

IN VITRO ANTIOXIDANT ACTIVITY OF EXTRACTS FROM THE LEAVES OF *FELICIA MURICATA* THUNB. AN UNDERUTILIZED MEDICINAL PLANT IN THE EASTERN CAPE PROVINCE, SOUTH AFRICA

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Abstract

Felicia muricata is a medicinal plant used for the management of different human and livestock diseases in the Eastern Cape Province of South Africa. The antioxidant potential of the leaves from this herb was investigated using its water, methanol, acetone and ethanol extracts. All the extracts were rich in phenols, proanthocyanidins and flavonols but low in flavonoids. The water extract exhibited low DPPH scavenging activity while the methanol, acetone and ethanol extracts showed higher activities. Again all the extracts showed high ABTS scavenging activity with a correlation between total phenolic content ($R^2=0.9965$), DPPH ($R^2=0.982$) and ABTS ($R^2=0.927$). Traditionally, however, plant extracts are prepared with water as infusions, decoction and poultice. Our results have shown that both the water and ethanol extracts from *Felicia muricata* displayed strong antioxidant activity. Therefore, it would seem likely that both solvents were able to extract those compounds which are responsible for the antioxidant activity of *F. muricata*.

Key words: *Felicia muricata*, antioxidant, DPPH, ABTS, radical scavenging activity.

List of abbreviations: BHT = butylated hydroxytoluene, DPPH = 1,1-diphenyl-2-picryl-hydrazyl, ABTS = 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, AIDS = Acquired Immune Deficiency Syndrome, $FeCl_3$ = ferric chloride.

Introduction

Medicinal plants typically contain several different pharmacologically active compounds that may act individually, additively or in synergy to improve health (Azaizeh et al., 2003; Gurib-Fakim, 2006). Bitters for example, are known to stimulate digestion while phenolic compounds could be responsible for anti-inflammatory and anti-oxidative activity of plant extracts (Torras et al., 2005; Afolayan et al., 2007; Diouf et al., 2009). The use of herbal remedies as alternative medicine plays a significant role in the cultures and beliefs of the indigenous populations of South Africa (Hutchings et al., 1996). However, there is dearth of information on the efficacy and more importantly, the toxicity of these remedies (Steenkamp et al., 2005). There is therefore, the need for more scientific validation of these claims.

Free radicals in the body contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS (Kumpulainen and Salonen, 1999; Pourmorad et al., 2006). Recently there has been an upsurge of interest in the therapeutic potentials of medicinal plants as antioxidants in reducing free radical induced tissue damages. Besides, well known and traditionally used natural antioxidants from tea, wine fruits, vegetables, spices and many other plant species have been investigated in the search for novel antioxidants (Koleva et al., 2002; Oke and Hamburger, 2002). There is still the demand to find information concerning the antioxidant potential of more plant species.

Felicia muricata Thunb. (Asteraceae), locally known as Ihbosisi in the Eastern Cape of South Africa, is a small drought resistant perennial aromatic herb growing up to 20 cm in height. The leaves are arranged in alternate fashion and are characterized by long and cylindrical non-glandular trichomes, tapering into a sharp point, all running parallel to the surface of the leaves in the direction of the leaf apex (Ashafa et al., 2008a). It is used by the people of the Eastern Cape Province in the management of headaches, stomach catarrh, pains and inflammation (Hutchings, 1989; Hutchings and van Staden, 1994; McGaw et al., 1997). Information gathered during our preliminary investigation on the local uses of the species also revealed its medicinal importance for the treatment of stomach ache and cancer. Extracts from the plant have been reported to show 80-90% inhibitory activity against cyclooxygenase, an important enzyme in the prostaglandin biosynthesis pathway (McGaw et al., 1997; Okoli and Akah, 2004), which may be responsible for the anti-inflammatory activity of this plant. Also, extracts from this herb have been found to possess strong antibacterial and antifungal activity (Ashafa et al., 2008b). Recently, its essential oil was found to be rich in limonene, alpha-pinene, beta-pinene, myrcene and cis-lachnophyllum ester (Ashafa et al., 2008c). These compounds have been reported to exhibit strong anticancer and antimicrobial activity (Sokmen et al., 2003; Deba et al., 2008). The aqueous extract from this herb was found to possess strong anti-inflammatory, antinociceptive and antipyretic activities in animal model (Ashafa et al., 2009a). Similarly, the aqueous extract had no significant effects on the haematology, liver and kidney functions indices of Wistar

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rats (Ashafa et al., 2009b). At the beginning of our study on the medicinal potential of this species, no information was available in the literature on its antioxidant activity. This paper reports on the antioxidant activity of *F. muricata* to further validate the use of the herb for the treatment of headache, pains and inflammation.

Materials and Methods

Plant materials

Plants used for this study were collected in August 2008 from several populations of *F. muricata* growing within the premises of Alice campus of the University of Fort Hare (33°11.10'S and 7° 10.60'E; altitude 695m). The mean annual rainfall of the area is about 700 mm and temperature range of 13 to 25°C. The species was authenticated by Mr Tony Dold, Selmar Schonland Herbarium, Rhodes University, South Africa. A voucher specimen (AshafaMed.2007/1) had been deposited in the Giffen Herbarium of the University of Fort Hare.

Extract preparations

The separated leaves were air dried at room temperature (30°C) and pulverized before extraction. Powdered plant material (40 g each) was separately extracted in acetone, methanol, ethanol and water for 48 h at 30°C, on an orbital shaker (Stuart Scientific Orbital Shaker, UK). The acetone, ethanol and methanol used were of high analytical grade. The extracts were filtered through Whatman no. 1 filter paper and the filtrate was evaporated to dryness under reduced pressure at 40°C using a rotary evaporator (Laborota 4000-efficient, Heidolph, Germany). The water extract was freeze-dried using Savant Refrigerated Vapor Trap, (RVT4104, USA). The freeze-dried extract was stored at 4°C before bioassay. The different extracts were re-dissolved in methanol to the required concentrations for the bioassay analysis.

Chemicals

All chemicals [(butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), gallic acid, rutin, catechin, quercetin, ferric chloride (FeCl₃), potassium ferricyanide and 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)] and reagents were analytical grades or purest quality purchased from Sigma, Aldrich (Johannesburg, South Africa).

Determination of total phenolics

Total phenol contents in the plant extracts were determined by the modified Folin-Ciocalteu method (Wolfe et al., 2003). An aliquot of the extract (known concentration) was mixed with 5 ml Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 ml (75 g/l) of sodium carbonate. The tubes were vortexed for 15 seconds and allowed to stand for 30 minutes at 40°C for colour development. Absorbance was read at 765 nm using a spectrophotometer (Beckman, DU 7400, USA). Extracts were evaluated at a final concentration of 1 mg/ml. Total phenolic content was expressed as mg/g gallic acid equivalent using the equation obtained from a calibration curve of gallic acid.¹⁸

Determination of total flavonoids

Total flavonoids were estimated using the method of Ordon-ez et al. (2006). Half a ml of 2% AlCl₃ ethanolic solution was added to 0.5 ml of the extracts. After one hour at room temperature, the absorbance was read at 420 nm. The development of yellow colour was taken as indication of the presence of flavonoids. Extract samples were evaluated at final concentration of 1 mg/ml. Total flavonoid content was calculated as quercetin equivalent (mg/g) using the equation obtained from the calibration curve.

Determination of total flavonols

The flavonols content was determined by the method of Kumaran and Karunakaran (2007). Twenty gram of aluminum trichloride and 50 g of sodium acetate anhydrous powder were separately dissolved in little quantity of distilled water and made up to 1.0 L with distilled water respectively. The rutin calibration curve was prepared by mixing 2 ml of varying concentrations of rutin (0.2 - 0.1 mg/ml) with 2 ml (20 g/L) aluminum trichloride and 6 ml (50 g/L) sodium acetate. The absorption at 440 nm was read after 2.5 h at 20°C. The same procedure was carried out with 2 ml of plant extract (0.1/ 1.0 mg/ml) instead of rutin solution. All determinations were carried out in triplicates. The flavonols content was expressed in rutin equivalents (mg/g) using the equation obtained from the calibration curve

Determination of total proanthocyanidins

Determination of proanthocyanidin was done using the procedure described by Sun et al. (1998). A volume of 0.5 ml of 1 mg/ml of extract solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid; the mixture was allowed to stand for 15 min for colour development. The absorbance was then measured at 500 nm. Total proanthocyanidin content was expressed as catechin equivalents (mg/g) using the equation based on the calibration curve.

Antioxidant activity assay

DPPH radical scavenging assay

The effect of extracts on DPPH radical was estimated using the method of Liyana-Pathiranan and Shahidi, (2005). A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this was mixed with 1.0 ml of different concentrations (0.02 – 0.1 mg) of the extract in methanol. The reaction mixture was vortexed thoroughly and left in the dark at room temperature 27°C for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. BHT was used as reference. The ability to scavenge DPPH radical was calculated by the following equation:

DPPH radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample}) / (Abs_{control})] \times 100$ where $Abs_{control}$ is the absorbance of DPPH radical + methanol; Abs_{sample} is the absorbance of DPPH radical + sample extract /standard.

ABTS radical scavenging assay

The method of Re et al. (1999), was adopted for this experiment. The stock solutions included 7 mM ABTS⁺ solution and 2.4 mM potassium persulfate solution. Varying concentrations of the plant extracts (1.0 ml) was allowed to react with 1 ml of the ABTS⁺ solution and the absorbance taken at 734 nm after 7 min using the spectrophotometer (Beckman, DU 7400, USA). The ABTS⁺ scavenging capacity of the extract was compared with that of standard (BHT) antioxidant and percentage inhibition was calculated thus.

ABTS radical scavenging activity (%) = $[(Abs_{control} - Abs_{sample}) / (Abs_{control})] \times 100$ where $Abs_{control}$ is the absorbance of ABTS radical + methanol; Abs_{sample} is the absorbance of ABTS radical + sample extract /standard.

Statistical analysis

The experimental results were expressed as mean \pm standard deviation (SD) of three replicates. EC₅₀ values (concentration at which 50% inhibition was achieved) were obtained from the regression plots. Where applicable, the results were treated to a one way analysis of variance (ANOVA) and the significant difference ($P < 0.05$) between means was determined by LSD using SPSS version 15.0 for Windows.

Results and Discussion

Total phenolic, flavonoid, proanthocyanidin and flavonol contents

The acetone leaf extract of *F. muricata* possessed the highest phenolic content when compared with methanol, ethanol and water extracts (Table 1). Generally, the phenolic content of all the extracts was considerably high, which could be a major contributing factor to the strong antioxidant activity of this plant extracts. According to Afolayan et al. (2007), high phenolic content of plant extracts could be responsible for their antioxidant activity. This is reflected in the correlation between the phenolic content ($R^2=0.9965$), ABTS⁺ ($R^2=0.927$), and DPPH ($R^2=0.982$). The study also revealed that all the extracts were rich in proanthocyanidins and flavonols but low in flavonoids. Proanthocyanidins are a group of flavonoids with strong antioxidant properties. They may reduce the risk of cardiovascular disease, cancer and protect against urinary tract infections (Howell, 2002; Steinberg et al., 2003). The strong antioxidant activity exhibited by the extracts from *F. muricata* could be attributed to the high phenols, proanthocyanidins and flavonols present in this species. Phenolic compounds are known for their antioxidant activity. Such activity is related to their redox properties in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Zheng and Wang, 2001; Miliauskas et al., 2004). They are also believed to have inhibitory effect on carcinogenesis. Flavonoids as one of the most diverse and widespread groups of natural compounds are probably the most important natural phenolics (Agrawal, 1989). These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties (Tung et al., 2007), such properties are especially distinct for flavonols (Miliauskas et al., 2004).

ABTS radical scavenging activity

Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm that decreases with the scavenging of the proton radicals (Mathew and Abraham, 2006). The

Table 1: Total phenolic, flavonoid, proanthocyanidin and flavonol contents of the shoot extracts from *Felicia muricata*. Data in $\bar{X} \pm S.D.$, each expressed per g of plant extract.

Extracts	Total phenol (mg gallic acid g ⁻¹)	Total flavonoids (mg quercetin g ⁻¹)	Proanthocyanidin (mg catechin g ⁻¹)	Total flavonols (mg rutin g ⁻¹)
Acetone	121.8 ± 0.03	42.0 ± 0.03	202.8 ± 0.004	266.3 ± 0.005
Methanol	62.2 ± 0.01	29.5 ± 0.02	118.6 ± 0.006	143.9 ± 0.01
Ethanol	60.4 ± 0.01	30.4 ± 0.03	137.2 ± 0.006	149.2 ± 0.01
Water	83.4 ± 0.03	25.4 ± 0.01	74.9 ± 0.02	41.6 ± 0.03

Table 2: EC₅₀ values and DPPH activity of the leaf extracts from *F. muricata*. Data in $\bar{X} \pm S.D.$

Leaf extracts	EC ₅₀ (µg/mL)	DPPH (%)
Water	-	17.57 ± 0.02
Methanol	70	93.92 ± 0.02
Acetone	410	59.89 ± 0.02
Ethanol	120	92.55 ± 0.03

extracts from *F. muricata* leaves were fast and effective scavengers of ABTS⁺ radical (Fig 1). This activity is comparable with that of BHT, the standard antioxidant used in this study. The percentage inhibition was 94.55%, 99.21%, 98.66%, 97.27% and 99.27% in water, methanol, acetone, ethanol and BHT respectively at 0.05 mg/mL, the highest concentrations tested. Higher concentrations of the extracts were more effective in quenching free radicals in the system. The high correlation between ABTS⁺ radical scavenging activity of the extracts and the total phenolic content ($R^2=0.9965$) implies that the phenolic compounds may contribute directly to the antioxidative action of these extracts.

DPPH radical scavenging activity

The effect of antioxidant on DPPH is believed to be due to their hydrogen-donating ability (Baumann et al., 1979). The DPPH assay measures the antioxidant activity of water soluble phenolics (Chun et al., 2005). Figure 2 shows the dose-response curve of DPPH radical scavenging activity of the different extracts of *F. muricata* leaves compared with BHT. It was observed that the methanol extract had the highest activity, followed by ethanol, acetone and water respectively. At the concentration of 0.1 mg/mL, the scavenging activity of methanol extract reached 69.36%, but at the same concentration, that of ethanol, acetone and water extracts were 42.85%, 13.81% and 6.65% respectively. Although the DPPH radical scavenging abilities of the extracts were significantly lower than that of BHT, it was evident that the extracts showed proton-donating ability and this could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants. The result also showed that the methanol extract had the least EC₅₀ value (Table 2). The lower the EC₅₀ value of a compound, the higher its radical scavenging activity (Maisuthisakul et al., 2007). Thus the methanol extract of this plant possesses the strongest ability to scavenge DPPH radical as compared to other extracts.

The scavenging of ABTS by the extracts was found to be higher than that of DPPH. Several factors like stereoselectivity of the radicals or the solubility of the extract in different testing systems have been reported to affect the capacity of extracts to react and quench different radicals (Yu et al., 2002). Similarly Wang et al. (1998), found that some compounds that have ABTS⁺ scavenging activity could not scavenge DPPH. A correlation between these two models was obvious in our study ($R^2=0.927$ ABTS⁺, $R^2=0.982$ DPPH). This further showed the potential of the extracts to scavenge different free radicals in different systems, indicating that they may be useful therapeutic agents for treating radical-related pathologic damage.

Antioxidants are important in the prevention of human diseases. Compounds with antioxidants activity may function as free radical scavengers, complexers of pro-oxidants metals, reducing agents, and quenchers of single-oxygen formation or reactive oxygen species, thereby protecting the body from degenerative diseases such as cancer. The reactive oxygen species (ROS) are harmful byproducts generated during normal cellular metabolism or from toxic insult. They lead to a state of oxidative stress that contributes to the pathogenesis of a number of human diseases by damaging lipids, proteins and DNA (Steenkamp et al., 2005). This has inspired much interest in antioxidant activity of phytochemicals. In this study, the choice of ethanol as an

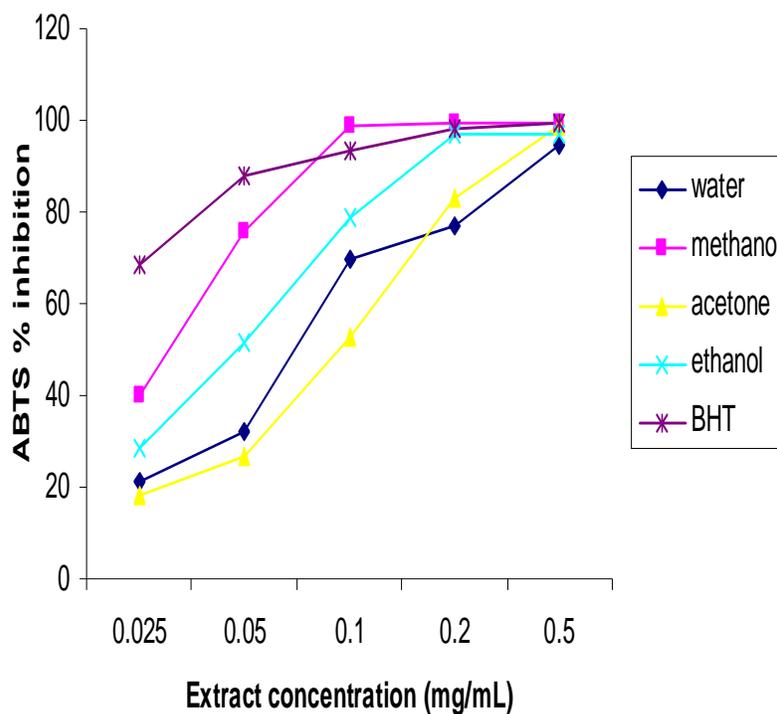


Figure 1: ABTS radical scavenging activities of extracts from the aerial parts of *F. muricata*

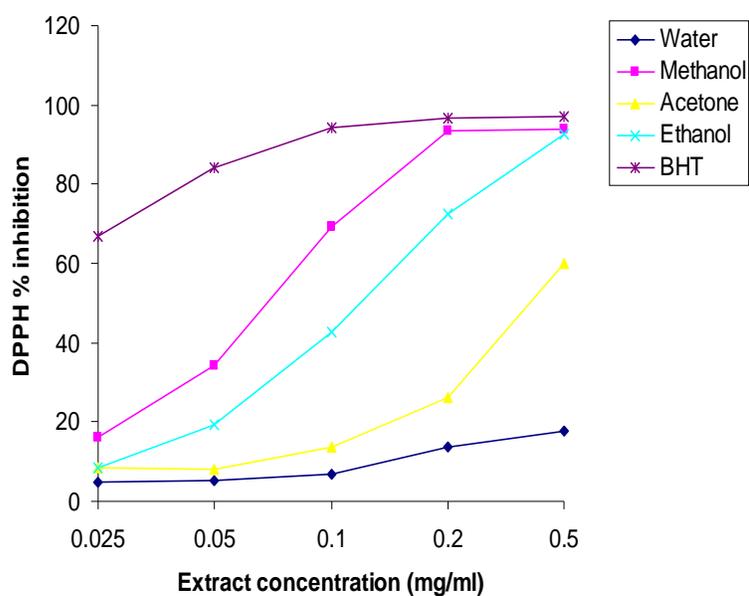


Figure 2: DPPH radical scavenging activities of extracts from the aerial parts of *F. muricata*

extractant was because of its availability and affordability at the time of this study. Traditionally, however, plant extracts are prepared with water as infusions, decoction and poultice. Our results have shown that both the water and ethanol extracts from *Felicia muricata* displayed strong antioxidant activity. Therefore, it would seem likely that both solvents were able to extract those compounds which are responsible for the antioxidant activity of the plant. This activity could partly explain why the plant is used in the traditional medicine practice of the Eastern Cape Province of South Africa.

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