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Cynara scolymus L. leaves, popularly known as globe artichoke, have cholagogue and choleric properties which are attributed to caffeoylquinic acid derivatives. Although, there are already pharmacopoeial methods for quality control of artichoke dry leaf extract, it only shows the assessment of chlorogenic acid determination. This study aimed to adapt and validate a method for simultaneous determination of neochlorogenic, chlorogenic, cryptochlorogenic, cynarin and isochlorogenic acids A and C in tablets or capsules of globe artichoke leaf extract herbal medicines. Also, it evaluated the content of these caffeoylquinic acid derivatives in three commercial products and leaf extract. The validation considered the parameters of selectivity, precision, linearity, quantification and detection limits and accuracy. The method proved to be selective, precise and accurate. Commercial products and leaf extract had significantly different concentrations of standard compounds which may bring an impact on therapeutic outcomes. The method proposed was suitable for the identification and quantification of caffeoylquinic acid derivatives in tablets and capsules and could be used in quality control, ensuring the safety and efficacy of commercially prepared herbal medicines.

Key words: Herbal medicines, quality control, *Cynara scolymus*.

INTRODUCTION

Over the last years, the search for natural treatments, including herbal medicines, has been growing due to the potential value for health and well-being. Commonly, these treatments are mistakenly considered to be risk-free, only because they are “natural”. However, adverse events from herbal medicines have been reported and therefore deserve attention. The World Health Organization (WHO) emphasizes the importance of providing traditional medicine with quality, safety, and

efficacy to ensure access to health for the entire population (Robinson and Zhang, 2011; WHO, 2013).

Several factors such as climatic variations, growing conditions, and extractive processes can cause a wide variability of the chemical substances contained in the leaves, which can contribute to alterations in quality, safety and efficacy of the herbal medicines. High-performance liquid chromatography (HPLC) allows the separation, identification and quantification of these

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Table 1. Mobile phase gradient.

Time (min)	Pump A (%)	Pump B (%)
0 – 1	92	8
1 – 20	92 → 75	8 → 25
20 – 33	75	25
33 – 35	75 → 0	25 → 100
35 – 40	0 → 92	100 → 8

substances, and thus it is a valuable tool for the quality control of these products (Tistaert et al., 2011; Govindaraghavan and Sucher, 2015; Applequist and Miller, 2013).

The plant species, *Cynara scolymus* L. is popularly known as globe artichoke and belongs to the Asteraceae family (Trópicos, 2016). This species is widely used in the traditional medicine for the treatment of digestive disorders (Boughrara and Belgacen, 2015; Nogueira et al., 2016; Lima et al., 2016; Moradi et al., 2016). The scientific literature mainly reports its effects on liver diseases and often associates its effects to the presence of phenolic compounds such as caffeoylquinic acid derivatives (Pereira et al., 2016; Mohamed et al., 2016; Colak et al., 2016; Mocelin et al., 2016).

Although, there are already pharmacopoeial methods for quality control of artichoke dry leaf extract, it lacks further information regarding the determination of compounds other than chlorogenic acid. This study aimed to adapt and validate a method for simultaneous determination of neochlorogenic, chlorogenic and cryptochlorogenic acids, isochlorogenic acid A and C, and cynarin in tablets or capsules of artichoke leaf extract herbal medicines. The used method was based on European Pharmacopoeia - 7th edition (2011) for identification and quantification of chlorogenic acid in the globe artichoke dry leaf extract. Also, this method was used to evaluate the content of caffeoylquinic acid derivatives in three commercial herbal products and a dry leaf extract of *Cynara scolymus* L.

MATERIALS AND METHODS

Reagents and standards

All solvents were HPLC grade purchased from Tedia® and Sigma-Aldrich®. The water was purified using a Milli-Q system (Millipore). The standards 3-O-caffeoylquinic acid (chlorogenic acid), 4-O-caffeoylquinic acid (cryptochlorogenic acid), 5-O-caffeoylquinic acid (neochlorogenic acid) and 1,5-di-O-caffeoylquinic acid (cynarin) were obtained from Sigma-Aldrich®; 3,5-dicaffeoylquinic acid (isochlorogenic acid A) and 4,5-dicaffeoylquinic acid (isochlorogenic acid C) were purchased from ChromaDex®.

Plant material

C. scolymus L. dried leaves (batch number: 1004154) were obtained from *Panizza Fitoterápicos* Laboratory.

Herbal medicines

Three commercial products (named A, B and C) were obtained from the local market in Brasilia, Brazil. The products presented 200, 335 and 300 mg of *C. scolymus* dry leaf extract per tablet or capsule, respectively. Products A and B were purchased in the tablet dosage form, while the product C was purchased in the capsule dosage form. The manufacturer did not provide information about the harvest and extraction processes.

Preparation of *Cynara scolymus* L. extract

The dried leaves of *C. scolymus* L. were powdered in a knife mill (Marcone, MA580) and submitted to infusion. The infusion was performed in the proportion of 1 g of the powdered dried leaves to 10 mL of distilled water at 70°C. The extract was filtered, cooled down and then lyophilized (SP Scientific, Advantage Plus XL-70).

HPLC analysis

HPLC analysis was carried out using the chromatograph LaChrom Elite (Hitachi, Tokyo, Japan) equipped with L2455 DAD detector, L2200 injector, L2130 pump and L2300 column oven. Data were obtained with EZChrom Elite software, version 3.3.2 SP1. The method was adapted from the quality control monography of artichoke dry leaf extract presented by European Pharmacopoeia - 7th edition (EDQM, 2011). It was equipped with a C18 column (250 x 4.6 mm, five µm) with the temperature maintained at 40°C, flow rate of 1.2 mL/min, injection volume of 25 µL and detection at 330 nm. The mobile phase was composed of a gradient of phosphoric acid 0.5% (pump A) and phosphoric acid 0.5% in acetonitrile (pump B) (Table 1).

Sample preparation

Commercial herbal medicines were prepared at a concentration of 4 mg/mL. Using not less than 20 tablets or capsules, an accurately weighed portion of the powder, equivalent to 100 mg of the labeled amount of artichoke dry leaf extract, was solubilized in 25 mL of a mixture of methanol and water (3:7). The solution was filtered through a 0.45 µm membrane filter.

C. scolymus L. leaf extract (LE) was prepared at a concentration of 5 mg/mL by solubilizing the extract in a mixture of methanol and water (3:7). Also, the solution was filtered through a 0.45 µm membrane filter.

Standard solution

All standard solutions were freshly prepared at the concentration of 25 µg/mL in methanol.

Method validation

The method was validated considering the parameters of selectivity, precision, linearity, quantification and detection limits and accuracy. ICH guidelines and Brazil's regulations were used as reference (ICH, 1996; Brazil, 2003).

Selectivity

Selectivity evaluation was performed in triplicate by acidic and basic hydrolysis induction. Samples were solubilized in hydrochloric acid (HCl) 1 M or sodium hydroxide (NaOH) 1 M and incubated at 60°C

Table 2. Peak areas variations of samples with acidic and basic hydrolysis compared to samples without hydrolysis

Peaks	Acidic hydrolysis	Basic hydrolysis
Neochlorogenic acid	- 20.21%	CD
Chlorogenic acid	- 90.31%	CD
Cryptochlorogenic acid	- 45.99%	CD
Cynarin	+ 18.10%	- 66.25%
Isochlorogenic acid A	CD	CD
Isochlorogenic acid C	CD	CD

*CD: Complete degradation.

for 1 h in a water bath. After cooling to room temperature, samples were neutralized and diluted in methanol to a concentration of 4 mg/mL. The method selectivity was given by the comparison of retention time and peak areas related to samples with and without hydrolysis.

Linearity

The linear relation between peak areas and concentration of markers were performed by three analytic curves with six concentrations for each standard compound. Samples were prepared at concentrations of 1, 2.5, 5, 10, 25 and 50 µg/mL. The linearity equations were calculated by linear regression, using the software GraphPad Prism® Version 6.0.

Limit of detection (LOD) and limit of quantification (LOQ)

Both LOD and LOQ were determined from slope (s) and standard deviation of Y-intercept (SD) of the linearity curve, according to the following equations:

$$LOD = \frac{SD \times 3}{s} \quad LOQ = \frac{SD \times 10}{s}$$

Precision

Precision was determined by repeatability (intraday precision). Six replicates of the same sample (4 mg/mL) were analyzed on the same day. Precision was expressed as relative standard deviation (RSD), not accepting results higher than 5% (Brasil, 2003).

Accuracy

The accuracy was performed by standard addition method with three concentrations (80, 100 and 120%) in triplicate. These concentrations were achieved with 500 µL of artichoke herbal medicine sample added to 500 µL of a mix containing all markers at the concentrations required to obtain the final theoretical concentration of 80, 100 and 120%. Accuracy was expressed as the ratio between the determined concentration mean and the corresponding theoretical concentration.

Evaluation of commercial products and leaf extract

Three commercial products and the leaf extract were evaluated for the presence of the caffeoylquinic acid derivatives, using the validated method. Identification of the markers was carried out by

comparing the retention times and UV spectra of the peaks presented in the sample with the peaks of the standards. The amount of each marker in the sample was calculated two different ways: expressed as chlorogenic acid, calculated by applying the peak areas in the formula obtained by the linearity curve of chlorogenic acid; and calculated by applying the peak areas of each marker in the formula obtained by the linearity curve of the corresponding standard.

Statistical analysis

All the statistical analyses were performed using the software GraphPad Prism® 6.0. Data were expressed as mean ± standard deviation (SD). The one-way ANOVA followed by Tukey post-test was carried out to compare the content of markers between the commercial products. Results with p -value less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Selectivity

The induction of acidic and basic hydrolysis led to partial or complete peak degradation (Table 2) and formation of degradation products, but there was no co-elution of these products with the peaks of interest. Although, the cynarin peak presented an area increase after acid hydrolysis, this growth showed no statistical significance ($p > 0.005$ at t-Student test). These results demonstrate the ability of the method to accurately measure a compound in the presence of others substances or degradation products.

Linearity and limit of detection (LOD) and limit of quantification (LOQ)

The peak area versus concentration of each marker showed a linear response in a range of 1 to 50 µg/mL. The correlation coefficients (r) were higher than 0.99, as required by the guidelines. The limits of detection and quantitation represent the lowest amount of the markers which can be detected and quantified, respectively (Table 3).

Table 3. Linearity, limit of detection (LOD) and limit of quantification (LOQ).

Markers	<i>r</i>	Equation	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
Neochlorogenic acid	0.997	$Y = 191859x - 103767$	1.05	3.49
Chlorogenic acid	0.999	$Y = 203957x - 17409$	0.20	0.68
Cryptochlorogenic acid	0.995	$Y = 268182x + 13926$	0.74	2.47
Cynarin	0.996	$Y = 355651x + 105198$	0.27	0.89
Isochlorogenic acid A	0.998	$Y = 516598x - 17972$	0.73	2.44
Isochlorogenic acid C	0.998	$Y = 343767x + 48808$	0.80	2.66

Table 4. Method precision.

Markers	RSD (%)
Neochlorogenic acid	1.65
Chlorogenic acid	2.22
Cryptochlorogenic acid	0.90
Cynarin	1.96
Isochlorogenic acid A	2.94
Isochlorogenic acid C	3.31

RSD: Relative standard deviation.

Precision

All values of intra-day precision, performed on the same day, with the same analyst and same instrumentation, were within acceptable limits with relative standard deviation not greater than 5%, demonstrating the method repeatability (Brasil, 2003). Results are presented in Table 4.

Accuracy

The accuracy, which represents the agreement between the obtained results and the accepted true value, was performed by the standard addition method. Regarding the standards chlorogenic acid, cryptochlorogenic acid, cynarin, isochlorogenic acid A and isochlorogenic acid C, the accuracy values were within the acceptable limits with relative standard errors not greater than 15%. However, relative standard error values of neochlorogenic acid were between 17 and 19%, which are higher than the acceptable limits. Therefore, the method has no proper accuracy for this standard. The results are shown in Table 5.

Evaluation of commercial products and dry leaf extract

After method validation, it was possible to obtain the chromatographic profiles, identify and quantify the caffeoylquinic acid derivatives in commercial globe artichoke herbal medicines and globe artichoke leaf

extract (LE). Caffeoylquinic acid derivatives were identified by retention times and UV spectra (Figure 1). The chromatographic profile of LE showed a major peak corresponding to chlorogenic acid, minor peaks corresponding to neochlorogenic acid, cryptochlorogenic acid, isochlorogenic acid A and C and other minor peaks of unidentified substances (Figure 2). All commercial products presented neochlorogenic, chlorogenic, cryptochlorogenic, isochlorogenic acids A and C. However, commercial product B had more peaks in relation to the other products and was the only one that presented cynarin (Figure 3).

The literature often associates the therapeutic effects of the leaves of *C. scolymus* with cynarin, emphasizing it as the main compound of the species. However, these studies were carried out mainly in European Countries (Pagano et al., 2016; Petrovic et al., 2008; Falco et al., 2015; Kaymaz et al., 2017). In this study, the absence of cynarin was noted in the leaf extract and two of the three commercial herbal medicines. Also, previous studies performed with globe artichoke leaves grown in Brazil reported only traces of cynarin (Noldin et al., 2003; Costa et al., 2005). Moreover, the high chemical instability of cynarin makes it difficult to detect it in the final product (Guimarães et al., 2007; Carvalho et al., 2004). Further studies are needed to elucidate the presence of cynarin in globe artichoke leaves grown in Brazil, and the therapeutic implications of its absence.

Regarding the concentration of chemical markers, a significant difference was observed among the herbal medicines and mainly between the herbal medicines and the leaf extract. The leaf extract showed a significantly higher amount of chlorogenic acid and, therefore, a higher total amount of caffeoylquinic acid derivatives. The commercial herbal medicine B showed a proportion of chemical constituents closer to the leaf extract in relation to the others products (Table 6). These differences found in the chemical composition can be attributed to several factors including harvesting, climatic variations and extractive process (Tistaert et al., 2011; Govindaraghavan and Sucher, 2015; Applequist and Miller, 2013). Therefore, it is important to establish the standardization of extracts with the proper foundation of safety and efficacy.

Although, information regarding the extractive process is necessary for the registration of the herbal medicine by

Table 5. Accuracy and relative standard error (E%).

Theoretical concentration ($\mu\text{g/mL}$)	Determined concentration ($\mu\text{g/mL}$)		Accuracy (%)	E (%)
	\pm SD			
Neochlorogenic acid				
5.38 (80%)	6.34 \pm 0.09		117.83	17.83
6.72 (100%)	8.05 \pm 0.03		119.00	19.00
8.06 (120%)	9.47 \pm 0.04		117.53	17.53
Chlorogenic acid				
33.12 (80%)	35.86 \pm 0.13		108.26	8.26
41.40 (100%)	46.33 \pm 0.04		111.90	11.90
49.69 (120%)	54.71 \pm 0.18		110.11	10.11
Cryptochlorogenic acid				
2.64 (80%)	2.62 \pm 0.07		99.08	0.92
3.30 (100%)	3.37 \pm 0.003		102.12	2.12
3.96 (120%)	4.02 \pm 0.002		101.53	1.53
Cynarin				
1.10 (80%)	1.23 \pm 0.005		111.82	11.82
1.37 (100%)	1.53 \pm 0.005		111.40	11.40
1.64 (120%)	1.77 \pm 0.004		108.08	8.08
Isochlorogenic acid A				
17.77 (80%)	14.78 \pm 0.05		83.16	16.84
22.21 (100%)	19.10 \pm 0.02		86.00	14.00
26.66 (120%)	23.16 \pm 0.09		86.88	13.12
Isochlorogenic acid C				
8.22 (80%)	7.98 \pm 0.01		97.11	2.89
10.28 (100%)	10.09 \pm 0.01		98.20	1.80
12.33 (120%)	11.90 \pm 0.01		96.55	3.45

Determined concentration represented by mean \pm standard deviation (SD). Accuracy was expressed as the ratio between the determined concentration mean and the corresponding theoretical concentration.

Table 6. Concentration of markers per 100 mg of extract.

Markers	Concentration (mg/100 mg of extract)			
	A	B	C	LE
Neochlorogenic acid	0.195 \pm 0.011	0.122 ^b \pm 0.010	0.208 \pm 0.005	0.079 ^a \pm 0.007
Chlorogenic acid	0.373 \pm 0.019	1.608 \pm 0.147	0.412 \pm 0.012	6.000 ^a \pm 0.485
Cryptochlorogenic acid	0.116 \pm 0.005	0.134 \pm 0.012	0.148 \pm 0.004	0.113 \pm 0.013
Cynarin	-	0.066 \pm 0.004	-	-
Isochlorogenic acid A	0.080 \pm 0.002	0.582 ^c \pm 0.046	0.078 \pm 0.003	0.365 ^a \pm 0.034
Isochlorogenic acid C	0.101 \pm 0.004	0.168 \pm 0.016	0.105 \pm 0.003	0.051 ^a \pm 0.005
Total	0.865	2.68	0.95	6.61

Concentration of markers per 100 mg extract. Data represented by mean \pm standard deviation (SD). LE: Leaf extract. Comparisons carried out by one-way ANOVA followed by Tukey post-test. a: different from A, B and C ($p < 0.05$); b: different from C ($p < 0.001$); c: different from A and C ($p < 0.005$).

the regulatory agency in Brazil (Anvisa), they are not usually provided transparently by the industry. Therefore, this information should be made available to health professionals in the commercial product professional leaflets, which could strengthen the confidence of

professionals in prescribing herbal medicines (Evans and Avila, 2016).

The results were also expressed as chlorogenic acid (Table 7), to allow the comparisons with the requirements established by Anvisa. According to Anvisa's regulation,

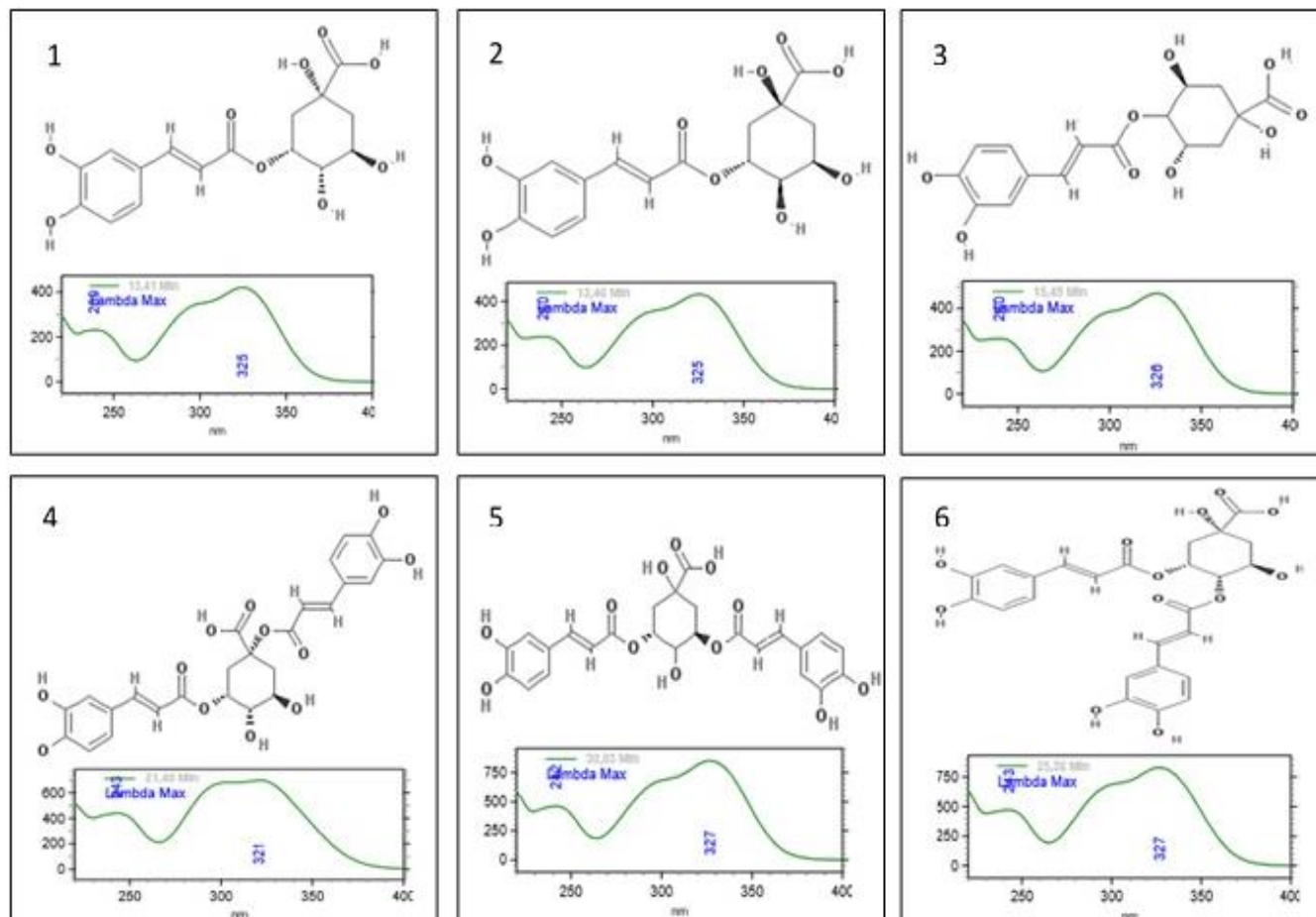


Figure 1. UV Spectra at 330 nm and chemical formulas. 1: Neochlorogenic acid; 2: chlorogenic acid; 3: cryptochlorogenic acid; 4: cynarin; 5 isochlorogenic acid A; 6: isochlorogenic acid C.

Table 7. Concentration of markers per 100 mg of extract expressed as chlorogenic acid.

Markers	Concentration (mg/100 mg of extract)			
	A	B	C	LE
Neochlorogenic acid	0.173 ± 0.010	0.104 ^b ± 0.009	0.185 ± 0.005	0.066 ^a ± 0.007
Chlorogenic acid	0.373 ± 0.019	1.608 ^c ± 0.147	0.412 ± 0.012	6.000 ^a ± 0.485
Cryptochlorogenic acid	0.156 ± 0.007	0.180 ± 0.015	0.198 ± 0.005	0.151 ± 0.018
Cynarin	-	0.130 ± 0.006	-	-
Isochlorogenic acid A	0.203 ± 0.006	1.473 ^c ± 0.117	0.198 ± 0.008	0.925 ^a ± 0.086
Isochlorogenic acid C	0.179 ± 0.006	0.292 ^c ± 0.026	0.184 ± 0.004	0.093 ^a ± 0.008
TOTAL	1.08	3.79	1.18	7,23

Concentration of markers per 100 mg of extract expressed as the equivalent of chlorogenic acid. Data represented by mean ± standard deviation (SD). LE: Leaf extract. Comparisons carried out by one-way ANOVA followed by Tukey post-test. a: different from A, B and C ($p < 0.005$); b: different from C ($p < 0.005$); c: different from A and C ($p < 0.005$).

the active markers can vary by ±15% in relation to the labeled amount (Brazil, 2014). According to the products leaflets, each capsule or tablet in commercial products A and B contained, respectively, 1.4 and 7 mg of

caffeoylquinic acid derivatives, expressed as chlorogenic acid equivalent. The commercial herbal medicines A and B showed a variation of 55 and 81.14%, respectively, in comparison with the labeled amount, which is higher than

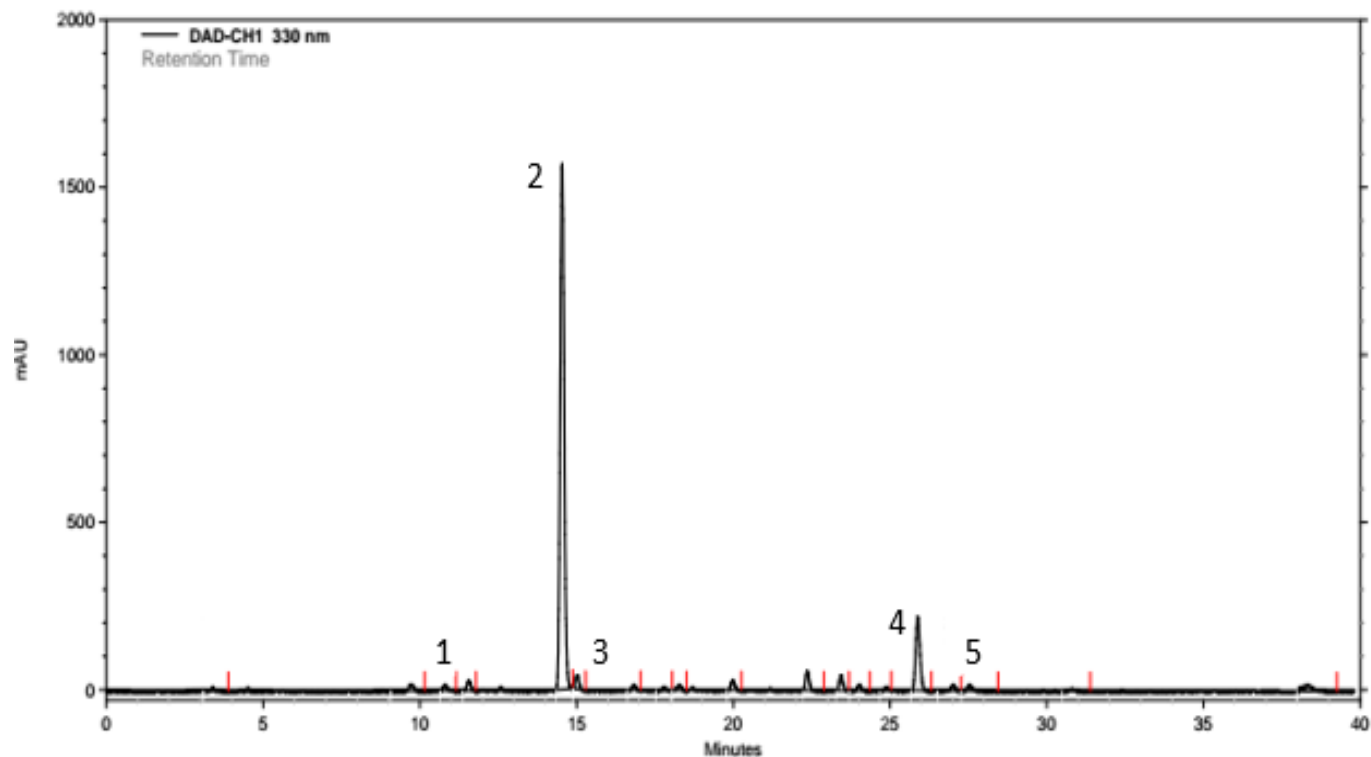


Figure 2. Chromatographic profile of *C. scolymus* L. leaf dry extract, obtained by HPLC-DAD with detection at 330 nm. 1: Neochlorogenic acid; 2: chlorogenic acid; 3: cryptochlorogenic acid; 4: isochlorogenic acid A; 5: isochlorogenic acid C.

the allowable variation. Herbal medicine C should have 1.5 mg of cynarin per capsule. However, as previously discussed, the analysis showed no detectable amounts of cynarin in the sample.

The Brazilian regulatory agency recommends a daily intake of 24 to 48 mg of caffeoylquinic acid derivatives expressed as chlorogenic acid (Brasil, 2014). According to the products leaflets, six capsules or tablets should be administered per day. The commercial products A and C showed lower amounts of caffeoylquinic acid derivatives in the finished product as compared to the recommended daily dose (13.02 and 21.18 mg, respectively), while product B showed a higher amount (76.08 mg). Therefore, all herbal medicines were in disagreement with the dose established by Anvisa. Besides demonstrating a clear noncompliance with the Brazilian sanitary regulation, these results can represent a negative impact on therapeutic outcomes and the occurrence of adverse events, which include, mainly, digestive disorders (EDQM, 2011).

Conclusion

Considering the synergistic action of plant constituents, an analysis focused on multiple markers, may be more representative in terms of quality (Xie and Leung, 2009;

Guo et al., 2016; Long et al., 2015). The proposed method for the simultaneous identification and quantification of chlorogenic, cryptochlorogenic, neochlorogenic, isochlorogenic acids A and C and cynarin can be a suitable tool for quality assessment of globe artichoke herbal medicines. A considerable variability in the proportion of chemical constituents was observed in the three samples of herbal medicines containing globe artichoke dry leaf extract. This variation can potentially represent public health risks, and this study emphasizes the importance of monitoring the quality of herbal medicines to ensure safety and efficacy of these products.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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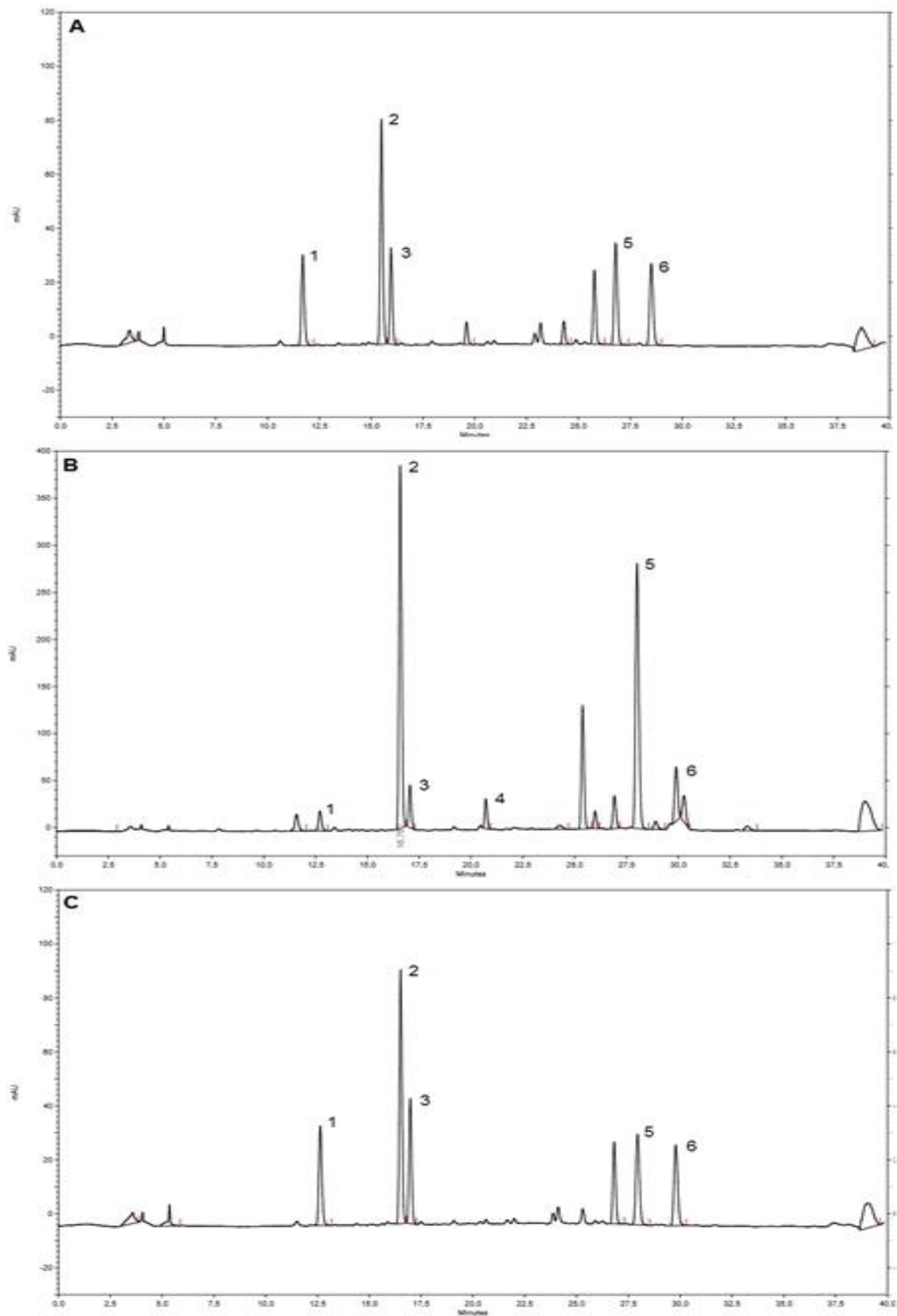


Figure 3. Chromatographic profiles of commercial products A, B and C, obtained by HPLC-DAD with detection at 330 nm. 1: Neochlorogenic acid; 2: chlorogenic acid; 3: cryptochlorogenic acid; 4: cynarin; 5: isochlorogenic acid A; 6: isochlorogenic acid C.

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Full Length Research Paper

Study of antibacterial and antioxidant activities of four common Nepalese kitchen spices

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Given the alarming incidence of antibiotic resistance in bacteria of medical importance and multiple side effects associated with the modern day chemotherapeutics, there is a constant need for new and effective therapeutic agents that could be easily extracted from our daily used Nepalese culinary. To study the antibacterial and antioxidant activity of common spices, locally available Clove (*Eugenia caryophyllus*), Cinnamon (*Cinnamomum zylancium*), Cumin (*Cumin cyminum*) and Timur (*Zanthoxylum alatum*) were subjected to cold extraction using ethanol and was assayed through agar well diffusion method and DPPH radical scavenging activity for different concentration gradient (100 to 500 µg). *Zanthoxylum* and *Eugenia* have showed potent antimicrobial activity against *Proteus vulgaris* and *Pseudomonas aeruginosa* followed by *Cinnamomum* and *Cumin* against *Pseudomonas aeruginosa*. All the extracts showed effective antimicrobial activity against gram positive and gram negative bacteria, however *Escherichia coli* remains ineffective towards any of the concentration of spices. DPPH radical scavenging activity showed effective antioxidant activity of the spices in the following order: *Eugenia* (93.84%) > *Cumin* (90.4%), *Zanthoxylum* (88.73%) > *Cinnamomum* (87.23%). Hence, our present study demonstrated that the ethanolic extract of different spices, *Eugenia* being the most effective, possess potent antibacterial and antioxidant activity and can be further analyzed for antimicrobial therapeutics and pharmacological evaluation.

Key words: 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, *Eugenia*, *Zanthoxylum*, *Cinnamon*, *Cumin*.

INTRODUCTION

The alarming rise of antibiotic resistance has been a global concern. The reason behind such situation has been notified as the haphazard use of the antimicrobial drugs which are excessively used during therapeutic treatment of infections. Additionally, the problems associated with modern day chemotherapeutics has a

gamut of adverse effect from hypersensitivity, immune-suppression to allergic reactions (Agrawal et al., 1996).

Natural products from plants and its significant parts has been proved to have lesser side effects in comparison to chemotherapeutics, hence they possess potential in being used as an 'alternative remedy' (Tepe

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et al., 2004). In developing countries modern-day antibiotics remains out of reach and 'plant-derived' drugs are their vital resources for treating infections (Thippeswamy et al., 2005). Plants products such as essential oil have been corroborated to have efficient effect against bacteria, virus, fungi and insects in addition to antioxidants properties. The active compounds cognate to these beneficial effects are most essentially secondary plant metabolites like glycosides, alkaloids and more and are profusely present in plant genre like spices and herbs (Pangsatabam et al., 2015; Saranraj et al., 2014).

With the most relevant notion, 'Kitchen spices' has been defines as the "vegetative products or mixture free from extraneous matter used for flavoring or imparting aroma in food". These spices are usually derivatives from different parts of plants like barks, leaves, fruits, buds, rhizomes and resins (Green et al., 1999).

The present research has mainly focused on four different spices used on daily basis in Nepalese Kitchen. The common spices like Clove (*Eugenia caryophyllus*), Cinnamon (*Cinnamomum zylancium*), Cumin (*Cumin cyminum*) and Timur (*Zanthoxylum alatum*) are used for the purpose of seeking its antimicrobial and antioxidant properties in the present study.

Cinnamon, also referred as *C. zylancium*, possess several potential health benefits. Antimicrobial activity, anti-inflammatory properties, controlled blood sugar, depletion of cardiovascular and colonic cancer along with the boosted cognitive function are reported in several scholars for cinnamon (Khan et al., 2003; Mata et al., 2007; Shen et al., 2010).

Another effective spice is clove, which is mostly used as agent for flavoring food items or herbal supplement. Clove has also been found to be utilized as a topical treatment in case of pain in tooth. Other usage of clove comprises the treatment for oral cavity inflammation, supplement during common cold or cough (Consumer information from USDA, 1997). Cumin also exhibits potential activity against microbes and fungi along with effective antioxidants activity. These properties are mainly found to be correlated with the content of phenol in cumin (Taira et al., 1992; Shetty et al., 1994; Zhang, 2004).

Lastly, extensive application of Timur has been seen in home-remedy for its carminative, antihelmintic and stomachic properties. The plant parts mainly fruits and seeds are widely linked with home-remedy as aromatic tonic during dyspepsia or fever. In addition, properties like anti-odor, disinfectant and antiseptic has enlarged its spectrum of application from dental troubles to lotion for skin problems like scabies (Green et al., 1999).

MATERIALS AND METHODS

Species collection

All the spices namely Clove (*E. caryophyllus*, family Myrtaceae),

Cinnamon (*C. zylancium*, family Lauraceae), Cumin (*C. cyminum*, family Apicaceae) and Timur (*Z. alatum*, family Rutaceae) were collected from local market of Kathmandu Nepal.

Chemicals and standards

The chemicals used were ethanol (Thermo-Fisher Scientific, India), DPPH and ascorbic acid (Sigma Aldrich, USA). All other chemicals used were of the highest commercially available grade. For absorption measurement, double beam U-2800 UV-visible spectrometer, HITACHI, Japan, was used.

Preparation of extracts

The spices were ground in clean and sterilized grinder and the fine powder was taken. 20 g of sample was weighed and soaked in 200 ml ethanol (for 48 h). After that it was stirred in a magnetic stirrer for 30 min and filtered by using Whatman No. 1 filter paper. The filtrate was taken and concentrated to dryness in rotatory evaporator at reduced pressure. Dry extract was dissolved in mother solvent to make the stock solution of 10 mg/ml.

Collection and maintenance of culture

All the pure cultures of the test organisms were collected from National Public Health Laboratory, Kathmandu, Nepal. Six different bacterial strains, *Staphylococcus aureus* (ATCC 25923), *Proteus vulgaris* (ATCC 15028), *Escherichia coli* (ATCC 25922), *Klebsiella pneumonia* (ATCC 700603), *Salmonella typhi* (ATCC 14028) and *Pseudomonas aeruginosa* (ATCC 27853) were taken. These organisms were then sub cultured in a sterile nutrient agar and incubated at 37°C for 24 h to obtain fresh culture.

Assay of antibacterial activity

Spices are believed to possess antibacterial activity and thus its assay was done by well diffusion method. At first the 24 h culture of six bacterial strains *S. aureus* (ATCC 25923), *P. vulgaris* (ATCC 15028), *E. coli* (ATCC 25922), *K. pneumonia* (ATCC 700603), *S. typhi* (ATCC 14028) and *P. aeruginosa* (ATCC 27853) were taken and cultured in nutrient broth. The sterile plates of Mueller-Hinton Agar (MHA) were prepared. A McFarland 0.5 standard was prepared and the bacterial suspension was compared to the 0.5 McFarland standards to adjust the turbidity of the inoculums for the susceptibility test. The bacteria were transferred into Petri-plates containing solidified agar using sterile cotton swab. The swab was used to spread the bacteria on the media in a confluent lawn. Wells were prepared by punching the agar plate already inoculated with a pure culture of the test organism with the help of sterile borer of 6 mm. Wells were made at the equidistance on the Petri plate. Then 40, 50, 60 and 70 µl of samples were added in wells from the stock solution of 10 mg/ml. In the two wells, 50 µl of ethanol (as negative control) and 50 µl of reference antibiotic solution (as positive control) were added. Gentamycin (1%) and Vancomycin (1%) were used as reference antibiotic for Gram-negative bacteria and Gram-positive bacteria, respectively. Diffusion of extracts, antibiotics and methanol were allowed at room temperature for 1 h. All the plates were then covered with lids and incubated at 37°C for 24 h and zone of inhibitions (ZOIs) were measured. For each sample all the experiments were performed thrice and obtained results were averaged.

1, 1-Diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity

1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) radical scavenging

Table 1. Antibacterial activity of Nepalese kitchen spices extract.

Sample	Amount of sample (µg)	Zone of Inhibition (mm)					
		<i>S. typhi</i> (ATCC 4028)	<i>E. coli</i> (ATCC 5922)	<i>P. vulgaris</i> (ATCC 15028)	<i>K. pneumonia</i> (ATCC 700603)	<i>P. aeruginosa</i> (ATCC 27853)	<i>S. aureus</i> (ATCC 25923)
<i>Zanthoxylum</i>	100	8±1	-	12±2	9±1	6±1	7±1
	200	12±1	-	12±1	11±1	7±1	10±1
	300	14±2	-	16±2	12±1	12±1	14±2
	400	14±2	6±1	20±3	12±1	12±1	14±2
	500	16±1	7±1	24±3	16±3	16±2	16±1
<i>Eugenia</i>	100	10±1	8±1	9±1	10±1	10±1	10±1
	200	12±2	8±1	9±1	11±1	14±2	11±2
	300	14±1	10±1	10±1	14±1	26±3	14±2
	400	14±2	14±2	12±2	16±2	26±3	16±2
	500	16±2	14±1	14±2	20±3	26±3	20±3
<i>Cinnamomum</i>	100	-	-	-	-	-	8±1
	200	-	-	-	-	10±1	10±1
	300	8±1	-	6±1	7±1	12±2	11±2
	400	10±1	7±1	8±1	9±1	14±2	11±2
	500	10±1	8±1	8±1	10±1	16±2	14±2
<i>Cumin</i>	100	-	-	-	-	10±1	6±1
	200	8±1	-	-	-	12±2	6±1
	300	12±2	-	8±1	-	14±2	10±2
	400	12±2	6±1	10±1	7±1	14±2	11±2
	500	12±2	6±1	11±2	8±1	20±3	14±1
Gentamycin (1%)		16±2	18±2	19±1	18±2	22±2	-
Vancomycin (1%)		-	-	-	-	-	20±3

activity was measured according to the method of Ilhami et al. (2005). Extract solutions were prepared by dissolving of different dry extract in methanol to produce a solution of 10 mg/ml. 600 µM DPPH was dissolved in 300 ml methanol and used as stock solution. The plant extract in methanol was taken in various concentrations, that is, 10, 20, 30, 40 and 50 µl from stock of 10 mg/ml (100, 200, 300, 400, and 500 µg) whose final volume was maintained 1 ml and were mixed with an aliquot of 2 ml of 600 µM DPPH solution in methanol and incubated at 25°C for 30 min. Absorbance of the test mixture was read at 517 nm using a spectrophotometer against a DPPH control containing only 1 ml of methanol in place of the extract. All experiments were performed thrice and the results were averaged. Ascorbic acid was used as a standard. Percentage inhibition was calculated using the following expression

$$\% \text{ Inhibition} = (A_{\text{control}} - A_{\text{sample}} / A_{\text{control}}) \times 100$$

Where, A_{control} and A_{sample} stand for absorbance of the control and absorbance of tested extract solution respectively.

RESULTS

Antibacterial activity

The result of antibacterial activity of spices is shown in

Table 1. The antibacterial potential may be due to various phytochemicals present in them. *Zanthoxylum* was most effective against *Proteus vulgaris* showing maximum zone of inhibition of 24±3 mm when 500 µg extract was used. *Eugenia* was most effective against *P. aeruginosa* showing maximum zone of inhibition of 26±3 when 300 to 500 µg extract was used. Similarly, *Cinnamomum* and *Cumin* were most effective against *P. aeruginosa* showing maximum zone of inhibition of 16±2 and 20±3 mm respectively when 500 µg extract was used. All the extracts were least effective against *E. coli* than other strains used.

Evaluation of antioxidant assay

DPPH radical scavenging activity

DPPH radical scavenging activity of Nepalese kitchen spices extracts at different concentrations (100 to 500 µg) was measured, with ascorbic acid as the standard. The DPPH radical scavenging activity is shown in Figure 1 and extent of radical scavenging was found to increase generally with increasing concentrations for all samples.

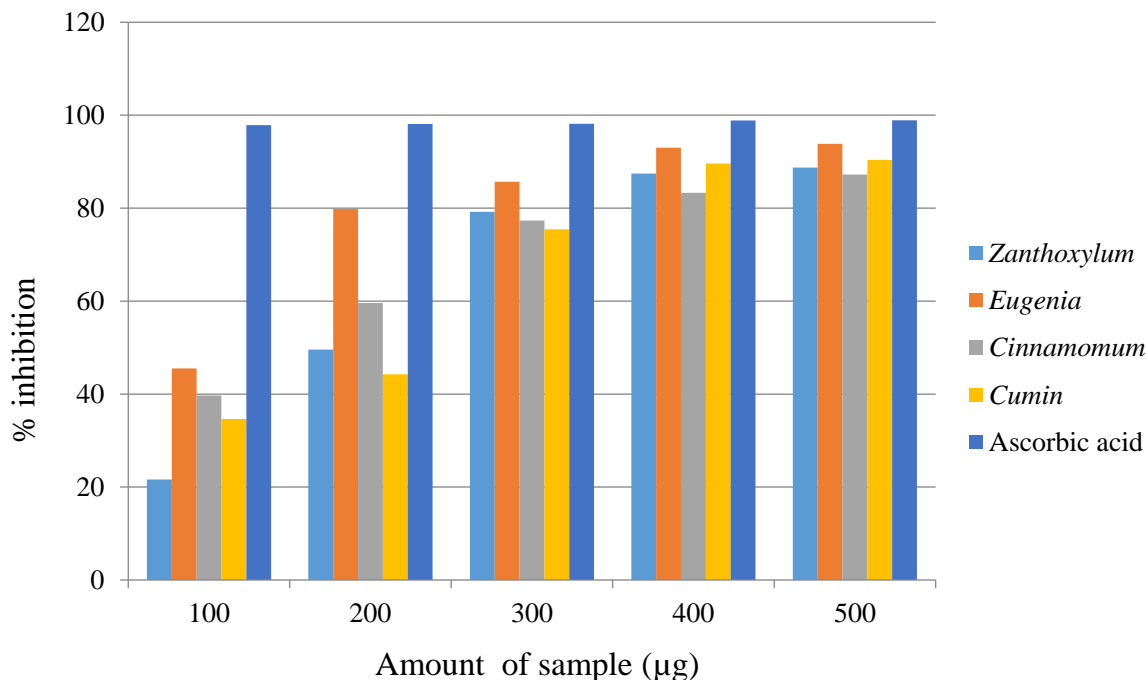


Figure 1. Antioxidant activity of Nepalese kitchen spices extract.

The results shows *Eugenia* (500 µg) exhibited the highest radical scavenging activity with 93.84%±4.51 followed by the *Cumin* (500 µg) extract with 90.4%±5.87, *Zanthoxylum* (500 µg) extract with 88.73%±5.64 and *Cinnamomum* (500 µg) extract with 87.23%±7.23.

Statistic

The results are expressed as mean ± standard deviation (SD).

DISCUSSION

Spices are believed to possess antibacterial activity and antioxidant activity and thus it was assayed through well diffusion method and DPPH radical scavenging activity. It was found that the ethanolic extract of different spices possess efficient antibacterial and antioxidant activity in a concentration dependent manner.

The beneficial medicinal effects of plant materials typically results from the secondary products present in the plant although, it is usually not attributed to a single compound but a combination of the metabolites. The medicinal actions of plants are unique to a particular plant species or group, consistent with the concept that the combination of secondary products in a particular plant is taxonomically distinct (Shen et al., 2010). Spices and their phytochemicals have become an exciting research topic because of their important and observed biological

activities and because they are used extensively in the culinary, industrial and pharmacological fields. These biological activities are mainly attributed to their antioxidant and antibacterial properties due to their high polyphenol content (Gaur, 1990; Mata et al., 2007).

The growth of new infectious disease and emergence of several infections appears to have been controlled, but the increase in multidrug resistant bacteria is followed which poses a serious threat to human and animal and creates the necessity for studies directed towards the development of new antimicrobials.. Plants can be the best source for such bioactive compounds (Rasooli, 2007). So, since both gram positive as well as gram negative bacteria are found to be sensitive against spice extract (Table 1), spices can be efficient source for deriving those compounds. Antioxidants are capable of protecting cells from free radical damage, act as chemo preventive agents by inhibiting the generation of free radicals and play an important role in neutralizing oxidative damage (Ouattara et al., 1997; Pokhrel et al., 2015). DPPH assay is well known method for the evaluation of free radical scavenging activity (Rasooli, 2007). Antioxidant activity may be due to various phytochemicals such as phenols and flavonoids which are well known for their antioxidant potential due to hydrogen donating property of their hydroxyl groups. Strong DPPH activity of clove might be related to phenolic antioxidant components such as eugenol, isoeugenol, etc. (Tepe et al., 2004). Dorman et al. (2000) identified as much as 94% phenyl propanols in clove oil of which eugenol was 91% of phenyl propanols (Duke et

al., 2003).

Conclusion

The study conducted has shown that crude ethanolic extracts of multiple kitchen spices found in Nepal possess effective antibacterial activity against both Gram positive and Gram negative bacteria. Also the extract has been found to contain effective antioxidant capacity. On further exploration of the bioactive constituents present in those plants development of novel antibacterial and antioxidant compounds could be possible.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests.

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