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Exploring phenolic contents, antioxidant and in vitro anti-inflammatory activities of *Ficus hispida* L.f. leaves

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ABSTRACT

Ficus hispida L.f. (FH) is among the widely cultivated tree species in Asian regions. The aim of this work was to determine phenolic and flavonoid contents, antioxidant, in vitro anti-inflammatory activities of FH extracts prepared with methanol (ME), ethanol (ET) and ethyl acetate (EA). The extract obtained from ethyl acetate contained the highest total phenolic and flavonoid contents (92.75 ±5.14 mg GAE/g and 5.67 ±0.65 mg QE/g), followed by ME and ET. The capacity to scavenge ABTS free radical of the extracts followed the order: EA > ME > ET while their DPPH activity differed insignificantly. ME exerted significantly higher reducing power activity in comparison with ET and EA. The inhibitory effect of EA and ME on bovine albumin denaturation (IC_{50} = 362.84 ± 29.05 and 386.95 ± 11.10 µg/mL, respectively) were higher than that of ET. Correlation analysis showed a high positive correlation between total phenolic content and ABTS activity. The findings of the study would be useful for development of new nature-derived agents for prevention and treatment of diseases.

Keywords: *Ficus hispida, phenolics, antioxidant, anti-inflammatory, albumin denaturation*

1. Introduction

Ficus hispida L.f. (FH), belonging to the Moraceae family, is widely cultivated in India and other Asian countries (Cheng *et al.*, 2020). This plant has been used in traditional medicine to prevention and treatment of various diseases, such as diarrhea, hepatitis, ulcers, leukoderma and hemorrhage (Ali and Chaudhary, 2011). Research revealed that

FH exhibited multiple bioactivities, such as anti-inflammatory, hepatoprotective and cardioprotective effects (Mandal *et al.*, 2000; Shanmugarajan *et al.*, 2008; Anasane and Chaturvedi, 2017). It is suggested that these bioactivities are attributable to different classes of phytochemicals in the plant, such as triterpenoids, alkaloids and flavonoids (Shanmugarajan *et al.*, 2008; Shi *et al.*, 2016).

In Vietnam, FH, also known as “Ngái”, is commonly found in many provinces. The tree often grows along water sources in the margins of primary forests, the composition of secondary forests or hilly areas. In the lowlands, it often grows on banks of ponds or in bushes. All parts of the plants, particularly leaves, have been often used as folklore remedies in treatment of fever, malaria, furuncle, oedema, diarrheal food poisoning. Despite multiple medicinal uses of the plant, limited information about its chemical composition and bioactivities important to human health is available. In the present study, phenolic contents, antioxidant and anti-inflammatory properties of FH leaf extracts were investigated in hope that the findings will give a better understanding of how these chemical components contribute to FH’s health benefits and open a new way to develop this plant species as therapeutic agents in prevention and treatment of diseases.

2. Materials and methods

2.1. Chemicals

Folin – Ciocalteu reagent was obtained from Merck KGaA (Darmstadt, Germany). Organic solvents (99.5%, ACS grade) were purchased from Fisher Scientific (Pittsburg, Pennsylvania, USA). The two reagents ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and DPPH (2,2-diphenyl-1-picrylhydrazyl) were purchased from Sisco Research Laboratories (Maharashtra, India) and Sigma-Aldrich (Missouri, USA), respectively. Diclofenac (> 99%) was obtained from National Institute of Drug Quality Control (Hanoi, Vietnam).

2.2. Crude extracts:

Leaves of FH collected in gardens located in Ho Chi Minh city were carefully washed under a running tap water, followed by air-drying until the moisture ≤ 8%. The dried sample was powdered and extracted with various solvents (methanol, ethanol and ethyl acetate). In detail, the mixture of the sample and each solvent (1:10, g/mL) in a screw-capped tube was vortexed for 1 min, and then sonicated for 60 min to facilitate the extraction. Afterwards, the mixture was filtered through a Whatman filter paper, the filtrate was collected and evaporated under reduced pressure. The yields of the metanolic, ethanolic and ethyl acetate extracts were 7.9, 4.0 and 8.7%, respectively. The residue obtained was used for determination of total phenolic and flavonoid contents and estimate bioactivities. The study was carried out in summer of the year 2022.

2.3. Total phenolic and flavonoid contents:

Total phenolic (TPC) and flavonoid (TFC) contents of the crude extracts were estimated using the methods previously described by (Vu, 2022).

2.4. Antioxidant activities:

ABTS assay. A mixture (1:1, v/v) of 7 mM ABTS and 2.45mM potassium persulphate in phosphate-buffered saline was kept at 25 °C in darkness for 12 h. This mixture (3mL) was then allowed to react with 0.1mL of a diluted FH extract and its absorbance was measured at 734nm (Leung *et al.*, 2009).

DPPH assay. A solution of DPPH (40µg/mL) prepared in 80% methanol beforehand was mixed with a diluted FH extract (2:3, v/v), followed by incubation at 37 °C for 30 min in darkness. The absorbance was analyzed at 517 nm in a spectrophotometer (Xiao *et al.*, 2014).

Reducing power assay. A diluted FH extract (0.2mL) mixed with phosphate buffer (0.5mL) and potassium ferricyanide (1%, 0.5mL) underwent incubation for 20 min at 50 °C. After adding trichloroacetic acid (10%, 0.5mL), the mixture was centrifuged for 10 min. The supernatant (0.8mL) obtained was mixed with ferric chloride (1%, 0.4mL) in a tube containing water (2mL). The absorbance was analyzed at 700nm in a spectrophotometer (Xiao *et al.*, 2014).

Ascorbic acid was used as a reference standardin all the assays. The results were presented as mg ascorbic acid equivalents per g of extract (mg AAE/g).

2.5. Inhibition of bovine albumin

An FH extract in DMSO (5%) mixed with bovine serum albumin solution (0.16%) and sodium acetate buffer (pH 5.5) at a ratio of 1:1:2 (v/v) was incubated at 37 °C for 45 min, followed by heating to 67 °C for 3 min. The mixture was then cooled down to room temperature, and its absorbance was spectrophotometrically determined at 660nm. The percentage inhibition of albumin denaturation was calculated as follows:

$$\% \text{ Inhibition} = (A_0 - A_s)/A_0 \times 100\%$$

where, A_0 and A_s are the absorbance of the blank and sample/standard. Diclofenac was used as a reference standard. The results were shown as IC_{50} (µg/mL).

2.6. Statistical analysis

One-way analysis of variance (ANOVA) was performed on the data obtained to compare phenolic levels between the samples. Significance differences between mean values were determined with the Tukey HSD test ($p < 0.05$). Correlation analysis and principal component analysis were implemented using XLSTAT 2016 (Addinsoft, France). All the experiments were conducted in triplicate and the results were expressed as mean ± standard deviation.

3. Results and discussion

3.1. Determination of phenolic and flavonoid contents in FH

As seen in Table 1, the FH extract obtained with ethyl acetate (EA) had the highest phenolic content (92.75 ± 5.14mg GAE/g), followed by the methanolic extract (ME). The amount of phenolics in EA was approximately twice as much as that in the ethanolic extract (ET). In addition, EA contained a significantly greater TFC (5.67 ± 0.65mg QE/g) than the others. No significant difference in TFC between ME and ET was observed ($p > 0.05$). The results demonstrated that extraction solvents affected phenolic and flavonoid contents in the resultant extracts. Previously, TPC values of Egyptian *Ficus* species leaf extracts obtained with ethyl acetate ranged between 63.61 and 131.38mg GAE/g (Abdel-Hameed, 2009). In another study, leaf extract of *Ficus racemose* was shown to contain 22.81mg of quercetin equivalents per gram of extract (Sumi *et al.*, 2016). In general, the results of the present study revealed leaf extracts of FH were composed of comparable phenolic and flavonoid amounts with those of the other *Ficus* species reported in prior research. Phenolics are naturally occurring compounds which have attracted a lot of attention because of their potential health endorsing properties. The better understanding of these components could help develop therapeutic agents for prevention and treatment of ailments.

TABLE 1. Total phenolic and flavonoid contents of the FH extracts

	ME	ET	EA
TPC, mg GAE/g	61.46 ± 0.73 b	47.22 ± 1.33 c	92.75 ± 5.14 a
TFC, mg QE/g	3.27 ± 0.32 b	2.75 ± 0.08 b	5.67 ± 0.65 a

Data are presented as mean ± standard deviation. Different letters indicated statistically significant differences among the extracts ($p < 0.05$).

3.2. Antioxidant activity

Antioxidant compounds have the potential to scavenge radicals via hydrogen donation. In this research, antioxidant activity was estimated by measuring the capacity to scavenge ABTS and DPPH radicals in the presence of the extracts. As displayed in Figure 1, EA had the highest ABTS radical scavenging activity (3.62 ± 0.25mg AAE/g), followed by ME (2.81 ± 0.25mg AAE/g) and ET (1.85 ± 0.22mg AAE/g). Regarding the antioxidant activity determined by DPPH assay, no significant differences in the activity were noted among the three extracts ($p > 0.05$). Prior research showed extracts of two *Ficus* species collected in Portugal had the comparable antioxidant activity determined by these assays (Teixeira *et al.*, 2009). Furthermore, the study suggested that rutin, chlorogenic acid and psoralen could contribute to the activity of these species.

Reducing power activity was determined by transformation of Fe^{3+} to Fe^{2+} in the presence of the extracts. This assay is often employed to assess antioxidant activity of extracts rich in phenolics. In this study, the reducing power activity was evaluated at three different concentrations of the extracts (1, 2 and 4mg/mL) and the results are presented in Figure 2. The methanolic extracts all exerted significantly higher reducing power activity in comparison with the others prepared with ethanol and ethyl acetate.

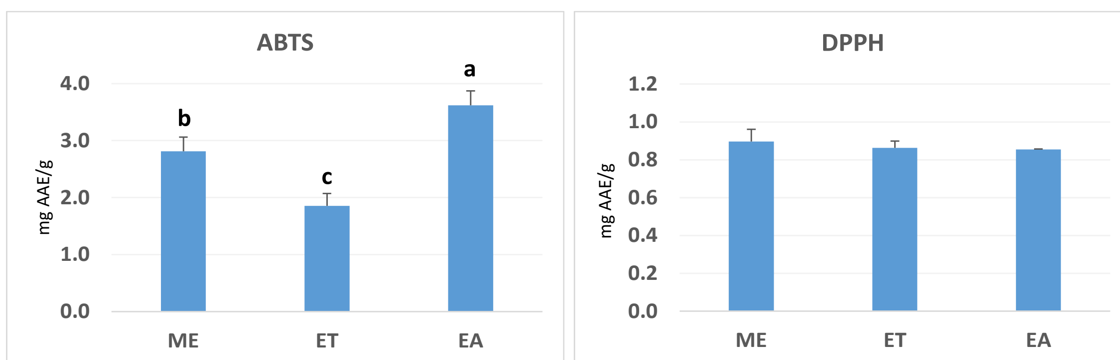

Figure 1. Antioxidant activity (mg AAE/g) of the extracts at the concentration of 4 mg/mL determined by ABTS and DPPH assays. Different letters show statistically significant differences among the extracts ($p < 0.05$).

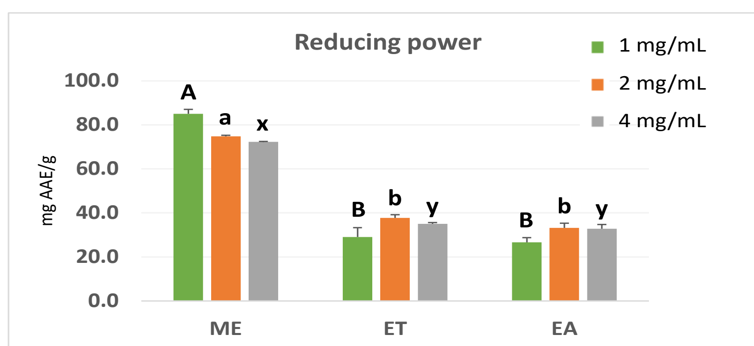

Figure 2. Antioxidant activity (mg AAE/g) determined by reducing power assays. Different letters show statistically significant differences among the extracts ($p < 0.05$). A, B, a, b, x and y correspond to the 1, 2 and 4mg/mL extracts.

3.3. In vitro anti-inflammatory activity

Heat is able to denature proteins, stripping these molecules of their physical, chemical and biological properties. Thus, the denaturation of proteins may be regarded as a marker for inflammation (Ruiz-Ruiz *et al.*, 2017). This research assessed potential anti-inflammatory properties of the FH extracts by predicting their ability to suppress bovine albumin denaturation. Figure 3 portrays the inhibition curves of the extracts on albumin denaturation. The results showed that ME and EA had significantly lower IC_{50} values (362.84 ± 29.05 and 386.95 ± 11.10µg/mL, respectively) compared to ET (530.06 ± 51.34 µg/mL). Statistically, no significant difference in IC_{50} was noted between ME and EA. This means that they possessed a significantly stronger inhibitory effect than ET did. The exact mechanism by which the extracts protected the protein from heat-induced denaturation is not clear. Perhaps, it could be due to interactions between their phenolic components, such as flavonoids, phenolics acid and tannins, and aliphatic regions surrounding lysine residue located on the albumin molecules (Williams *et al.*, 2008).

Prior research reported leaf extracts of FH reduced inflammation by about 64% in comparison with 45% of the positive control (diclofenac) (Vishnoi and Jha, 2004).

Vanillic acid present in FH leaves possessed an anti-inflammatory activity related to antioxidant effect and NF-κB-associated inhibition of pro-inflammatory cytokine release (Calixto-Campos *et al.*, 2015). In a review discussing pharmacological properties of FH, it was shown that most of the investigations into anti-inflammatory potential were focused on bark of the plant (Cheng *et al.*, 2020). The findings of the present study could hopefully contribute to our understanding of anti-inflammatory effects of FH leaves.

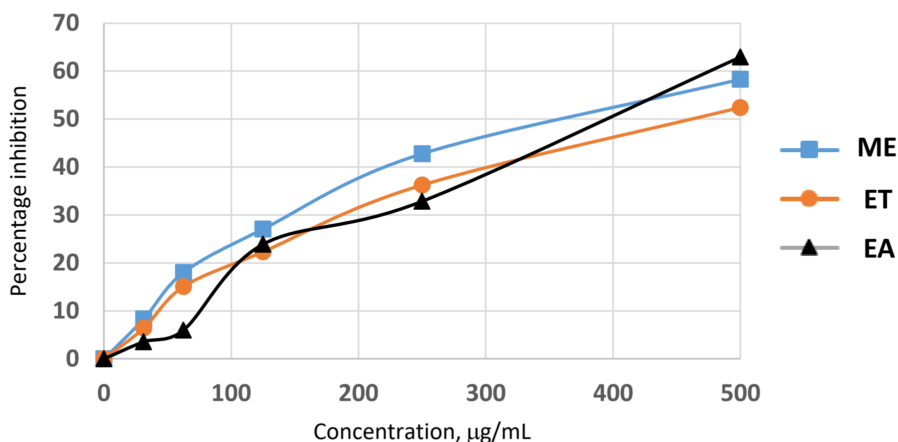

Figure 3. Inhibitory effect of the extracts on albumin denaturation

3.4. Correlation analysis

Pearson's correlation analysis was carried out to examine the relationships between TPC, TFC and bioactivities of the FH extracts. The results showed that TPC had a strong positive correlation with antioxidant activity estimated by ABTS assay (r = 0.938) (Table 2). These hint at possible contribution of phenolic constituents to radical scavenging activity of FH extracts. Beyond this, no relationships were observed between phenolic/flavonoids and the other bioactivities. Reportedly, the ABTS assay is based on the release of a blue/green $ABTS^{\bullet+}$, which is suitable for analyzing hydrophilic and lipophilic antioxidant components while the DPPH assay is more applicable to hydrophobic compounds (Kim and Lee, 2020). It is also noted that ABTS activity and inhibition of albumin denaturation were moderately positively correlated (r = 0.725).

TABLE 2. Pearson's correlation matrix

	TPC	TFC	ABTS	DPPH	RP*	Anti-inflammatory activity**
TPC	**1**	**0.709**	**0.938**	-0.189	-0.254	0.532
TFC		**1**	-0.840	-0.157	-0.487	-0.918
ABTS			**1**	-0.103	0.000	**0.725**
DPPH				**1**	0.440	0.002
RP					**1**	0.580
Anti-inflammatory activity						**1**

Values in bold are different from 0 with a significance level $\alpha = 0.05$

*: reducing power activity of the extracts at 4mg/mL.

**: reciprocal of IC_{50} was used to perform the correlation analysis.

3.5. Principal component analysis

Principal component analysis was carried out to graphically highlight the similarities and dissimilarities in data about phenolics/flavonoids and bioactivities of FH leaves. As illustrated in Figure 4, principal component 1 (F1) accounting for 56.88% of the total variability is associated with TPC, TFC and anti-inflammatory activity. This expounds the distant positioning of ET extract from the others. Principal component 2 (F2) formed by DPPH and reducing power activity explains 29.37% of the total variance.

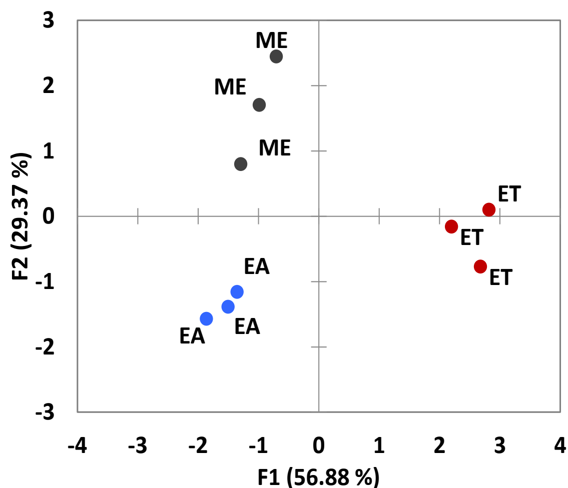

Figure 4. Principal component analysis of the data about phenolics/flavonoids and bioactivities of FH leaves.

4. Conclusion

In summary, phenolic and flavonoid contents, antioxidant and anti-inflammatory activities of FH leaf extracts obtained from various extraction solvents with different polarity were revealed. These extractants had influences on phenolics, flavonoids and bioactivities of the plant extracts. Ethyl acetate was much more effective in extracting phenolics. The extract obtained with ethyl acetate exhibited the highest ability to scavenge ABTS free radicals. Methanol and ethyl acetate extracts presented significantly higher capacity to inhibit bovine albumin denaturation compared to ethanol. Correlation analysis demonstrated a strong positive relationship between TPC and ABTS antioxidant activity. The implementation of principal component analysis facilitates a visible differentiation in phenolic, flavonoid contents and bioactivities among the extracts. The study improves our understanding of potential health endorsing properties of FH, helping development of nature-derived agents for disease prevention and treatment.

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