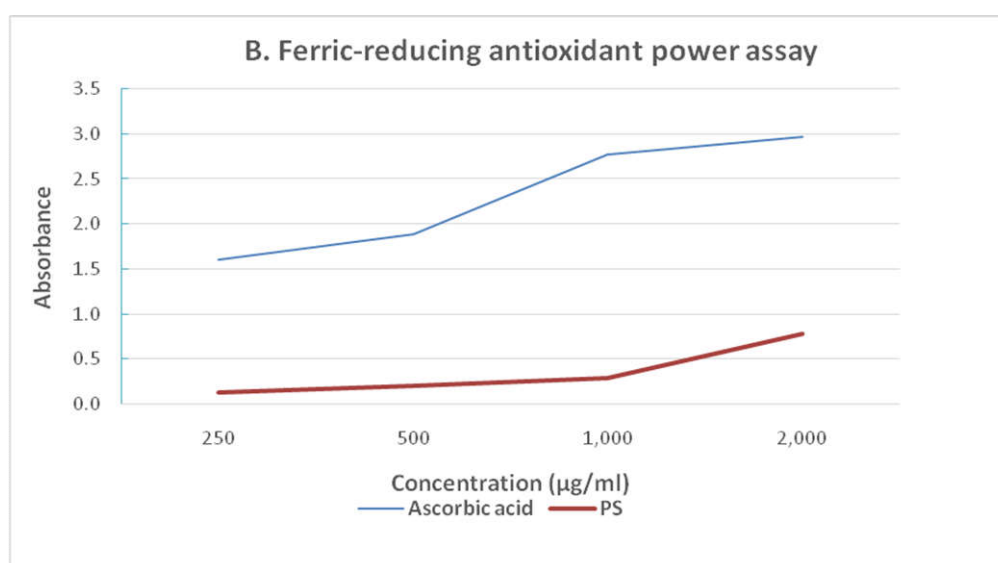


Figure.3: Antibacterial activity of *P. crispa*; where CI =Clinically isolated Munich, Amp= Ampicillin, NA= ≥ 8 (not active), mm= millimeter

Figure.4: Antifungal activity of *P. crispa*; mm= millimeter.Fig. 5: DPPH activity of *P. crispa* and ascorbic acidFig. 6: Reducing (Ferric) assay of *P. crispa* and ascorbic acid

DISCUSSION

Plant morphology is highly useful in visual identification of medicinal plant and also a first step of research work. In present study morphology of leaves, stem, roots and flowers were carried out and data mention is useful to visual investigation of plant and selection. It was found that the plant has yellow to green in colour with leaves: oval-lanceolate, stem: branch from base, root: fibrous and flower: involucre bracts (approximately Ray floret=Disc floret) which are characteristic features of the family Asteraceae[23].

The microscopic study is a cheapest and simplest method to identify the natural drug mainly leaf anatomy and powder of any parts or whole plant (herbs) are highly practiced [24]. In the present work microscopic evaluation of leaf was carried out and showed the typical structure with upper and lower epidermis with palisade and spongy parenchyma. Aerial part of *P. crispa* powder was studied in the present work. The microscopic characters of powder contain long covering trichome, fibers, spiral vessels and the specific structure of pollen grains. These microscopical studies revealed some of useful characters to the easily identification of this plant and also employed to detect the adulteration in future identifications because these old procedure is still one of the simplest and cheapest methods for identifying the adulterants [25]. The physicochemical parameters such as ash values used to find out quality, authenticity, and purity of powder drugs and these properties are important for quantitative standardization of such drugs [26]. Moisture content generally related to chances of microbial growth in crude drugs, generally less value of moisture content lesser chance to microbial growths during storage [27]. The extractive values are primarily useful for the detection of adulterated drugs and are useful to estimate the chemical constituents present in powder drug as well as assist in evaluation of solubility of drugs [28].

The phytochemical study was investigated as the presence of the active phytochemical present in ME mainly. These compounds are well known for their hepatoprotective, wound healing, cardioprotective, neuroprotective, anticancer, antimicrobial, antiviral and antioxidant effects[29]. The antimicrobial activity of *P. crispa* ME may be due to the presence of phytochemical compounds and the presence of these compounds usually justify the use of this plant for treatment of infections caused by pathogens [30]. The ME of *P. crispa* was showing an antibacterial and antifungal activity and the used microorganisms in present studies are previously well documented for different types of infectious diseases [31]. The *P. crispa* showed better inhibitory action on *C. albicans* than *A. niger*. The *Candida* species are the most common cause of fungal infections of a range fatal to severe skin diseases. *C. albicans* is the most common agent among them[32].

The compounds found in phytochemical study has been already evaluated for free radical scavenging and reducing power [33,34]. Antioxidant has a vital role in the prevention of most of human ailments. In the present work, the DPPH free radical antioxidative activity was employed to determine the scavenging potentials of *P. crispa* methanolic extract and showed that, change in colour during the reaction. The gradual decrease in rate of colour indicated the scavenging potentials of the ME. The scavenging activity of *P. crispa* ME may help in protective of body from several diseases [35]. The ferric reducing ability (FRAP) is a novel method for assessing antioxidant activity, in this ferric reduce to ferrous ion at low pH caused the formation of ferrous complex [36]. In present study ME of *P. crispa* subjected to determine the reducing potential. In FRAP, Ferric ion (Yellow) is changed into a ferrous-tripyridyltriazine complex (blue colored) during the reducing activity. In the complex mixture the absorption change was associated with the total reducing power [37] and may be linked with flavonoids and phenolic contents present in ME, the highest FRAP activity also signifying the presence of a richer flavonoids and phenolic compounds [38].

CONCLUSION

The Present work may help in identification and authenticate of *P. crispa* plant. The antimicrobial and antioxidant activities explore the medicinal potential of this plant due to presence of active constituents and suggested that, more research will required to isolate the active components and demonstrate for the possible biological evaluation.

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