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In vitro phytochemical and antibacterial activity of traditional hemorrhoid herbal medicine

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Abstract. The phytochemical properties and antimicrobial activity of the herbal mixture used in folk medicine for thousands of years against hemorrhoids in aria Turkey, were highlighted in this manuscript. The aim of this study was to determine the *In vitro* phytochemical and antibacterial activity of traditional hemorrhoid herbal medicine. The results were as expected, it was not surprising, so the information from folk medicine was validated.
Keyword*: Hemorrhoids, herbal drugs, traditional treatment.*

Introduction

The uses of traditional herbal mixture in treatment of the patients have been widely discussed for over hundreds of years in the developing and developed countries [QAZI and MOLVI, 2016]. Regarding the use of herbal medicine, the first documents date back to 5.000 years in China [RIVERA *et al*., 2013].

Especially, herbal medicine has been used by 75–80 % of the world population for healing purposes in the developing countries because of being cheap, without side any side effects and locally available [PAL and SHUKLA, 2003].

The use of herbal medicines/mixtures for the therapeutic purpose has been declared increasing day by day by World Health Organization (WHO) [PAL and SHUKLA, 2003; BOADU and ASASE, 2017]. Despite of the development and spread of modern medicine, these approaches to the preparation and use of herbal medicine between folks are still in existence today [PAL and SHUKLA, 2003].

52 species belonging to 28 plant families were reported to use for remedy of 42 human diseases and ailments [BOADU and ASASE, 2017].

The global herbal market is estimated to be US $83 billion annually by the WHO [RIVERA *et al*., 2013].

Hemorrhoids (*Bavāsir*) are known to be swollen veins located around the anus or in the lower rectum and can be either internal or external.

In Turkey, about 50% of adults at the age of 50 are shown the symptoms of hemorrhoids. 241 plant taxa used as medicine against hemorrhoids in various regions of Turkey were reported [ERBAY and SARİ, 2018].

In this study, the phytochemical properties and antimicrobial activity of the herbal mixture used as medicine against hemorrhoids in Gaziantep and Sanliurfa, South–eastern Anatolia of Turkey are aimed to investigate.

Material and methods

Sample preparation

Herbal mixture which is used traditionally in hemorrhoid treatments in South–eastern Anatolia of Turkey, is prepared by 13 plants including *Curcuma longa*, *Zingiber officinale*, *Terminalia chebula*, *Aquilaria agallocha*, *Cortex cinchonae*, *Alpinia officinarum*, *Cinnamomum verum*, *Liquidambar orientalis*, *Poix regine*, *Pistacia lentiscus*, *Thuja*, *Myristica fragrans*, *Pinus halepensis resinae*.

This mixture in cream consistency was extracted by methanol, ethyl acetate, distilled water and hexane solvent (1:10

(w/v)) at room temperature for 3 days under shaking conditions.

After evaporation of solvents, samples were suspended in methanol at the 100 mg/mL final concentration. Phytochemical and antibacterial analyses were continued with 10, 25, 50, 75 and 100 mg/mL concentrations of this mixture.

Total antioxidant activity

The antioxidant activity of this traditional mixture was determined by DPPH Radical Scavenging Method. This method is based on the reduction of DPPH, a pink stable compound, and measuring the decrease in colour spectrophotometrically.

In brief, DPPH (0.025 g/L) in methanol was prepared. 3.9 mL of this solution was added to 100 µL of the extracts in methanol at different concentrations. After shaken vigorously, the mixture was incubated at room temperature for 120 min.

Then, absorbance (A) was measured at 515 nm by using spectrophotometer.

$$\%= [(A_{Control}–A_{sample})/A_{control})] \cdot 100$$

The inhibition of DPPH was calculated as percent by following equation [UCAN TURKMEN and MERCIMEK TAKCI, 2018. BUTNARIU and CAUNII, 2014].

Total phenolic compounds

Quantification of the total phenolic compounds in samples was analysed by colorimetric reaction of the Folin–Ciocalteu reagent. Based on the work of Stankovic [STANKOVIC, 2011], 0.5 mL of the per sample was mixed with 2.5 mL of 10% Folin–Ciocalteus reagent and 2.5 mL 7.5% $NaHCO_3$**.**

After shaking, the mixture was left at 45ºC for 45 min. in a water bath.

The reaction mixture was measured at 765 nm on a spectrophotometer. The absorbance results are expressed with gallic acid equivalent [BUTNARIU, 2014].

Total flavonoid contents

The flavonoids content of samples was detected by the aluminium chloride method using catechol as standard. After diluting with distilled water at the rate of 1:6, 1 mL of extracts was added to 0.3 mL of 5% $NaNO_2$ solution and this reaction mixture was allowed to stand for 5 min. Then 0.6 mL of 10% $AlCl_3.6H_2O$ solution was added to the mixture and incubated for 5 min. followed by adding 2 mL of 1 M NaOH solution. The final volume of the reaction mixture was adjusted to 10 mL with distilled water. After incubating 15 min. the absorbance of samples was measured at 510 nm with UV–VIS spectrophotometer. The total flavonoid content was calculated by a catechol calibration curve [SHARM and VIG, 2013].

Statistical analysis

In order to determine significant differences between the samples, the software SPSS 22.0 for Windows (SPSS Inc., Chicago, IL, USA) was performed variance analysis (ANOVA) and Tukey multiple comparison tests. Each spectrophotometric analysis was repeated at least three times.

Antibacterial activity

The antibacterial activity of this herbal mixture was researched by using Kirby–Bauer disk diffusion susceptibility test [BAUER *et al.*, 1966].

For this method, 7 clinical strains obtained from Kilis State Hospital (*Bacillus subtilis*, *Klebsiella* spp., *Shigella* spp., *S. aureus*, *E. coli*, *E. faecalis*, Methicillin resistance *S. aureus*) were tested. The density of cultures in growth Luria–Bertani (LB) broth for 24 h was adjusted to 0.5 MacFarland turbidity.

The antibacterial activity was performed on Mueller Hinton Agar.

The sterile antimicrobial blank discs impregnated with 20 µL of the extracts were strategically placed away from each other. Methanol solvent and erythromycin standard antibiotic were used as negative and positive control, respectively.

Following the incubation at 37 ºC for 24 h, the clear zones around the discs were evaluated as antibacterial activity.

Minimal Inhibition Concentration (MIC) of the extracts that showed antibacterial activity against test microorganisms was determined.

This analysis was performed based on fact that the lowest inhibitory concentration determines to effective on test microorganisms.

In this test, the 10, 25, 50 and 75 mg/mL concentration of the methanolic and distilled water extracts were investigated for their inhibitory effects against *B. subtilis*, *Klebsiella* spp., *Shigella* spp., *S. aureus*, *E. coli*, MRSA, *E. faecalis*.

Results and discussion

Phytochemical properties of herbal mixture extracts

Flavonoids, secondary metabolite of plants, are bioactive phenolic compounds. The antioxidant activity of these compounds is a result of mechanisms such as scavenging free radicals, suppressing ROS production via enzymes inhibition and protecting through antioxidant defences [MOHISENI, 2017].

Especially, these antioxidant activities of herbal resources are brought prospective clinical application areas for designing/developing effective synthetic drugs for human and animal treatments [GUHA et al., 2011].

In this present study, all quantitative examination of phytochemical analysis were found statistically significant (P<0.05). The highest DPPH radical scavenging activity was determined in the polar extracts (methanol and distilled water) by comparison with non–polar extracts (hexane and ethyl acetate).

According to results (Table 1), % inhibition values ranged from 2.87 to 86.67%. Methanol and distilled water extracts at the 100 mg/mL concentrations showed 86.67±0.06% DPPH inhibition.

Table 1.

Antioxidant activity (DPPH scavenging) of herbal extracts

Concentration \ Solvents	Methanol	Distilled water	Hexane	Ethyl acetate
10 mg/mL	79.02±0.32^{d}	79.91±0.06^{e}	2.87±2.74^{c}	75.45±1.98^{c}
25 mg/mL	83.74±0.06^{c}	83.61±0.06^{d}	2.93±0.00^{c}	82.65±0.13^{b}
50 mg/mL	84.25±0.06^{b}	84.25±0.06^{c}	12.63±2.81^{b}	85.91±0.45^{a}
75 mg/mL	86.22±0.26^{a}	86.16±0.32^{b}	25.77±3.70^{a}	86.54±0.06^{a}
100 mg/mL	86.67±0.06^{a}	86.67±0.06^{a}	28.70±1.79^{a}	86.61±0.00^{a}

*The presented dates are mean of triplicate determinations (n=3), ± standard deviation. The difference between the values expressed by the different symbols in table (a–e, a–d and a–c) is significant (p<0.05).

The amounts of total phenolic and flavonoid compounds extracted from herbal mixture were detected to change based on polarity of solvents. The maximum amount of phenolic compounds was observed as 4.301±0.16 mg GAE/g in methanol extract, followed by distilled water extract (2.825±0.166 mg GAE/g) (Table 2).

Table 2.

Total phenolic contents of herbal extracts

Concentration \ Solvents	Methanol	Distilled water	Hexane	Ethyl acetate
10 mg/mL	1.24±0.12^{d}	1.54±0.01^{c}	0.12±0.01^{d}	0.16±0.02^{d}
25 mg/mL	2.72±0.15^{c}	2.27±0.01^{b}	0.27±0.00^{c}	0.54±0.13^{c}
50 mg/mL	3.50±0.06^{b}	2.56±0.04^{a}	0.52±0.06^{b}	0.88±0.08^{b}
75 mg/mL	3.72±0.07^{b}	2.76±0.01^{a}	0.61±0.04^{b}	1.01±0.06^{a}
100 mg/mL	4.30±0.16^{a}	2.82±0.01^{a}	0.76±0.05^{a}	1.06±0.17^{a}

*The presented dates are mean of triplicate determinations (n=3), ± standard deviation. The difference between the values expressed by the different symbols in table (a–d and a–c) is significant (p<0.05).

Methanolic and aqueous extracts among the tested extracts had the highest total flavonoid content (1.34±0.042 and 0.86±0.033 mg RE/g) (Table 3).

The antioxidant activity of herbal mixture is dependent on the solubility of phenolic substances in tested solvents.

Due to hydrophobic characteristics of these compounds, the polarity of solvent plays an important role in increasing the extraction yield.

In current medical approaches, the treatment of hemorrhoids has recommended phototherapy because of

failure in management of hemorrhoids complications such as inflammation, bleeding, pain and defecation, discomfort.

According to this literature knowledge, the herbal mixtures, having anti–inflammatory, antioxidant, analgesic and wound healing, may be used as an effective medicine for hemorrhoid treatment [DEHDARI *et al.*, 2018].

In brief, the high antioxidant activity of our herbal mixture with hemorrhoids healing effects was indicated the large of pharmacological effects.

Table 3.

Total flavonoid compounds of herbal extracts

Concentration / Solvents	Methanol	Distilled water	Hexane	Ethyl acetate
10 mg/mL	0.24±0.01^{e}	0.16±0.01^{e}	0.14±0.01^{d}	0.19±0.04^{d}
25 mg/mL	0.55±0.02^{d}	0.30±0.01^{d}	0.24±0.05^{d}	0.26±0.01^{cd}
50 mg/mL	0.83±0.05^{c}	0.47±0.02^{c}	0.45±0.05^{c}	0.33±0.02^{c}
75 mg/mL	1.00±0.04^{b}	0.76±0.01^{b}	0.63±0.02^{b}	0.51±0.04^{b}
100 mg/mL	1.34±0.04^{a}	0.86±0.03^{a}	0.78±0.03^{a}	0.82±0.02^{a}

*The presented dates are mean of triplicate determinations (n=3), ± standard deviation. The difference between the values expressed by the different symbols in table (a–e and a–d) is significant ($p<0.05$).

Hemorrhoid healing properties of plant species consisted of this herbal mixture are separately reported in literature [ADAM *et al.*, 2017; GURUNG and DE, 2017; HASHEMPUR *et al.*, 2017; DEHDARI, 2018].

But the previous studies which may be compared to phytochemical and antibacterial activity of this mixture were not encountered.

However, apart from the topical management for hemorrhoid symptoms in the published literature, many herbal mixtures have been used orally.

Main reason of an oral treatment, flavonoid compounds in herbal mixture is the most common phlebotomic agent reducing venous capacity and capillary permeability, increasing vascular tone and having anti–inflammatory effect.

This herbal mixture used in hemorrhoid treatments in South–eastern Anatolia of Turkey, is consumed as an oral suspension.

Antibacterial activity of extracts

Ethyl acetate and hexane extracts not showed antibacterial activity against test bacteria.

Klebsiella spp was more resistance to all the herbal extracts (Table 4). Only methanolic extract was effective on *Bacillus subtilis* (Table 4).

Table 4.

Antibacterial activity of the extracts at the 100 mg/mL concentrations (in terms of mm)

Solvents	Methanol	Ethyl acetate	Hexane	Distilled water	Negative control	Erythromycin
B. subtilis	9	–*	–*	–*	–*	8
Klebsiella spp.	–*	–*	–*	–*	–*	–*
Shigella spp.	7	–*	–*	15	–*	11
S. aureus	10	–*	–*	10	–*	24
E. coli	7	–*	–*	9	–*	9
MRSA	8	–*	–*	14	–*	20
E. faecalis	10	–*	–*	7.5	–*	30

–*: Any inhibition zone was not observed on MHA plates.

Distilled water extract was active against test bacteria with average zone diameters ranging from 6 to 11 mm.

Methanolic extract had higher antibacterial activity against Gram–positive with MICs of 10 and 100 mg/mL (Table 5).

But this MIC value on Gram–negative bacteria was limited with 100 mg/mL. The Gram–negative bacteria were observed to be more resistance to the herbal extracts than the Gram–positive bacteria.

The mean MIC of methanolic and distilled water extracts against *E. coli* was 100 mg/mL.

So, these bacteria were relatively resistance to herbal mixture extracts. The average MIC of the methanolic extract against *S. aureus* and *E. faecalis* was ranging from 10 to 100 mg/mL with 6–10 mm zone diameters.

Table 5.

MIC values of methanol extract (in terms of mm)

Methanol extract	75 mg/mL	50 mg/mL	25 mg/mL	10 mg/mL
B. subtilis	7	–*	–*	–*
Klebsiella spp.	–*	–*	–*	–*
Shigella spp.	–*	–*	–*	–*
S. aureus	8	7	6.5	6
E. coli	–*	–*	–*	–*
MRSA	7	6	–*	–*
E. faecalis	9	7.5	7	7

–*: Any inhibition zone was not observed on MHA plates.

Distilled water extract against Gram–positive and Gram–negative bacteria had a high MIC of 50–100 mg/mL compared with the methanolic extracts (Table 6).

Besides, distilled water extracts was showed the highest inhibition zones with 15 mm zone diameter against *Shigella* spp.

The inhibition effects of aqueous extract at 100 and 75 mg/mL concentrations were higher than standard antibiotic.

Table 6.

MIC values of distilled water extract (in terms of mm)

Distilled water extract	75 mg/mL	50 mg/mL	25 mg/mL	10 mg/mL
B. subtilis	–*	–*	–*	–*
Klebsiella spp.	–*	–*	–*	–*
Shigella spp.	10	9	–*	–*
S. aureus	9	7	–*	–*
E. coli	–*	–*	–*	–*
MRSA	12	10	–*	–*
E. faecalis	7	6	–*	–*

–*: Any inhibition zone was not observed on MHA plates.

The management of infected hemorrhoids requires medical treatment such as antibiotic reducing complications.

Therefore, the methanolic and distilled water herbal extracts with antibacterial activity may be stated to reduce the risk of infection.

Conclusions

Because of the numerous antioxidant compounds of herbal mixtures, they are a potential candidate for designing/developing drugs.

However, our knowledge about the application areas and uses of this mixture is still limited.

Before herbal mixture is considered as a food additive in diet, bioactive substances of this mixture are need to examine.

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