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PCR-DGGE ANALYSIS OF THE YEAST POPULATION ASSOCIATED WITH NATURAL FERMENTATION OF TABERNA

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ABSTRACT

In this study, yeast community associated with the natural fermentation of Taberna, an alcoholic beverage made from palm sap, was determined using PCR-denaturing gradient gel electrophoresis (PCR-DGGE) of the D1 region of the 26S rRNA gene. This technique was complemented by cloning and sequencing of DGGE bands. The experiments were performed in triplicate. Each palm tree (palm tree I, II and III) represented an experimental unit. Fourteen batches from each palm tree were analyzed. These molecular methods allowed a rapid monitoring of yeast population associated with Taberna fermentation. Most frequent yeast species were *Hanseniaspora guilliermondii*, *Saccharomyces cerevisiae* and *Pichia kudriavzevii* (*Issatchenkia orientalis*), followed by *Candida tropicalis* and *Kazachstania exigua*. In addition, *Meyerozyma guilliermondii*, *Candida akabanensis*, *Candida blattae*, *Candida intermedia*, *Pichia kluyveri* and *Trichosporon moniliiforme* were also detected. In the first batches, only 5 yeast species were identified, from the second batch the number of species increased, in some batches ten yeast species were identified. The implementation of PCR-DGGE to describe yeast population in fermentation of Taberna revealed new members (*C. akabanensis, C. blattae and T. moniliiforme*) related with this palm wine. Results showed that PCR-DGGE is a good technique for characterizing yeast population structure. Findings allowed us gain important information about yeast community structure of Taberna fermentation.

Keywords: Palm wine, *Acrocomia aculeata*, Yeast, DGGE, Taberna

INTRODUCTION

Taberna is a traditional palm wine produced by the natural fermentation of the sap obtained from a palm tree named *Acrocomia aculeata* ("coyol" palm tree). This beverage is consumed as a refreshing and alcoholic drink in small villages in southeast of Mexico and Central America (**Balick, 1990; Alcántara-Hernández *et al*., 2010**). The production process of Taberna has been previously reported by **Santiago-Urbina *et al.* (2013, 2015)**. Palm sap is obtained from the stem of felled palm trees through a series of steps which involve the perforation in the apical part of the stem. The receptacle is known as canoe (place where the sap is accumulated and naturally fermented), which is scraped every day. The main sugar of the coyol palm sap is sucrose, about 11% (**Santiago-Urbina *et al*., 2013**). Two batches of Taberna are harvested daily. Each one has a different chemical composition: ethanol content (0.21 to 4.78%), sugar content (1 to 8%), lactic acid (0.05 to 0.5% w/v), acetic acid (0.01-0.24% w/v) and pH (6 to 4) (**Santiago-Urbina *et al*., 2013**). The fermentation of Taberna is a natural and non-controlled process that involves lactic acid bacteria, acetic acid bacteria and yeast (**Alcántara-Hernández *et al*., 2010; Santiago-Urbina *et al*., 2015**). Yeasts have been reported to be important in the fermentation process of different types of palm wine such as those produced in Ghana, Cameroon and Burkina Faso (**Amoa-Awua *et al.*, 2007; Stringini *et al*., 2009; Ouoba *et al.*, 2012**). There is a limited knowledge about the yeasts population on Taberna fermentation. Based on isolation and PCR-RFLP analysis followed by sequencing of the domain D1/D2 of the 26S rDNA **Santiago-Urbina *et al.* (2015)** have identified *Kazachstania unispora*, *K. exigua*, *C. tropicalis, C. intermedia*, *M. guilliermondii*, *P. kudriavzevii* (*I. orientalis*), *H. guilliermondii* and *P. kluyveri* as yeast species involved in this fermentation. Although, culture-dependent techniques provide significant insight, it may not reflect the complete yeast population. **Ampe *et al.* (1999)** have reported that at least 25-50% of the active microbial community of fermented foods cannot be cultivated in the laboratories conditions. To complete and overcome this challenge, a culture-independent molecular approach using Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) has been employed, and it is a well documented method for analysis of microbial communities in fermented foods (**Stringini *et al*., 2009; Osimani *et al*., 2015; Puerari *et al*., 2015; Valera *et al*., 2015**). PCR-DGGE is a molecular tool used and recommended for microbial community structure analysis (**Neilson *et al*., 2013**). The objective of this study was to identify the yeast population associated with Taberna fermentations using PCR-DGGE as a culture-independent technique. The information obtained in this investigation will help to improve the knowledge about the microbiology of this palm wine.

MATERIAL AND METHODS

Taberna samples

Taberna production is similar to fed-batch fermentation, because the sap flow is constant and accumulated in the canoe until it is full. Fermented sap (Taberna) is harvested in the morning and in the afternoon. In the morning when the receptacle is empty, the producer scrapes the walls of the canoe in order to amplify the size of it. In this work each sample collected corresponds to the final product (Taberna) of a batch, i.e. when the canoe is full (12 h approximately). Experiments were conducted in triplicate. Each palm tree (I, II and III) corresponded to an experimental unit. Samples of Taberna were collected from 14 batches from each palm tree. In addition, one sample of fresh sap (unfermented sap taken immediately after the producer opened the canoe) was evaluated with the aim of determining the initial yeast population. Samples were harvested in March 2012 in Benito Juárez, Chiapas, Mexico as described in **Santiago-Urbina *et al.* (2013, 2015)**. From each batch, approximately 50 mL of palm sap was taken directly from the canoe. The samples were collected into pre-sterilized 50-mL Falcon tubes in the morning (6:00 am), before the canoe scraping was performed.

Extraction of total microbial DNA

The Taberna samples were processed as follows: in order to remove the residue of palm tree bark in the palm wine, 50-mL of sample was filtered through sterile gauze. Then, 10-mL of each filtered sample was centrifuged at 11 600 x g at 4 °C for 10 min. After that, the pellets were washed using wash solution 1 (50 mM Tris-HCl pH 8.3, 200 mM NaCl, 5 mM Na_2EDTA, 0.05% Triton X-100), wash solution 2 (50 mM Tris-HCl pH 8.3, 200 mM NaCl, 5 mM Na_2EDTA) and wash solution 3 (10 mM Tris-HCl pH 8.3, 0.1 mM Na_2EDTA) following the wash protocol described in **Fortin *et al.* (2004)**. Finally, the total DNA of the pellets was extracted using a MasterPure^{tm}Yeast DNA Purification kit (Epicentre® an Illumina company, USA, Madison) according to the manufacturer's instructions.

PCR-DGGE protocol

Thirty nanograms of Total DNA were used as template in PCR amplification of the D1 region of the 26S rRNA gene using the primers NL1GC and LS2 **(Cocolin *et al.*, 2000)**. PCR was performed in total volume of 50 µL containing 1x PCR Buffer, 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphates (dNTPs), 0.2 µM of each primer and 2.5 U of *Taq polymerase*. The amplification reactions were performed in a thermal cycler (PIKO 24 thermal cycler, Thermo Scientific, Finland , Vantaa) as follows: 5 min at 95 °C, 35 cycles of 1 min at 95 °C, 45 s at 55 °C, 1 min at 72 °C, and then a final step at 72 °C for 7 min. Five microliters of each PCR product was analyzed by electrophoresis in 1% (w/v) agarose gel in 0.5x TBE (44.5 mM Tris (Sigma-Aldrich, USA, Missouri), 44.5 mM boric acid (Sigma-Aldrich), 1 mM Na_2-EDTA (Sigma-Aldrich)) containing 0.7 µg/mL of ethidium bromide (Bio-Rad, USA, California) at 100 V during 50 min, using a standard molecular weight marker (1 kb plus DNA ladder; Invitrogen, USA, California). Gels were photographed under transilluminated ultraviolet (UV) light with Kodak Molecular Imaging Software version 5.0 (Carestream Health, Inc, USA, New York). The PCR products were analyzed by DGGE using a Bio-Rad Dcode^{tm} universal mutation detection system (Bio-Rad). PCR samples were directly applied onto 8% (w/v) polyacrylamide gels (0.8 mm) in a running buffer (1x TAE) containing 0.04 mM Tris base; 0.02 M acetic acid, glacial; 1 mM EDTA, pH 8. Denaturing conditions were first determined using a perpendicular gradient gel and DNA of the yeast species previously isolated and identified. The gels were prepared with a denaturing gradient from 25 to 60% of urea and formamide (100% corresponds to 7 M urea and 40% (w/v) formamide) and an acrylamide and bis-acrylamide ratio of 19:1 (poliacrylamyde mixing powder, Sigma-Aldrich). Electrophoresis was performed at a constant voltage and temperature of 70 V and 60 °C for 17 h. After electrophoresis the gels were stained using a silver-staining method described by **Byun *et al.* (2009)**, photographed and analyzed with Kodak Molecular Imaging Software version 5.0 (Carestream Health, Inc.). Batches were grouped based on the DGGE profiles using unweighted pair group average (UPGMA) cluster analysis based on the Dice similarity coefficient using the PAST software, version 2.17c.

DGGE band sequencing

DGGE bands of interest were excised from the gels with a sterile scalpel and placed into sterile Eppendorf tubes containing 50 µL of sterile deionized water. Tubes were incubated overnight at 4 ºC to allow diffusion of DNA. Ten nanograms of each eluate were then used as a DNA template for a PCR using the primers NL1 and LS2 and the conditions described above. The PCR products were purified using a DNA Clean and Concentrator^{tm}-5 (Zymoresearch, USA, California), and sent for sequencing to Laragen Company (USA, California). Sequences were aligned in GenBank using the Blast program **(Altschul *et al.*, 1997)** for identification.

Cloning of the PCR products of DGGE bands

When many ambiguous peaks in the sequences were obtained, the bands were then cloned by using CloneJet PCR cloning Kit (Thermo scientific, Lithuania). Purified PCR products amplified from eluted DGGE bands were ligated into pJET1.2/blunt Cloning Vector according to the manufacturer's instructions (CloneJet PCR Cloning kit, thermo scientific, Lithuania), and transformed into *Escherichia coli* DH5α cells by chemotransformation. To confirm the presence of insert, five representative colonies were randomly selected and picked. The picked colonies were resuspended in 20 µL of mix PCR containing 1x PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphates (dNTPs), 0.2 µM of each primer and 0.5 U of *Taq polymerase*. The amplification reactions were performed in a thermal cycler (PIKO 24 thermal cycler, Thermo Scientific) as follows: 5 min at 95°C, 35 cycles of 1 min at 95°C, 45 s at 55 °C, 1 min at 72 °C, and then a final step at 72 °C for 7 min. Five microliters of each PCR product were analyzed by electrophoresis in agarose gel (1% (w/v)) as described above. Positive clones were confirmed by the amplification of a PCR product of approximately 340 bp length containing the 250 bp D1 region of the 26S rRNA gene. Reamplification, sequencing, alignment and identification of cloned PCR products were performed as described previously in DGGE band sequencing section.

Principal component analysis (PCA) and frequency percentage

Data of the yeast species identified in each batch was converted to a presence (1)-absence (0) matrix, and subjected to principal component analysis to investigate the relationships between yeast species and batches from each palm tree. Analysis was performed using the free software package PAST (Paleontology statistics, http://folk.uio.no/ohammer/past/).
To study the species distribution in our samples, the method proposed by **Solieri *et al.* (2006)** was used. The frequency was estimated as the number of positive samples for a species divided by the total number of samples expressed as a percentage.

RESULTS

Yeast community fingerprinting patterns

Fourteen batches of Taberna from each palm tree (palm tree I, palm tree II and palm tree III) and one sample of fresh sap were analyzed through PCR-DGGE. DGGE fingerprints obtained from the total DNA extracted from each sample are shown in Fig. 1. Each vertical lane represents a Taberna sample corresponding to one batch, and each band or spot represents yeast species. A total of 29 bands with different position in the DGGE were obtained. The DGGE fingerprints of batches from palm tree I consisted of 21 bands, resembling to those of palm tree III. Whereas, the profile of the batches in palm tree II involved 19 bands. Fourteen bands (a, b, d, e, f, g, h, k, l, q, t, u, x and z) were common in the batches of the three palm trees, and fifteen bands were distributed among them (bands c, i, j, o, r were present only in palm tree I; bands oo and nn were present in palm tree II; bands v, w, bb, cc were present in palm tree III; band n was present in palm trees I and III; band p was present in palm trees I and II; bands aa and dd were present in palms II and III). Cluster analysis was carried out using DGGE data to estimate the order of relatedness among the different batches (Fig. 2). DGGE profiles from each batch were mainly clustered in the groups A, B, E, F and H, which had equal or greater similarity than 65 % (Fig. 2). First batches from each palm tree were grouped in A (batches 2-8), B (batches 2-6) and H (batches 4-10) clusters. Batches (10-14) from the palm tree I were grouped in cluster E, and batches (9-13) from the palm tree II were grouped in cluster F. A low similarity (35%) was obtained between cluster K (including the first batch of each palm tree) and the cluster L (including most the final batches) (Fig. 2). The cluster analysis showed that the similarities between batches from the same palm tree (55-100%) are most closed than the similarities among batches from different palm trees (33-60%). To determine the identity of each band in the DGGE profile of the samples, the DNA in the band was eluted, reamplified and sequenced.

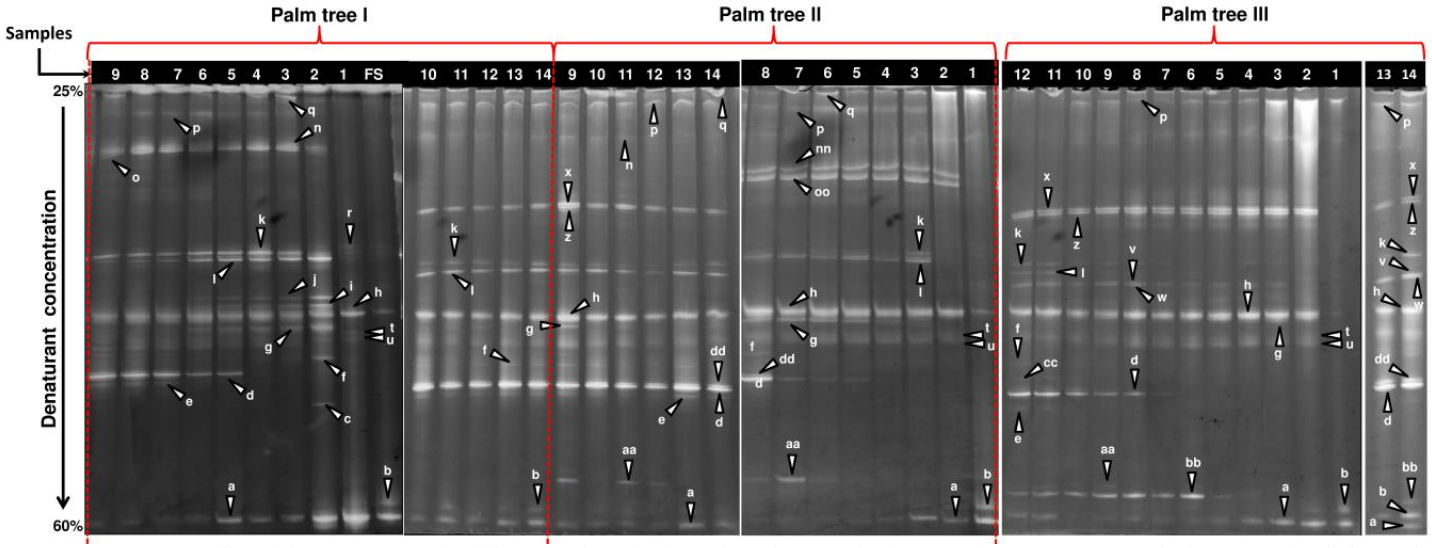

Figure 1 DGGE profiles of the yeasts present in different batches of Taberna from three coyol palm tree. Line FS represents to fresh sap (FS) for all three palm trees, because the sap from each palm tree was mixed (for more volume) and only the one mixed sample was analyzed. Lanes 1 to 14 indicate the number of batch. Band a–*P. kudriavzevii*, band b–*C. akabanensis*, *M. guilliermondii* and *S. cerevisiae*, band c–*K. exigua*, band d–*S. cerevisiae*, band e–*S. cerevisiae*, band f–*K. exigua* and *P. kluyveri*, band g–*C. tropicalis*, band h–*H. guilliermondii*, band i–*M. guilliermondii*, band j–*C. akabanensis*, band k–*S. cerevisiae*, band l–*S. cerevisiae*, band n–*S. cerevisiae*, band o–*H. guilliermondii*, band p–*H. guilliermondii*, band q–*S. cerevisiae*, band r–*M. guilliermondii*, band t–*K. exigua* and *C. tropicalis*, band u–*H. guilliermondii*, band v–*H. guilliermondii*, band w–*H. guilliermondii*, band x–*C. akabanensis*, *H. guilliermondii* and *C. blattae*, band z–*H. guilliermondii*, band aa–*T. moniliiforme*, *M. guilliermon*dii and *H. guilliermondii*, band bb–*K.exigua*, band cc–*H. guilliermondii* and *S. cerevisiae*, band dd–*S. cerevisiae*, band nn–*H. guilliermondii*, band oo–*H. guilliermondii*, *C. blattae* and *C. intermedia*

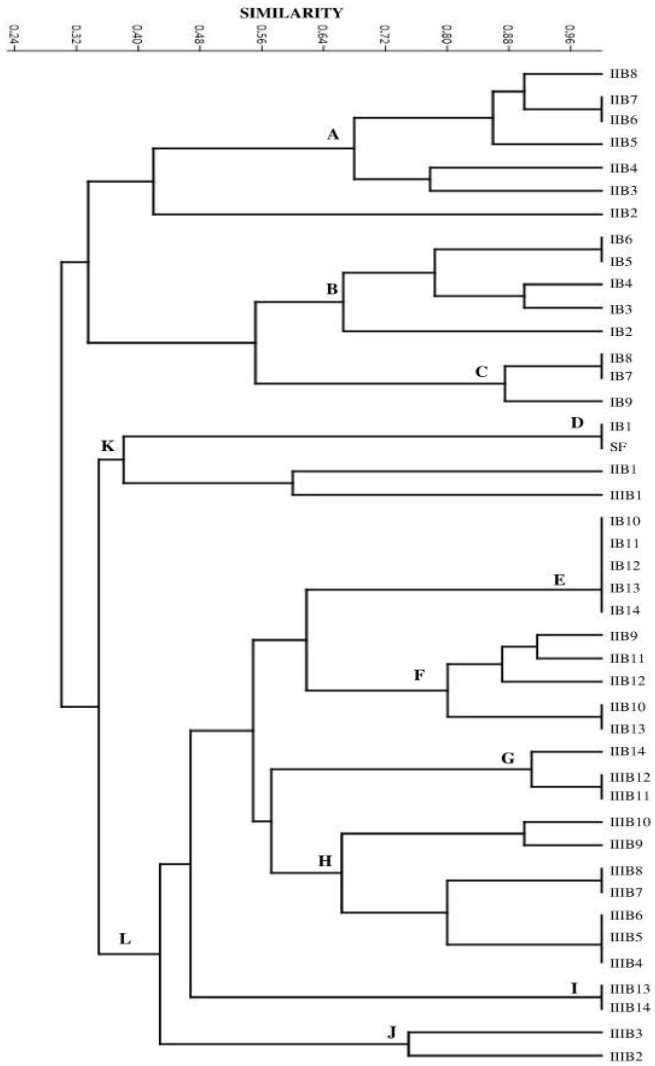

Figure 2 Dendogram based on UPGMA clustering of Dice similarity coefficients of yeasts community PCR-DGGE fingerprintings of different batches of Taberna. I–palm tree I, II–palm tree II, III–palm tree III, B–Batch, 1 to 14–number of batch. A, B, C, D, E, F, G, H, I, J–groups ≥65% of similarity.

Yeast identification by sequencing bands

From excised bands across all samples and gels, it was possible to assign an identity to 19 of 29 total sequences. The closest relative of the sequences obtained are shown in Table 1. Bands d, e, k, l, n, q and dd were related with *S. cerevisiae*. While, bands h, o, p, u, w, z and nn were identified as *H. guilliermondii*, the bands i and r belonging to the species *M. guilliermondii* and the band bb corresponds to *K. exigua*. The results showed multiband profiles associated with single species; DNA sequences had the same phylogenetic assignment, although their migration profile on the DGGE was different. On the other hand, the resulting sequences of the bands a, b, f, g, t, v, x, aa, cc and oo failed due to the presence of many ambiguous peaks. To determine the identity of these bands in the DGGE profile of the samples, the DNA in the bands were cloned, and five clones for each band were selected in order to identify clones with the insert of interest. In this step, bands a, b, f, g, t, v, aa and cc presented clones with insert of 350 bp and insert with 200 bp. The latter is an undesirable fragment, which can be an artefact of PCR and their presence may be associated with the multiple sequences for a single band. In addition, the results showed that a single band may consist of two or three yeast species as indicated in the Table 2. With the cloning of DGGE band, it was possible to identify *P. kudriavzevii*, *P. kluyveri*, *C. tropicalis*, *C. blattae* and *T. moniliiforme*.

In the fresh sap of the coyol palm, the bands a, b, h, k and r were detected (Fig. 1, lane 1). Band "a" corresponds to *P. kudriavzevii*, while band "b" represented three yeast species: *M. guilliermondii*, *C. akabanensis* and *S. cerevisiae*. These results indicate the co-migration of different DNA fragments in the same position. Band "h" was identified as *H. guilliermondii*, band "k" was related with *S. cerevisiae* and band "r" was associated with *M. guilliermondii*. From batch 3, an increase in the number of bands was observed (Fig. 1, lane 3). Thus, bands f, g, l, n, q, t and u were detected in the batches of the three palm trees. Additionally, bands c, i and j were observed in the batch of palm tree I, as well as bands nn and oo in palm tree II. Band "f" contained two species, *K. exigua* and *P. kluyveri*, while band "g" was assigned to *C. tropicalis*, which predominated in the batches of palm tree I and II. Bands n, l, k and q represented a multiband profile associated with *S. cerevisiae*. Similarly, as happened with yeast species *H. guilliermondii* (Table 1), *Pichia kudriavzevii* (band a) and *S. cerevisiae* (k and l) were present from samples 3 to 15 in the three palm trees. Thus, in fresh sap *P. kudriavzevii*, *H. guilliermondii*, *S. cerevisiae*, *M. guilliermondii* and *C. akabanensis* were identified. Batches (10, 11, 12, 13 and 14) from the palm tree I had the same yeast population as batches (2, 3, 4, 5, 6, 7, 8, 13 and 14) from the palm tree II (Fig. 1), *S. cerevisiae*, *H. guilliermondii*, *P. kudriavzevii*, *C. tropicalis*, *K. exigua*, *C. blattae*, *C. akabanensis* and *M. guilliermondii* were identified. Batches 8, 9, 11 and 12 from the palm tree II, as well as batch 8 from palm tree III presented 10 yeast species (Fig. 1).

Table 1 Identities of bands obtained of the yeast community from Taberna samples

Band[a]	Closest relative	Identity %[b]	Source[c]
c	*Kazachstania exigua*	100	HM106427.1
d	*Saccharomyces cerevisiae*	99	KM609510.1
e	*Saccharomyces cerevisiae*	99	KJ530642.1
h	*Hanseniaspora guilliermondii*	86	KM210521.1
i	*Meyerozyma guilliermondii*	92	KM609507.1
j	*Candida akabanensis*	98	AB499000.1
k	*Saccharomyces cerevisiae*	98	KM655850.1
l	*Saccharomyces cerevisiae*	94	KJ530642.1
n	*Saccharomyces cerevisiae*	99	KJ530642.1
o	*Hanseniaspora guilliermondii*	100	KM210521.1
p	*Hanseniaspora guilliermondii*	96	GQ249098.1
q	*Saccharomyces cerevisiae*	99	JX068683.1
r	*Meyerozyma guilliermondii*	100	HM771258.1
u	*Hanseniaspora guilliermondii*	99	KM210521.1
w	*Hanseniaspora guilliermondii*	90	KM210521.1
z	*Hanseniaspora guilliermondii*	94	KM210521.1
bb	*Kazachstania exigua*	99	FJ527857.1
dd	*Saccharomyces cerevisiae*	99	KJ530642.1
nn	*Hanseniaspora guilliermondii*	94	KM210521.1

a–The letter correspond to the band letter in Fig. 1.
b–Percentage of identical nucleotides in the sequence obtained from the DGGE band and the sequence of the closest relative found in the GenBank database.
c–Accession number of the sequence of the closest relative found by a blast search.

Table 2 Identification of clone sequences from DGGE bands of Taberna samples

Band[a]	Clone[b]	Closest relative	Identity %[c]	Source[d]
a	1	*Pichia kudriavzevii*	100	LC015645.1
b	1	*Candida akabanensis*	99	AB499000.1
	2	*Meyerozyma guilliermondii*	100	HM771258.1
	3	*Saccharomyces cerevisiae*	96	AY526109.1
f	1	*Kazachstania exigua*	100	HM106427.1
	2	*Kazachstania exigua*	99	JQ585744.1
	3	*Pichia kluyveri*	99	KJ569591.1
g	1	*Candida tropicalis*	95	EF644469.1
t	1	*Kazachstania exigua*	90	JQ585744.1
	2	*Candida tropicalis*	90	KM013373.1
v	1	*Meyerozyma guilliermondii*	99	KF241563.1
x	1	*Candida akabanensis*	93	AB499000.1
	2	*Candida akabanensis*	90	AB499000.1
	3	*Candida akabanensis*	92	AB772160.1
	4	*Hanseniaspora guilliermondii*	100	KM210521.1
	5	*Candida blattae*	99	DQ655694.1
aa	1	*Trichosporon moniliiforme*	99	KF826528.1
	2	*Meyerozyma guilliermondii*	99	KM609507.1
	3	*Hanseniaspora guilliermondii*	99	KM210521.1
	4	*Hanseniaspora guilliermondii*	99	KM210521.1
cc	1	*Saccharomyces cerevisiae*	100	HG425339.1
	2	*Hanseniaspora guilliermondii*	100	KM210521.1
oo	1	*Hanseniaspora guilliermondii*	97	KM210521.1
	2	*Candida blattae*	98	DQ655694.1
	3	*Candida blattae*	99	DQ655694.1
	4	*Candida blattae*	98	DQ655694.1
	5	*Candida intermedia*	90	KJ794663.1

a–The letter corresponds to the band letter in Fig. 1.
b–The number corresponds to the number of clones obtained with an insert of 350 bp from each band.
c–Percentage of identical nucleotides in the sequence obtained from the clones and the sequence of the closest relative found in the GenBank database
d–Accession number of the sequence of the closest relative found by a blast search.

Principal component analysis (PCA)

Principal component analysis was performed using the presence-absence of the yeast species identified in each batch of Taberna production. Principal components 1 and 2 together explained 57.15% of the yeast species variation that occurred between batches and palm trees (Fig. 3). *Pichia kudriavzevii*, *H. guilliermondii* and *S. cerevisiae* were the dominant species. The first batch (PIB1, PIIB1, PIIIB1 and FS) from each palm tree was correlated with *C. akabanensis*, and batches P1B9, P1B7, P1B8, PIIB14, PIIB10, PIIB13 and cluster b (Fig. 3) were correlated with *P. kluyveri*. Batches from the palm tree II (PIIB3, PIIB4, PIIB5, PIIB6, PIIB7 and PIIB8) were strongly correlated with *C. intermedia*. The group including the samples FS, PIIIB1, PIIB1 and group comprising the batches PIB7, PIB8 and PIB9 were distinguished by the low number of species and because they were most distant from zero. As indicated by PCA, some batches from the same palm tree were grouped based on the yeast population similarity. This grouping showed more resemblance among initial, middle and final batches.

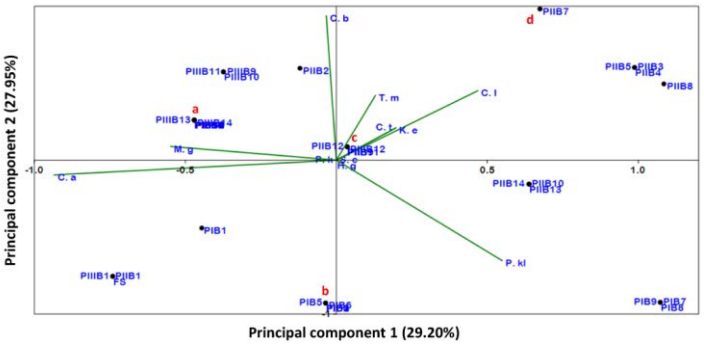

Figure 3 Principal component analysis of yeast species identified of different batches of Taberna production from three palm trees. C.b–*Candida blattae*, P. kl–*Pichia kluyveri*, S. c–*Saccharomyces cerevisiae*, H. g–*Hanseniaspora guilliermondii*, M. g–*Meyerozyma guilliermondii*, C. t–*Candida tropicalis*, C. a–*Candida akabanensis*, C. i–*Candida intermedia*, P. k– *Pichia kudriavzevii*, T. m–*Trichosporon moniliiforme*, K. e–*Kazachstania exigua*. Group a–(PIB10, PIB11, PIB12, PIB13, PIB14, PIIIB2, PIIIB3, PIIIB4, PIIIB5, PIIIB6, PIIIB7, PIIIB8, PIIIB13, PIIIB14), group b–(PIB2, PIB3, PIB4, PIB5, PIB6), group c–(PIIB9, PIIB11, PIIB12, PIIIB12), group d–(PIIB6, PIIB7)

Frequency percentage analysis

The frequency of positive samples of different species identified in batches from each palm tree is shown in Table 3. *Saccharomyces cerevisiae*, *H. guilliermondii* and *P. kudriavzevii* were common and abundant yeast species present in all batches of palm trees. In addition, in batches of palm tree I, *C. tropicalis* and *K. exigua* were detected with 93.33% frequency, followed by *C. akabanensis* (80%) and *M. guilliermondii* (66.67%). Other yeast species identified were *P. kluyveri* (53.33%) and *C. blattae* (33.33%). Furthermore, in batches of palm tree II, *C. tropicalis*, *C. blattae* and *K. exigua* were detected with a frequency of 86.67%, followed by *M. guilliermondii* (80%). Others species were also detected (Table 3). In batches of palm tree III, in addition to the three yeast species mentioned above, *M. guilliermondii* and *C. akabanensis* were detected with a frequency of 100%, followed by *C. tropicalis*, *C. blattae* y *K. exigua* (Table 3). *Candida intermedia* was detected only in one batch of palm tree II, and *T. moniliiforme* was found in batches of palm trees II and III. Yeast species with a low percentage of frequency (<35%) could be considered as sporadic strains in the process of Taberna production.

Table 3 Number of positive samples and frequency of yeasts species present in Taberna samples from three palm trees

Species	Palm tree I		Palm tree II		Palm tree III	
	Positive samples	Frequency (%)[a]	Positive samples	Frequency (%)[a]	Positive samples	Frequency (%)[a]
S. cerevisiae	15	100	15	100	15	100
H. guilliermondii	15	100	15	100	15	100
M. guilliermondii	10	66.67	12	80	15	100
C. akabanensis	12	80	9	60	15	100
C. tropicalis	14	93.33	13	86.67	13	86.67
C. blattae	5	33.33	13	86.67	13	86.67
C. intermedia	Nd	-	7	46.67	nd	-
P. kudriavzevii	15	100	15	100	15	100
P. kluyveri	8	53.33	7	46.67	3	20
K. exigua	14	93.33	13	86.67	13	86.67
T. moniliiforme	Nd	-	6	40	3	20

a–Frequency was calculated as the number of positive samples for a species divided by the total number of samples (15 samples) expressed as a percentage. nd: none detected

DISCUSSION

The PCR-DGGE analysis of yeast population in Taberna samples permitted the identification of 11 species named *S. cerevisiae*, *C. tropicalis*, *C. intermedia*, *C. akabanensis*, *C. blattae*, *K. exigua*, *P. kluyveri*, *P. kudriavzevii*, *H. guilliermondii*, *M. guilliermondii* and *T. moniliiforme*. Three yeast species (*C. blattae*, *C. akabanensis* and *T. moniliiforme*) were additionally identified to the species recently reported through culture-dependent techniques **(Santiago-Urbina *et al*., 2015)**. The distribution of these eleven yeast species among the batches of each palm tree, was similar but not identical, i.e. one or three yeast species were different in each batch. However, in some of these, the yeast population was identical. On the other hand, when the yeast population of each batch was compared among palm trees, a difference of one to four species was found, but in some cases, the population was identical. The growth and distribution of yeast species in Taberna fermentation is a process that depends on the types of vectors as inoculum source, and the environmental conditions. Both factors were detected at the site of the Taberna collection: environmental temperature around 38 ºC, and different insects, such as flies, ants, wasps, bees, dragonflies, mosquitoes, beetles and other species. **Nguyen *et al*. (2007)** suggest that *Drosophila* and small beetles are the first to inoculate fresh substrates. Furthermore, the material employed in scraping the canoe and in sap collection may be contributing to the dispersion of the yeasts between palm trees. Moreover, yeast can be distributed through the air, which acts as a support medium or carrier until the microorganisms fall into the substrate **(Garijo *et al*., 2008)**. Thus, the low diversity of species in the fresh sap can be attributed to limited environmental exposure, considering that this sample was collected immediately after the cavity in the stem (canoe) was created. *Saccharomyces cerevisiae*, *P. kudriavzevii*, and *H. guilliermondii* were the yeast species which predominated in all batches from the three palm trees, which was indicated by their frequency (Table 3). On the other hand, other yeast species appeared with less frequency among the samples. These results show that the sap fermentation is performed by a heterogeneous inoculum, which could be explained by different factors. First, during the collection and scraping process of the canoe, the microbiota adhered to the walls of the canoe is disrupted. Thus, the microbiota in the next batch could be different than that of the predecessor. Second, the microorganisms present in the sap and their growth can be related to their tolerance of the conditions of the sap in the canoe such as acidity, pH, ethanol content and oxygen availability **(Stringini *et al*., 2009; Santiago-Urbina *et al*., 2015)**, as well as the nutrient availability.

Seven genera (*Saccharomyces*, *Hanseniaspora*, *Candida*, *Meyerozima*, *Kazachstania*, *Pichia* and *Trichosporon*) were detected through of different batches sampled. These numbers of genera were higher than those detected in palm wine of Ghana (*Candida*, *Pichia*, *Saccharomyces* and *Zygosaccharomyces*) **(Stringini *et al*., 2009)** and lower than these (*Saccharomyces*, *Arthroascus*, *Issatchenkia*, *Candida*, *Trichosporon*, *Hanseniaspora*, *Kodamaea*, *Schizosaccharomyces*, *Trigonopsis* y *Galactomyces*) reported in Bandji (palm wine from Burkina Faso) **(Ouoba *et al*., 2012)**. The results showed that number of species was similar to these reported in other palm wine produced in different countries. Although several genera are reported in palm wine, only one or three of these are predominant. In the palm wine of Ghana and Burkina Faso, *S. cerevisiae* is the predominant yeast **(Stringini *et al*., 2009; Ouoba *et al*., 2012)**, and it is considered responsible for alcoholic fermentation **(Amoa-Awua *et al*., 2007; Stringini *et al*., 2009; Ouoba *et al*., 2012)**. In Taberna the presence of non-*Saccharomyces* yeast species could contribute to the particular organoleptic characteristics. For example, in wine production, *H. guilliermondii* has been reported to produce higher alcohol, esters, fatty acids, and heavy sulphur compounds **(Moreira *et al*., 2008, 2011)**. The environmental temperature in the place where the samples were collected was around 30 to 38°C. Thus, *Pichia kudriavzevii* and *C. tropicalis*, two yeast species reported as producers of ethanol at high temperature **(Jamai *et al*., 2001; Yuangsaard *et al*., 2013)** could be contributing to ethanol production in Taberna.

In general, PCR-DGGE analysis allowed a fast detection of the yeast species involved in the Taberna fermentation. However, the technique was not free of drawbacks; a multiple banding patterns were formed for a single species and comigration of band was found. *Saccharomyces cerevisiae* and *H. guilliermondii* yielded greater number of bands (11 and 9 bands, respectively) through DGGE. Similar results have been reported by **Arana-Sánchez *et al*. (2015)**, who found 7 bands associated with *Hanseniaspora* sp., and **Papalexandratou and De Vuyst (2011)** reported that the DGGE pattern of *S. cerevisiae* was composed of three bands. Multiple bands derived from a single sequence can be related with the GC-clamp primers, which undergo truncations, deletions, substitutions and insertions during PCR (**Rettedal *et al*., 2010**). Forty-base-GC-clamp primers containing multiple guanosines could form temporary quartets and four-stranded tetraplexes, leading truncations in subsequent cycles of PCR and premature elongation termination of PCR (**Keniry, 2001; Rettedal *et al*., 2010**), resulting products with different %GC and therefore T_m (**Rettedal *et al*., 2010**). Several drawbacks have been reported in some studies (**Nikolausz *et al*., 2005; Satokari *et al*., 2001; Hong *et al*., 2007; Thompson *et al*., 2002; Neilson *et al*., 2013**).

The findings suggested that PCR-DGGE is a good technique for the study of community structure, and it provides more precise information when coupled with cloning and sequencing of DGGE bands, allowing detection of multiband profile and comigration of bands. These techniques revealed the predominance of *H. guiliiermondii*, *S. cerevisiae* and *P. kudriavzevii*, followed by *C. tropicalis* and *K. exigua* in different batches of Taberna.

CONCLUSION

The present study provided new information about the yeast population associated with the Taberna fermentations, and defined the predominant yeast species determined by PCR-DGGE. This technique proved to be useful for study the structure of the yeast population. *Candida blattae*, *C. akabanensis* and *T. moniliiforme* were identified in the Taberna fermentation for the first time. These findings complement the information currently available on the microbiology of Taberna. The detection of predominant yeast species in all batches will be useful for the development of a yeasts starter culture to obtain a homogeneous final product.

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REFERENCES

Alcántara-Hernández, R. J., Rodríguez-Álvarez, J. A., Valenzuela-Encinas, F. A., Gutiérrez-Miceli, F. A., Castañón-González, H., Marsch, R., Ayora-Talavera, T., & Dendooven, L. (2010). The bacterial community in "taberna" a traditional beverage of Southern Mexico. *Letters in Applied Microbiology*, 51(5), 558-563. http://dx.doi.org/10.1111/j.1472-765x.2010.02934.x

Altschul, S. F., Madden, T. L., Shaffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped Blast and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, 25(17), 3389-3402. http://dx.doi.org/10.1093/nar/25.17.3389

Amoa-Awua, W. K., Sampson, E., & Tano-Debrah, K. (2007). Growth of yeasts, lactic and acetic acid bacteria in palm wine during tapping and fermentation from felled oil palm (*Elaeis guineensis*) in Ghana. *Journal of Applied Microbiology*, 102(2), 599-606. http://dx.doi.org/10.1111/j.1365-2672.2006.03074.x

Ampe, F., Ben Omar, N., Moizan, C., Wacher, C., & Guyot, P. J. (1999). Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations. *Applied and Environmental Microbiology*, 65, 5464-5473.

Balick, M. J. (1990). Production of coyol wine from *Acrocomia mexicana* (Arecaceae) in Honduras. *Economic Botany*, 44(1), 84-93. http://dx.doi.org/10.1007/bf02861070

Arana-Sánchez, A., Segura-García, L. E., Kirchmayr, M., Orozco-Ávila, I., Lugo-Cervantes, E., & Gschaedler-Mathis, A. (2015). Identification of predominant yeast associated with artisan Mexican cocoa fermentations using culture-dependent and culture-independent approaches. *World Journal of Microbiology and Biotechnology*, 31(2), 359-369. http://dx.doi.org/10.1007/s11274-014-1788-8

Byun, S. O., Fang, Q., Zhou, H., & Hickford, J. G. H. (2009). An effective method for silver-staining DNA in large numbers of polyacrylamide gels. *Analytical Biochemistry*, 385(1), 174-175. http://dx.doi.org/10.1016/j.ab.2008.10.024

Cocolin, L., Bisson, L. F., & Mills, D. A. (2000). Direct profiling of the yeasts dynamics in wine fermentations. *Fems Microbiology Letters*, 189(1), 81-87. http://dx.doi.org/10.1111/j.1574-6968.2000.tb09210.x

Fortin, N., Beaumier, D., Lee, K., & Greer, C. W. (2004). Soil washing improves the recovery of total community DNA from polluted and high organic content sediments. *Journal of Microbiological Methods*, 56(2), 181-191. http://dx.doi.org/10.1016/j.mimet.2003.10.006

Garijo, P., Santamaria, P., López, R., Sanz, S., Olarte, C., & Gutiérrez, A. R. (2008). The occurrence of fungi, yeasts and bacteria in the air of a Spanish winery during vintage. *International Journal of Food Microbiology*, 125(2), 141-145. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.03.014

Hong, H., Pruden, A., & Reardon, K. F. (2007). Comparison of CE-SSCP and DGGE for monitoring a complex microbial community remediating mine drainage. *Journal of Microbiological Methods*, 69(1), 52-64. http://dx.doi.org/10.1016/j.mimet.2006.11.016

Jamai, L., Sendide, K., Ettayebi, K., Errachidi, F., Hamdouni-Alami, O., TahriJouti, M. A., McDermott, T., & Ettayebi, M. (2001). Physiological difference during ethanol fermentation between calcium alginate-immobilized *Candida tropicalis* and *Saccharomyces cerevisiae*. *FEMS Microbiology Letters*, 204(2), 375-379. http://dx.doi.org/10.1111/j.1574-6968.2001.tb10913.x

Keniry, M. A. (2001) Quadruplex structure in nucleic acids. Biopolymers, 56(3), 123-146. http://dx.doi.org/10.1002/1097-0282(2000/2001)56:3<123::aid-bip10010>3.0.co;2-3

Moreira, N., Pina, C., Mendes, F., Couto, J. A., Hogg, T., & Vasconcelos, I. (2011). Volatile compounds contribution of *Hanseniaspora guilliermondii* and *Hanseniaspora uvarum* during red wine vinifications. *Food Control*, 22(5), 662-667. http://dx.doi.org/10.1016/j.foodcont.2010.07.025

Moreira, N., Mendes, F., Guedes de Pinho, P., Hogg, T., & Vasconcelos, I. (2008). Heavy sulphur compounds, higher alcohols and esters production profile of *Hanseniaspora uvarum* and *Hanseniaspora guilliermondii* grown as pure and mixed cultures in grape must. *International Journal of Food Microbiology*, 124(3), 231-238. http://dx.doi.org/10.1016/j.ijfoodmicro.2008.03.025

Neilson, J.W., Jordan, F.L., & Maier, M.R. (2013). Analysis of artifacts suggests DGGE should not be used for quantitative diversity analysis. *Journal of Microbiological Methods*, 92(3), 256-263. http://dx.doi.org/10.1016/j.mimet.2012.12.021

Nguyen, N. H., Suh, S-O., & Blackwell, M. (2007). Five novel *Candida* species in insect-associated yeast clades isolated from *Neuroptera* and others insects. *Mycologia*, 99(6), 842-858. http://dx.doi.org/10.3852/mycologia.99.6.842

Nikolausz, M., Sipos, R., Révész, S., Székely, A., & Márialigeti, K. (2005). Observation of bias associated with re-amplification of DNA isolated from denaturing gradient gels. *FEMS Microbiology Letters*, 244(2), 385-390. http://dx.doi.org/10.1016/j.femsle.2005.02.013

Osimani, A., Garofalo, C., Aquilanti, L., Milanović, V., & Clementi, F. (2015). Unpasteurized comercial boza as a source of microbial diversity. *International Journal of Food Microbiology*, 194, 62-70. http://dx.doi.org/10.1016/j.ijfoodmicro.2014.11.011

Ouoba, L., Kando, C., Parkouda, C., Sawadogo-Lingani, H., Diawara, B., & Sutherland, J. P. (2012). The microbiology of Bandji, palm wine of *Borassus akeassii* from Burkina Faso: identification and genotypic diversity of yeasts, lactic acid and acetic acid bacteria. *Journal of Applied Microbiology*, 113(6), 1428-1441. http://dx.doi.org/10.1111/jam.12014

Papalexandratou, Z., & De Vuyst, L. (2011). Assessment of the species composition of cocoa bean fermentations in different cocoa-producing regions using denaturing gradient gel electrophoresis. *Fems Yeast Research*, 11(7), 564-574. http://dx.doi.org/10.1111/j.1567-1364.2011.00747.x

Puerari, C., Magalhães-Guedes, K. T., & Schwan, R. F. (2015). Physicochemical and microbiological characterization of chicha, a rice-based fermented beverage produced by Umutina Brazilian Amerindians. *Food Microbiology*, 46, 210-217. http://dx.doi.org/10.1016/j.fm.2014.08.009

Rettedal, E. A., Clay, S., & Brözel, V. (2010). GC-clamp primer batches yield 16S rRNA gene amplicon pools with variable GC clamps, affecting denaturing gradient gel electrophoresis. *Fems Microbiology Letters*, 312(1), 55-62. http://dx.doi.org/10.1111/j.1574-6968.2010.02097.x

Santiago-Urbina, J. A., Verdugo-Valdez, A. G., & Ruíz-Terán, F. (2013). Physicochemical and microbiological changes during tapping of palm sap to produce an alcoholic beverage called "Taberna", which is produced in the south east of Mexico. *Food Control*, 33(1), 58-62. http://dx.doi.org/10.1016/j.foodcont.2013.02.010

Santiago-Urbina, J. A., Arias-García, J. A., & Ruiz-Terán, F. (2015). Yeast species associated with spontaneous fermentation of *taberna*, a traditional palm wine from the southeast of Mexico. *Annals of Microbiology*, 65(1), 287-296. http://dx.doi.org/10.1007/s13213-014-0861-8

Satokari, R.M., Vaughan, E.E., Akkermans, A. D. L., Saarela, M., & De Vos W. M. (2001). Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology*, 67(2), 504-513. http://dx.doi.org/10.1128/aem.67.2.504-513.2001

Solieri, L., Landi, S., De Vero, L., & Giudici, P. (2006). Molecular assessment of indigenous yeast population from traditional balsamic vinegar. *Journal of Applied Microbiology*. 101(1), 63-71. http://dx.doi.org/10.1111/j.1365-2672.2006.02906.x

Stringini, M., Comitini, F., Taccari, M., & Ciani, M. (2009). Yeast diversity during tapping and fermentation of palm wine from Cameroon. *Food Microbiology*, 26(4), 415-420. http://dx.doi.org/10.1016/j.fm.2009.02.006

Thompson, J. R., Marcelino, L. A., & Polz, M. F. (2002). Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. *Nucleic Acids Research*, 30(9), 2083-2088. http://dx.doi.org/10.1093/nar/30.9.2083

Valera, M. J., Torija, M. J., Mas, A., & Mateo, E. (2015). Acetic acid bacteria from biofilm of strawberry vinegar visualized by microscopy and detected by complementing culture-dependent and culture-independent techniques. *Food Microbiology*, 46, 452-462. http://dx.doi.org/10.1016/j.fm.2014.09.006

Yuangsaard, N., Yongmanitchai, W., Yamada, M., & Limtong, S. (2013). Selection and characterization of a newly isolated thermotolerant *Pichia kudriavzevii* strain for ethanol production at high temperature from cassava starch hydrolysate. *Antonie Van Leeuwenhoek*, 103(3), 577-588. http://dx.doi.org/10.1007/s10482-012-9842-8

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THE STUDY OF SEASONAL STEROID HORMONES IN MALE SIBERIAN STURGEON (*Acipenser baerii*) FOR DETERMINING GONADAL DEVELOPMENT STAGES

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ABSTRACT

The aim of this study is to investigate of steroid hormones of male Siberian sturgeon in different seasons. For this, blood sampling was taken seasonally from 11 male Siberian sturgeons (7 years old) and then steroid hormones including testosterone and 11-keto testosterone were analyzed by using ELISA. The results showed that testosterone had no significant differences during all the seasons ($p>0.05$) and the maximum level was in autumn. But 11ketotestesterone had significant differences between winter with other seasons ($p<0.05$). The min and max level of 11ketotestesterone was in autumn and winter, respectively. 11-keto testosterone hormone levels based on sexual maturation stages showed significant difference between stages III and IV with stage II ($p<0.05$). Also, there was significant differences between stage II with others in testosterone levels ($p<0.05$).

Keywords: Siberian sturgeon, testosterone, 11keto testosterone, sex determination

INTRODUCTION

Sturgeons are the oldest chondrostean fish called alive fossils **(Baker *et al*, 2005).** Today, the population of these fish is decreasing because of overfishing, pollution, habitat degradation and dams **(Keyvanshokooh *et al*, 2010; Moghim et al., 2002; Flynn et al., 2006; Billard and Lecointre., 2000).** The family of Acipenseridae has economical values such as the expensive caviar production. So from the point of aquaculture, the rearing whole female population is more profitable than mix population **(Hassanzadeh Saber *et al*, 2008; Keyvanshokooh *et al*, 2010).** Among the species of this family, Siberian sturgeon (*Acipenser baeri*) is one of the species that has advantages including easy adaptation with culture condition, resident to environmental changes **(Pyka & Kolman, 2003),** high growth rate, low maturity age and fast caviar production **(Adamek *et al.*, 2007).** So, to reach a successful management, it is important to know reproduction features **(Matsche *et al*, 2011).** *A. baerii* can reach a maximum length of 2 m and weight of 210 kg. However, it usually does not exceed 65 kg in weight with a maximum age of approximately 60 years. The spawning season is from May to June. *A. baerii* feeds predominantly on benthic organisms including chironomid larvae and river amphipods, isopods and polychaetes (**Sokolov and Vasil'ev, 1989**).
It is essential to know about sturgeons' reproduction cycle for optimal management of stocks and developing diagnostic methods of sex determination in low ages for aquaculture purposes **(Petochi *et al*, 2011).** Determining of reproduction status is difficult in sturgeons because they are sexually uniformic and don't have external dimorphism features and as well maturity age is late and have two or more reproduction cycle **(Barannikova *et al*, 2004).**
Reproduction in fishes regulates with a cascade of hormones along brain-pituitary- gonad called reproductive axis. Nervous gonadotropin releasing hormones directly affect pituitary gland and stimulate FSH and LH secretion. The most important steroid hormones are estradiol and 11-keto testosterone which play major roles in females and males, respectively. In males, 11-keto testosterone is the major regulator of spermatogenesis. FSH acts on sertoli cells and biosynthesises 11-keto testosterone by activating special enzymes like 11-β hydroxylase **(Cabrita et al., 2008).** Therefore, blood sex steroid hormones analysis is another gonadal development method which play important roles in gonadal development **(Scholz *et al*, 2009).** As mentioned above, the most important steroid hormones include testosterone (T), 11-keto testosterone (11-KT) and 17-β estradiol (E2). Plasma levels of these hormones in sturgeons are low before gonadal development stage and show considerable increase of testosterone and 11-keto testosterone levels by the cell division beginning in males. Also, in females and because of oocyte growth, testosterone and 17- β estradiol levels increase **(Barannikova *et al*, 2004; Davail- Cuisset *et al*, 2011).** Measuring of plasma sex steroid and glycophospholipoprotein levels has fewer stress compared with surgery and investigating of these hormones can be used in sex and gonadal development stages determination **(Craig *et al*, 2009).**

MATERIAL AND METHODS

Fish and culture condition

For this purpose, 23 individual of Siberian sturgeon (7 years) randomly selected in Shahid Dr. Beheshti Sturgeon Fish Propagation and Rearing Complex, tagged and transferred to fiberglass tanks (1× 1.8× 1.8 m^3) with 50 cm water depth. Water temperature was measured (16 C°).
First, fishes were anesthetized by clove powder (150 mg/lit) in water, then total length and weight measured by meter and scale, respectively, shown in table 1. Blood sampling was taken from caudal fin by using none heparinized syringe (5ml) and samples were centrifuged (4000 rpm in 10 min) and stored in -10 °C. ELISA procedure was used for male steroid hormones analysis (T & 11-KT, ng/ml) in endocrine gland research center in Tehran, Iran.

Data analysis

By general statistic and Excel, total length and weight mean and standard error were calculated. Independent samples t-test was performed to compare mean weight of females and males in each group and mann whitney test for T and 11-KT at 95% significance level, using SPSS 16.0 software. Data are presented as mean± standard error.

RESULT AND DISSCUSSION

The results showed that there were no significant differences in total length and weight of fish ($p> 0.05$). In table 1, total length and weight were given (Mean± SEM). The mean values of T and 11-KT are shown in table 2. Testosterone levels were 23±0.34, 22.3± 0.81, 23.5± 0.22 and 22.2± 0.58 (ng/ml) and 11-keto testosterone levels 1.35± 0.36, 1.38± 0.48, 1.01± 0.23 and 2.86± 0.4 (ng/ml) in spring, summer, autumn and winter, respectively. Testosterone hormone analysis demonstrated that there was no significant difference during all seasons ($p>0.05$)

and the highest level was in autumn. But in 11-keto testosterone, there was significant difference between winter with other seasons ($p<0.05$) and the highest and lowest levels were in winter and autumn, respectively (table.2).

The result of hormone analysis based on sexual maturity stages showed that testosterone had the highest and lowest levels in stages V and II, respectively (23.97 and 22.54 ng/ml, respectively). There was also significant difference between stage II with other stages ($p<0.05$). In other hand, the highest and lowest levels of 11-keto testosterone were in stages III and II (2.98 and 1.52 ng/ml, respectively). The results showed that there was significant difference between stages III and IV ($p<0.05$) (table.3).

Table 1 The mean of total length and weight of Siberian sturgeon 7 years old

Sex	No.	Total length (cm) (Mean ± SEM)	Total weight (kg) (mean± SEM)
female	12	100.57± 1.62	4.13± 0.18
male	11	94± 2.58	3.12± 0.34

Table 2 Steroid hormone levels in male Siberian sturgeon in different seasons

Hormone (ng/ml)	Spring (Mean±SEM)	Summer(Mean±SEM)	Autumn(mean±sem)	winter(mean±sem)
Testosterone(T)	23± 0.34 **a**	22.3± 0.81**a**	23.5± 0.22**a**	22.2± 0.58**a**
11-keto testosterone(11-KT)	1.35± 0.36**a**	1.38± 0.48**a**	1.01± 0.23**a**	2.86± 0.4**b**

Table 3 Steroid hormone levels in male Siberian sturgeon in different gonadal stages

Gonadal stage	Testosterone (Mean± SEM)	11-ketotestosterone (Mean± SEM)
II	22.54 ± 0.4**a**	1.52 ± 0.07**a**
III	23.6 ± 0.6**a**	2.98 ± 0.4**b**
IV	23.4 ± 0.2**a**	2.46 ± 0.3**b**
V	23.97± 0.1**a**	2.83 ± 0.1**b**

One of the gender and gonadal development stages determination is the measuring of steroid hormones such as testosterone, 11-keto testosterone and estradiol in wild sturgeons. In teleosts, dominant male androgens are testosterone and 11-keto testosterone that testosterone acts as a precursor of 11-keto testosterone and can participate in spermatogenesis process **(Aramli *et al*., 2013)**. Testosterone and 11-keto testosterone play roles in spermatogenesis and early spermatogenesis, respectively. Also, testosterone with enzymes such as 11-β hydroxylase and 11-β hydroxysteroid dehydrogenase converts to 11-keto testosterone. During spermatogenesis, androgen levels due to the decreasing conversion of 17β- hydroxyprogesteron to androgens and the change in the pathway of steroid to progestin formation decrease **(Barannikova *et al*., 2004)**.

In this research, 11-KT levels reach highest in winter and demonstrate an increasing trend in spring and summer, then a decreasing in autumn and finally increase in winter. Testosterone had a fluctuation trend during seasons which being increased in spring and autumn and decreased in summer and winter. Recently, plasma 11-KT level has been used for determining of immature Siberian sturgeons. According to **Cuisset *et al* (1991)**, 11-KT level used for immature Siberian sturgeon was 5 ng/ml, fish with 5ng/ml or more 11-KT classify as male; and fish with fewer than 5 ng/ml as female. If the threshold level decreased to 3ng/ml, immature females classification increases from 5% (4ng/ml) to 9% (3 ng/ml) for testosterone and 3% (4 ng/ml) to 7% (3 ng/ml) for 11-KT. Therefore, it seems that 4 ng/ml of testosterone or 11-KT can be used for immature females and males differentiation, but also should consider the error in immature males classification as female. However, in this paper 11-KT level was 2/86 ng/ml.

Sex steroids decrease after gonadal development near spawning and post spawning **(Craig *et al*., 2009; King *et al*., 1994; Rosenblum *et al*., 1987)**. In lake sturgeon, sex steroids increase in pre spawning and quickly decrease in post spawning **(McKinley *et al*., 1998)**. Similar results have been reported in other sturgeons about steroids and vitellogenin **(Amiri *et al*., 1996; Barannikova *et al*., 2004; Linarse-Casenave *et al*., 2003)**.

Despite of special sexual differences in productive hormone levels between male and female, these levels are not completely reliable just because of natural fluctuations during reproductive cycle. Vitellogenin produces in response to estradiol increasing during oocytes growth. Immature and males have much low or no vitellogenin. **Wildhabber *et al* (2007)** showed that with reproductive cycle existence, female vitellogenin level is 100 times higher than male. Since males mature in lower ages, and reproduce more than females, it is reasonable that more reproducing males than females exist during spawning cycle **(Craig *et al*., 2009)**. Although there is difference of steroid levels between species, plasma steroid profile is similar during sturgeon maturing and may be used for determining gender and maturing stages. Although there is error in classification of gender and maturing stages of white sturgeon by using plasma indexes, this method has advantages compared with biopsy **(Webb *et al*., 2002)**. According to **Bagheri *et al* (2008)**, the results showed that steroid hormone levels are influenced by gender, and testosterone in males and estradiol and progesterone in females were higher. Hormonal changes depend on environment temperature. The study on immature and mature *Acipenser sturio* in brackish water showed that the highest steroid hormones levels is testosterone. In immature fishes, estradiol and testosterone levels are so low that likely because of low gonadal development. Additionally, they showed seasonal fluctuation in immature fishes. Seasonal fluctuations of steroid hormones were seen not only in mature but also in immature fishes **(Davail- Cuisset *et al*, 2011)**.

Sex steroid hormone level measurements have little stress compared with surgery. Thus, hormone measuring can be used for determination of gender and gonadal development stage in the fishes **(Craig *et al*., 2009)**. In Persian sturgeon (*Acipenser persicus*) **(Viayeh *et al*., 2006)**, and shovelnose sturgeon (*Scaphirhynchus platorynchus*)**(Wildhaber *et al*., 2007)**, these hormones were reported by more than 90% accuracy for sexual stage determination. Also, it is reported that testosterone level in male white sturgeons in stage II were higher than females **(Webb *et al*., 2002).** This property results in early sex determination in different gonadal development stages. **Semenkova *et al* (2006)** confirmed this result but showed that effective and reliable application of this method needs investigating of reproductive status and testosterone, 11-keto testosterone as well as estradiol in different ages of male and females in various farms(pond, circular and warm water). **Chebanov and Gallich (2009)** stated that one of the endocrine method disadvantage is the high expense in field and laboratory conditions. Blood analysis requires related equipments, fish tagging system and employees for capturing as well as analysis time. However, **Sakomoto *et al* (2001)** suggested that changes in blood parameters among fishes can be influenced by other variables including sampling, capturing, correct handling way, captive condition and analysis method.

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REFERENCES

ADÁMEK, Z., PROKEŠ, M., BARUŠ, V., SUKOP, I. 2007. Diet and Growth of 1+ Siberian Sturgeon, *Acipenser baerii* in Alternative Pond Culture. *Turkish Journal of Fisheries and Aquatic Sciences*, 7(2), 153-160.

AMIRI, B.M., MAEBAYASHI, M., HARA, A., ADACHI, S., YAMAUCHI, K. 1996. Ovarian development and serum sex steroid and vitellogenin profiles in the female cultured sturgeon hybrid, the bester. *J. Fish Biol*, 48(4), 1164–1178. http://dx.doi.org/10.1111/j.1059-1996.tb01812.x

ARAMLI, M. S., KALBASSI, M. R., NAZARI, R.M. 2013. Comparative Study of Sex Steroid Levels of Persian Sturgeon, *Acipenser persicus* Males in Responding Negative and Positive to LHRH-A2 Hormone. *J Aquac Res Development*, 4(3), 177. http://dx.doi.org/10.4172/2155-9546.1000177

BAGHERI, T., HEDAYATI, A. A., JAFARIAN, A., ASKARI HESNI, M., MOVAHEDINIA, A. 2008. The study of seasonal fluctuation of steroid hormones in great sturgeon (*Huso huso*) cultured in brackish water condition. *Bulg. J. Agric. Sci,* 14(4), 432-435.

BAKER, D. W., WOOD, A. M., LITVAK, M. K., KIEFFER, J. D. 2005. Haematology of juvenile *Acipenser oxyrinchus* and *Acipenser brevirostrum* at rest and following forced activity. *Journal of Fish Biology*, 66, 208–221. http://dx.doi.org/10.1111/j.0022-1112.2005.00595.x

BARANNIKOVA, I. A., BAYUNOVA, L. V., SEMENKOVA, T. B. 2004. Serum levels of testosterone, 11-ketotestosterone and oestradiol-17β in three species of sturgeon during gonadal development and final maturation induced by hormonal treatment. *Journal of Fish Biology*, 64, 1330–1338. http://dx.doi.org/10.1111/j.1095-8649.2004.00395.x

BILLARD, R., LECOINTRE, G. 2000. Biology and conservation of sturgeon and paddlefish. *Reviews in fish Biology and fisheries*, 10(4). 355-392. http://dx.doi.org/10.1023/a:1012231526151

CABRITA, E., ROBLES, V., HERRAEZ, P. 2008. Methods in reproductive aquaculture: marine and freshwater species. CRC press, Taylor & Francis group. 17-18pp. ISBN 9780849380532

CHEBANOV, M. S and GALICH, E. V. 2009. Sturgeon hatchery manual. *FAO fisheries and aquaculture technical paper*. No.558. Ankara. 303 p. ISSN 2070-7010

CRAIG, J. M., PAPOULIAS, D. M, THOMAS, M. V., ANNIS, M. L., BOASE, J. 2009. Sex assignment of lake sturgeon (*Acipenser fluvescens*) based on plasma sex hormone and vitellogenin levels. *J. Appl. Ichthyol*, 25 (Suppl. 2), 60–67. http://dx.doi.org/10.1111/j.1439-0426.2009.01289.x

CUISSET, B., PELISSERO, C., NENEZ RODRIGUEZ, J., LE MENN, F. 1991. Use of plasma 11- ketotestosterone for sex determination in Siberian sturgeon *Acipenser baeri. In* A.P. Scott, S.P. Sumpter, D.E. Kime, and M.S. Rolfe, editors. *Reproductive physiology of fish* (Proceedings of the fourth international symposium) University of East Anglia, Norwich, UK, 272.

DAVAIL-CUISSET, B., ROUAULT, T., WILLIOT, P. 2011. Estradiol, testosterone, 11-ketotestosterone, 17, 20b-dihydroxy-4-pregnen-3-one and vitellogenin plasma levels in females of captive European sturgeon, *Acipenser sturio*. *J. Appl. Ichthyol*, 27, 666–672. http://dx.doi.org/10.1111/j.1439-0426.2011.01730.x

FLYNN, S. R., MATSUOKA, M., REITH, M., MARTIN-ROBICHAUD, D. J., BENFEY, T. J. 2006. Gynogenesis and sex determination in shortnose sturgeon, *Acipenser brevirostrum* Lesuere. *Aquaculture*, 253(1), 721–727. http://dx.doi.org/10.1016/j.aquaculture.2005.09.016

HASSANZADEH SABER, M., BARADARAN NOVEIRI, SH., POURKAZEMI, M., YARMOHAMMADI, M. 2008. Induction of gynogenesis in stellate sturgeon (*Acipenser stellatus* Pallas, 1771) and its verification using microsatellite markers. *Aquaculture Research*, 39, 1483-1487. http://dx.doi.org/10.1111/j.1365-2109.2008.02015.x

KEYVANSHOKOOH, S., GHARAEI, A. 2010. A review of sex determination and searches for sex-specific markers in sturgeon. *Aquaculture Research*, 41, e1-e7. http://dx.doi.org/10.1111/j.1365-2109.2009.02463.x

KING, W., THOMAS, P., HARRELL, R. M., HODSON, R. G., SULLIVAN, C. V., 1994. Plasma levels of gonadal steroids during final oocyte maturation of striped bass, *Morone saxatillis* L. *Gen. Comp. Endocrinol*, 95(2), 178–191. http://dx.doi.org/10.1006/gcen.1994.1115

LINARES-CASENAVE, J., KROLL, K. J., VAN EENENNAAM, J. P., DOROSHOV, S. I. 2003. Effect of ovarian stage on plasma vitellogenin and calcium in cultured white sturgeon. *Aquaculture* 221(1), 645–656. http://dx.doi.org/10.1016/s0044-8486(03)00134-0

MATSCHE, M. A., BAKAL, R. S., ROSEMARY, K. M. 2011. Use of laparoscopy to determine sex and reproductive status of shortnose sturgeon (*Acipenser brevirostrum*) and Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*). *J. Appl. Ichthyol*, 27, 627–636. http://dx.doi.org/10.1111/j.1439-0426.2011.01679.x

MCKINLEY, S., VAN DER KRAAK, G., POWER, G. 1998. Seasonal migrations and reproductive patterns in the lake sturgeon, *Acipenser fulvescens*, in the vicinity of hydroelectric stations in northern Ontario. *Environmental Biology of Fishes*, 51, 245-256. http://dx.doi.org/10.1023/A:10074930228238

MOGHIM, M., VAJIHI, A.R., VEHKINI, A., MASOUDIFARD, M. 2002. Determination of sex and maturity in *Acipenser stellatus* by using ultrasonography. *J. Appl. Ichthyol*, 18(4-5), 325–328. http://dx.doi.org/10.1046/j.1439-0426.2002.00423.x

PETOCHI, T., DI MARCO, P., DONADELLI, V., LONGOBARDI, A., CORSALINI, I., BERTOTTO, D., FINOIA, M.G., MARINO, G. 2011. Sex and reproductive stage identification of sturgeon hybrids (*Acipenser naccarii* ×*Acipenser baerii*) using different tools: ultrasounds, histology and sex steroids. *J. Appl. Ichthyol*, 27, 637–642. http://dx.doi.org/10.1111/j.1439-0426.2011.01715.x

PYKA, J., KOLMAN, R. 2003. Feeding intensity and growth of Siberian sturgeon *Acipenser baerii* Brandt in pond cultivation. *Arch. Pol. Fish*, 11(2), 287-294.

ROSENBLUM, P. M., PUDNEY, J. P., CALLARD, I. P. 1987. Gonadal morphology, enzyme histochemistry and plasma steroid levels during the annual reproductive cycle of male and female brown bullhead catfish, *Ictalurus nebulosus Lesueur*. *J. Fish Biol*, 31, 325–341. http://dx.doi.org/10.1111/j.1095-8649.1987.tb05239.x

SAKOMOTO, K., LEWBART, G.A., SMITH, T.M. 2001. Blood chemistry values of juvenile Red pacu, *Piaractus brachypomus*. *Vet Clinical Pathol*, 30(2), 50–52. http://dx.doi.org/10.1111/j.1939-165x.2001.tb00257.x

SCHOLZ, S., and KLÜVER, N. 2009. Effects of endocrine disrupters on sexual, gonadal development in fish, *sexual development* 3(2-3), 136-51. http://dx.doi.org/10.1159/000223078

SEMENKOVA, B., BAYUNOVA, L., WEBB, M., KOLMAKOV, N., ROMANOV, A., BARANNIKOVA, I. 2006. Effect of progestins on germinal vesicle break down in sturgeon follicles *in vitro*. *J Appl Ichthyol*, 22, 353-357. http://dx.doi.org/10.1111/j.1439-0426.2007.00983.x

SOKOLOV, L. I., VASILEV, V. P. 1989. *Acipenser baerii* Brandt – In:J.Holêik(ed). The freshwater fishes of Europe Vol.I, Pt II. General introduction to fishes, Acipenseriformes, AULA- Verlag, Wiesbaden, 263-284 pp.

VIAYEH, R. M., WEBB, M. A. H., HALLAJIAN, A., KAZEMI, R., YALI, M. P. 2006. Biochemical and morphometric parameters as indicators of sex and gonadal stages in maturing Persian sturgeon, *Acipenser persicus*. *J. Appl. Ichthyol*. 22, 364–368. http://dx.doi.org/10.1111/j.1439-0426.2007.00986.x

WEBB, M. A. H., FEIST, G. W., FOSTER, E. P., SCHRECK, C. B., FITZPATRICK, M. S. 2002. Potential classification of sex and stage of gonadal maturity of wild white sturgeon using blood plasma indicators. *T. Am. Fish. Soc*, 131(1), 132–142. http://dx.doi.org/10.1577/1548-8659

WILDHABER, M. L., PAPOULIAS, D. M., DE LONAY, A. J., TILLITT, D. E., BRYAN, J. L., ANNIS, M. L., 2007. Physical and hormonal examination of Missouri River shovelnose sturgeon reproductive stage: a reference guide. *J. Appl. Ichthyol*. 23, 382–401. http://dx.doi.org/10.11111/j.1439-0426.2007.00878.x

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GENETIC DIVERSITY ANALYSIS OF CASTOR (*RICINUS COMMUNIS* L.) USING SSR MARKERS

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ABSTRACT

The aim of this study was to assess genetic diversity within the set of 60 ricin genotypes using 5 SSR primers. Ten SSR primers revealed a total of 36 alleles ranging from 5 to 10 alleles per locus with a mean value of 7.20 alleles per locus. The PIC values ranged from 0.758 (Rco30) to 0.879 (Rco29) with an average value of 0.829 and the DI value ranged from 0.774 (Rco30) to 0.881 (Rco29) with an average value of 0.836. Probability of identity (PI) was low ranged from 0.002 (Rco29) to 0.015 (Rco30) with an average of 0.006. A dendrogram was constructed from a genetic distance matrix based on profiles of the 5 SSR loci using the unweighted pair-group method with the arithmetic average (UPGMA). According to analysis, the collection of 60 diverse accessions of castor bean was clustered into five clusters. Cluster 1 contained 14 genotypes, cluster 2 included 7 genotypes of ricin and cluster 3 contained 8 genotypes of ricin. Cluster 4 included 10 genotypes and cluster 5 contained 21 genotypes. We could not distinguish 4 genotypes grouped in cluster 1, RM-103 and RM-104 and genotypes RM-100 and RM-101, which are genetically the closest. Knowledge on the genetic diversity of castor can be used to future breeding programs for increased oil production to meet the ever increasing demand of castor oil for industrial uses as well as for biodiesel production.

Keywords: Castor; Dendrogram; Genetic diversity; Simple sequence repeat (SSR)

INTRODUCTION

Castor (*Ricinus communis* L.) is a cross-pollinated diploid (2n = 2x = 20) species belonging to the family *Euphorbiaceae* and genus *Ricinus*. Its seed oil has multifarious applications in production of wide industrial products ranging from medicines to lower molecular weight aviation fuels, fuel additives, biopolymers and biodiesel (**Ogunniyi, 2006**). Castor seeds contain around 50–55% oil which is rich in an unusual hydroxy fatty acid, ricinoleicacid which constitutes about 80–90% of the total fatty acids (**Jeong and Park, 2009**).
Knowledge of genetic variability is important for breeding programs to provide the basis for developing desirable genotypes. Genetic variability has been studied using molecular techniques, including amplified fragment length polymorphism (AFLP) (**Pecina-Quintero *et al*., 2013**), random amplified polymorphism DNA (RAPD) (**Petrovičová *et al*., 2015; Vivodík *et al*., 2015**), single nucleotide polymorphism (SNP) markers (**Foster et al., 2010**), simple sequence repeat (SSR) (**Tan *et al*., 2014**), start codon targeted polymorphism (SCoT) and inter simple sequencerepeat (ISSR) (**Kallamadi *et al*., 2015**). **Pecina-Quintero *et al*., (2013)** used four different AFLP primer pairs. In total, the four combinations of selective primers amplified 430 products, of which 228 were polymorphic. **Vivodík *et al*., (2014)** used 8 RAPD markers to detect genetic variability among the set of 40 castor genotypes. **Foster *et al*., (2010)** analyzed the population genetics of *R. communis* in a worldwide collection of plants from germplasm and determined the population genetic structure of 676 samples using single nucleotide polymorphisms (SNPs) at 48 loci. The goal of **Tan *et al*., (2014)** was to develop a more complete panel of SSR markers that can be used to construct a genetic map of castor bean and to examine genetic variation in this plant. The present investigation of **Kallamadi *et al*., (2015)** has been undertaken to assess the extent of genetic diversity in 31 accessions of castor using ISSR and SCoT primers. These markers are favourable as they exhibit high locus-specificity, high levels of variability, robustness towards genotyping, and a co-dominant mode of inheritance (**Woodhead *et al*., 2005**). So far, several investigations on the discrimination between crop genotypes using SSR markers have been carried out by **Siripiyasing *et al*., (2013); Fayyaz *et al*., (2014); Kanwal *et al*., (2014); Polat *et al*., (2015); Yousaf *et al*., (2015)**.
This study investigates the genetic diversity among 60 castor genotypes using 5 SSRs markers for the purpose of further breeding ricin.

MATERIAL AND METHODS

Plant material and DNA extraction

A total 60 castor genotypes (called RM-45 – RM-105) obtained from the breeding station Zeainvent Trnava Ltd. (Slovakia), were used in this study. Genotype of castor were grown in a cultivation box at temperature 27 °C and photoperiod 12 hours light and 12 hours dark. DNA of 60 genotypes of castor was extracted from leaves of 10 day old seedlings using the GeneJET Plant Genomic DNA Purification Mini Kit. Each sample was diluted to 20 ng in TE buffer (10 mmol Tris–HCl, pH 8.0 and 0.1 mmol EDTA, pH8.0), stored at -20 °C and resolved on agarose gel with the standard lambda DNA for determining the DNA concentration.

SSR and data analysis

Amplification of SSR fragments was performed according to **Bajay *et al*., (2009, 2011)** (Table 1). Polymerase chain reaction (PCR) were performed in 25 µl of a mixture containing 10.5 µl H_2O, 12.0 µl Master Mix (Genei, Bangalore, India), 0.75 µl of each primer (10 pmol) and 1 µl DNA (100 ng). Amplification was performed in a programmed thermocycler (Biometra, Germany) and amplification program consisted of an initial denaturing step at 94 °C for 1 min, followed by 35 cycles of amplification [94 °C (1 min), 1 min at the specific annealing temperature of each primer pair (Table 1), 72 °C (1 min)] and a final elongation step at 72 °C for 10 min. Amplification products were confirmed by electrophoresis in 7% denaturing polyacrylamide gels and silver stained and documented using gel documentation system Grab-It 1D for Windows. Data obtained from SSR analysis were scored as presence (1) or absence (0) of fragments for each castor genotype and entered into a matrix. Based on the similarity matrix, a dendogram showing the genetic relationships between genotypes was constructed using unweighted pair group method with arithmetic mean (UPGMA) by using the SPSS professional statistics version 17 software package. For the assessment of the polymorphism between castor genotypes and usability of SSR markers in their differentiation diversity index (DI) (**Weir, 1990**), the probability of identity (PI) (**Paetkau *et al*., 1995**) and polymorphic information content (PIC) (**Weber, 1990**) were used.

Table 1 List of SSR primers **(Bajay *et al*., 2009, 2011)**

Marker name	*Ta* (°C)	Repeat motif	Sequence of the primer (5´ - 3´)
Rco23	62	$(GA)_{15}(AG)_8$	F: CATGGATGTAGAGGGTCGAT R: CAGCCAAGCCAAAGATTTTC
Rco26	62	$(CT)_{19}$	F: TTGCTTGTCAAAGGGGAGTT R: TCATTTTGAGGGAGAAACCA
Rco29	60	$(GA)_7$	F: GGAGAAAAGAAAGGGAGAAGG R: GCCAAAAGCACACTTAATTTGA
Rco30	60	$(AG)_{19}$	F: TGAAACTTTGGAGCTTGGAGA R: GGTCCCACACATTCATACACA
Rco31	60	$(TC)_{12}(TCTA)_4(AC)_{10}$	F: ACAATGCGTGTGTCTGTGTG R: CCTCAACCCTTTGCTGTTTC

Ta- annealing temperature

RESULTS AND DISCUSSION

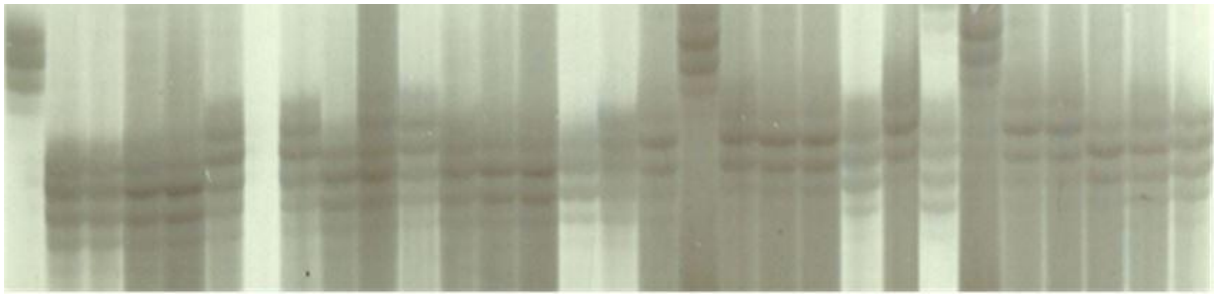
Five SSR primers were used for cultivar identification and estimation of the genetic relations among 60 ricin genotypes. All 5 SSR primers generated clear banding patterns with high polymorphism (Figure 1). Five SSR primers revealed a total of 36 alleles ranging from 5 (Rco30) to 10 (Rco29) alleles per locus with a mean value of 7.20 alleles per locus (Table 2). Results indicated the presence of wide genetic variability among different genotypes of castor. Variations in DNA sequences lead to polymorphism. Greater polymorphism is indicative of greater genetic diversity. The PIC values ranged from 0.758 (Rco30) to 0.879 (Rco29) with an average value of 0.829 and the DI value ranged from 0.774 (Rco30) to 0.881 (Rco29) with an average value of 0.836 (Table2). 100% of used SSR markers had PIC and DI values higher than 0.7 that means high polymorphism of chosen markers used for analysis. Probability of identity (PI) was low ranged from 0.002 (Rco29) to 0.015 (Rco30) with an average of 0.006 (Table 2).

A dendrogram was constructed from a genetic distance matrix based on profiles of the 5 SSR loci using the unweighted pair-group method with the arithmetic average (UPGMA). According to analysis, the collection of 60 diverse accessions of castor bean was clustered into five clusters. Cluster 1 contained 14 genotypes, cluster 2 included 7 genotypes of ricin and cluster 3 contained 8 genotypes of ricin. Cluster 4 included 10 genotypes and cluster 5 contained 21 genotypes (Figure 2). We could not distinguish 4 genotypes grouped in cluster 1, RM-103 - RM-104 and RM-100 - RM-101, which are genetically the closest.

Table 2 List of SSR primers, total number of bands and the statistical characteristics of the SSR markers used in castor.

Marker name	Number of alleles	DI	PIC	PI
Rco23	8	0.861	0.856	0.003
Rco26	6	0.822	0.816	0.006
Rco29	10	0.881	0.879	0.002
Rco30	5	0.774	0.758	0.015
Rco31	7	0.843	0.837	0.004
Average	7.20	0.836	0. 829	0.006

DI- diversity index, PIC- polymorphic information content, PI- probability of identity



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

Figure 1 PCR amplification products of 30 genotypes of castor produced by SSR marker Rco29. Lanes 1 - 30 are castor genotypes RM-45 – RM-74.

Similar results detected **Pecina-Quintero *et al*., (2013)** who used seven SSR markers and the profiles generated were collectively able to discriminate among 82 *R. communis* accessions and the six controls. **Kyoung-In *et al*., (2011)** used 28 SSR loci revealed polymorphisms in a castor bean collection consisting of 72 accessions. A total of 73 alleles were detected, with an average of 3.18 alleles per locus, and the polymorphism information content (PIC) ranged from 0.03 to 0.47 (mean = 0.26). **Gedil *et al*., (2009)** used-six primers for analysis of castor. The 6 SSR primers produced amplification products with alleles ranging from 1 to 2 for the parents and the hybrids. The present investigation of **Tan *et al*., (2013)** was to assess the genetic diversity in 58 accessions of castor. Seventy alleles were detected among the somaclones and their donors, with an average of 2.1 alleles per locus. Based on the profiles of the SSR loci, a dendrogram was constructed using the unweighted pair-group method with an arithmetic average (UPGMA). **Dhingani *et al*., (2012)** used 9 SSR primers for analysis of genetic diversity of castor. SSR analysis yielded 16 fragments, of which 11 were polymorphic, with an average PIC value of 0.87. SSR molecular markers have been used in population genetic studies **Yang *et al*., (2013); Žiarovská *et al*., (2013); Ahmad *et al*., (2014); Aslam *et al*., (2014); Maršálková *et al*., (2014); Lancíková *et al*., (2015)**.

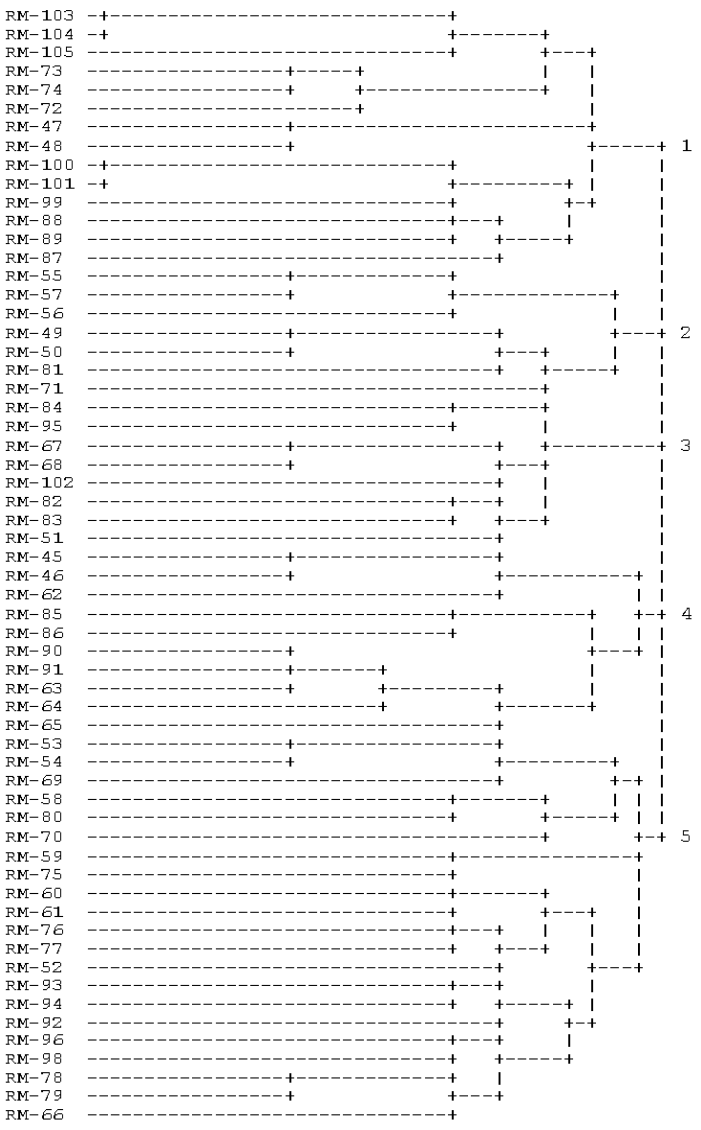


Figure 2 Dendrogram of 60 castor genotypes constructed based on 5 SSR markers.

CONCLUSION

In conclusion, a high level of genetic diversity exists among the castor accessions analyzed. According to analysis, the collection of 60 diverse accessions of castor bean was clustered into five clusters. We could not distinguish 4 genotypes grouped in cluster 1, RM-103 - RM-104 and RM-100 - RM-101, which are genetically the closest. A SSR marker system is a rapid and reliable method for cultivar identification that might also be used in quality control in certified seed production programs, to identify sources of seed contamination, and to maintain pure germplasm collections.

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REFERENCES

Ahmad, Z., Nisar, M., Mumtaz, A. S., Ghafoor, A. and Ali, S. (2014). SSR markers linked to seed size and seed weight in local and exotic chickpea germplasm reported from Pakistan. *Pak. J. Bot.*, 46(6), 2113-2120.

Aslam, K. and Arif, M. (2014). SSR analysis of chromosomes 3 and 7 of rice (*Oryza staiva* L.) associated with grain length. *Pak. J. Bot.,* 46(4), 1363-1372.

Bajay, M. M., Pinheiro, J. B., Batista, C. E. A., Nobrega, M. B. M., Zucchi, M. I. (2009). Development and characterization of microsatellite markers for castor (*Ricinus communis* L.), an important oleaginous species for biodiesel production. *Conserv.Genet.*, 1, 237-239. **http://dx.doi.org/10.1007/s12686-009-9058-z**

Bajay, M. M., Zucchi, M. I., Kiihl, T. A. M., Batista, C. E. A., Monteiro, M., Pinheiro, J. B. (2011). Development of a novel set of microsatellite markers for Castor bean, *Ricinus communis* (*Euphorbiaceae*). *Am. J. Bot.*, 98, 87-89. **http://dx.doi.org/10.3732/ajb.1000395**

Dhingani, R. M., Tomar, R. S., Parakhia, M. V., Patel, S. V. and Golakiya, B. A. (2012). Analysis of genetic diversity among different *Ricinus communis* genotypes for macrophomina root rot through RAPD and microsatellite markers. *International Journal of Plant Protection*, 5(1), 1-7.

Fayyaz, L., Farhatullah, Rabbani, M. A., Iqbal, S., Kanwal, M. and Nawaz, I. (2014). Genetic diversity analysis of *Brassica napus/Brassica campestris* progenies using microsatellite markers. *Pak. J. Bot.*, 46(3), 779-787.

Foster, J. T., Allan, G. J., Chan, A. P., Rabinowicz, P. D., Ravel, J., Jackson, P. J., Keim, P. (2010). Single nucleotide polymorphisms for assessing genetic diversity in castor bean (*Ricinus communis*). *BMC Plant Biol.*, 10, 13-18.

Gedil, M., Kolade, F., Raji, A., Ingelbrecht, I. and Dixon, A. (2009). Development of molecular genomic tools for verification of intergeneric hybrids between castor bean (*Ricinus communis* L.) and cassava (*Manihot esculenta* Crantz). *Journal of Food, Agriculture & Environment*, 7(2), 534-539.

Jeong, G. T., Park, D. H. (2009). Optimization of biodiesel production from castor oilusing response surface methodology. *Appl. Biochem. Biotech.*, 156, 1-11.

Kallamadia, P. R., Ganga Rao Nadigatlab, V. P. R., Mulpurib, S. (2015). Molecular diversity in castor (*Ricinus communis* L.). *Industrial Crops and Products*, 66, 271-281. **http://dx.doi.org/10.1016/j.indcrop.2014.12.061**

Kanwal, M., Farhatullah, Rabbani, M. A., Iqbal, S., Fayyaz, L. and Nawaz, I. (2014). The assessment of genetic diversity between and within brassica species and their wild relative (*Eruca sativa*) using SSR markers. *Pak. J. Bot.*, 46(4), 1515-1520.

Kyoung-In, S., Gi-An, L., Kyung-Ho, M., Do-Yoon, H., Yong-Jin, P., Jong-Wook, J., Sok-Young, L., Jae-Gyun, G., Chung-Kon, K., Myung-Chul, L. (2011). Isolation and characterization of 28 polymorphic SSR loci from Castor Bean (*Ricinus communis* L.). *J. Crop Sci. Biotech.*, 14(2), 97-103. **http://dx.doi.org/10.1007/s12892-010-0107-7**

Lancíková, V., Hlavačková, L., Žiarovská, J., Kubíková, H., Bežo, M., Ražná, K., Danchenko, M., Rashydov, N., Hajduch, M. (2015). Analysis of stability of trinucleotide TTC motifs in common flax planted in the Chernobyl area. *J Microbiol Biotech Food Sci.,* 4 (special issue 2), 70-72. **http://dx.doi: 10.15414/jmbfs.2015.4.special2.70-72**

Maršálková, L., Židek, R., Pokoradi, J., Golian, J., Belej, Ľ. (2014). Genetic diversity and relatedness among seven red deer (*Cervus Elaphus*) populations. *Potravinarstvo*, 8(1), 15-19. **http://dx.doi.org/10.5219/320**

Ogunniyi, D. S. (2006). Castor oil: a vital industrial raw material. *Bioresour. Technol.,* 97, 1086-1091.

Paetkau, D., Calvert, W., Stirling, I., Strobeck, C. (1995). Microsatellite analysis of population structure in Canadian polar bears. *Mol. Ecol.*, 4, 347-354.

Pecina-Quinteroa, V., Anaya-Lópeza, J. L., Núnez-Colína, C. A., Zamarripa-Colmenerob, A., Montes-Garcíac, N., Solís-Bonilla, J. L., Aguilar-Rangel, M. R. (2013). Assessing the genetic diversity of *castor bean* from Chiapas, México using SSR and AFLP markers. *Industrial Crops and Products*, 41, 134-143. **http://dx.doi.org/10.1016/j.indcrop.2012.04.033**

Petrovičová, L., Gálová, Z., Balážová, Ž., Vivodík, M., Wójcik-Jagła, M., Rapacz, M. (2015). Assessment of RAPD polymorphism in rye (*Secale Cereale* L.) genotypes. *J Microbiol Biotech Food Sci.,* 4 (special issue 2), 94-97. **http://dx.doi: 10.15414/jmbfs.2015.4.special2.94-97**

Polat, I., Turgutoglu, E. and Kurt, S. (2015). Determination of genomic diversity within mutant lemon (*Citrus limon* L.) and mandarin (*Citrus reticulata*) using molecular markers. *Pak. J. Bot.*, 47(3), 1095-1102.

Siripiyasing, P., Kaenratana, K., Mokkamul, P. and Chaveerach, A. (2013). Molecular assessment for genetic identification and stability of Cymbidium Sanderae (*Orchidaceae*). *Pak. J. Bot.*, 45(2), 519-523.

Tan, M., Yan, M., Wang, L., Yan, X., Fu, Ch., Wang, L. (2013). Replication of pistillate plants of *Ricinus communis* L. and investigationof the sex stability and genetic variation of the somaclones. *Industrial Crops and Products*, 50, 50–57. **http://dx.doi.org/10.1016/j.indcrop.2013.06.042**

Tan, M., Wu, K., Wang, L., Yan, M., Zhao, Z., Xu, J., Zeng, Y., Zhang, X., Fu, Ch., Xue, J., Wang, L. X., Yan, X. (2014). Developing and characterising *Ricinus communis* SSR markers by data mining of whole-genome sequences. *Mol Breeding*, 34, 893-904. **http://dx.doi.org/10.1007/s11032-014-0083-6**

Vivodík, M., Balážová, Ž., Gálová, Z. (2014). Dna analysis of ricin using RAPD technique. *J Microbiol Biotech Food Sci*., 3 (special issue 2), 181-183.

Yang, B., Ji, W., Wang, Ch., Xue, F., Ali, M., Wang, Y., Zhang, H. and Liu, X. (2013). Chromosome location and SSR markers of a stripe rust resistance gene from wheat (*Triticum aestivum*) line n9738. *Pak. J. Bot.,* 45(3), 719-724.

Yousaf, Z., Hu, W., Zhang, Y., Zeng, S. and Wang, Y. (2015). Systematic validation of medicinally important genus epimedium species based on microsatellite markers. *Pak. J. Bot.*, 44(2), 477-484.

Žiarovská, J., Ražná, K. and Labajová, M. (2013). Using of Inter Microsatellite Polymorphism to evaluate gamma-irradiated *Amaranth* mutants. *Emir. J. Food Agric.*, 25(9), 673-681. **http://dx.doi.org/10.9755/ejfa.v25i9.15879**

Weber, J. L. (1990). Informativeness of human (dC-dA)n x (dG-dT)n polymorphism. *Genomics*, 7: 524-530.

Weir, B. S. (1990). Genetic data analysis. *Methods for discrete population genetic data*. ISBN 0-87893-872-9

Woodhead, M., Russell, J., Squirrell, J., Hollingsworth, P. M., Mackenzie, K., Gibby, M., Powell, W. (2005). Comparative analysis of population genetic structure in *Athyrium distentifolium* (*Pteridophyta*) using AFLPs and SSRs from anonymous and transcribed gene regions. *Mol Ecol.*, 14, 1681-1695.

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IN VITRO ANTIOXIDANT ACTIVITY, ANTIMICROBIAL AND PRELIMINARY CYTOTOXIC ACTIVITY OF *CYNOMETRA RAMIFLORA*- A MANGROVE PLANT

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ABSTRACT

A mangrove medicinal plant *Cynometra ramiflora* (Family: Leguminosae) was selected to investigate the bioactivities namely antioxidant, antimicrobial and preliminary cytotoxic activity using methanol and chloroform extracts of the leaves and stems, respectively. In 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, 50% inhibitory concentration (IC_{50}) of the methanolic stem extract was found to be 31.62 µg.mL^{-1}. Reducing power of the same extract demonstrated consistent increase in a concentration-dependent manner and was comparable with quercetin while ferric reducing antioxidant power (FRAP) assay revealed potential total antioxidant capacity (84.0 mM Fe (II)/g of extract). In addition, the presence of total phenolics (96.2 mg GAE/g of extract), total flavonoids (166.4 mg QE/g of extract) and tannins content (80.4 mg GAE/g of extract) were determined in the methanolic stem extract. The chloroformic stem extract exhibited moderate antimicrobial activity against a number of bacterial strains while the MIC values of extracts were in the range from 62.5 to 500µg.mL^{-1}. The methanolic stem and leaf extracts demonstrated strong lethality in preliminary cytotoxicity assay using brine shrimp nauplii where the 50% lethal concentration (LC_{50}) values were 1.596 and 4.613 µg.mL^{-1} respectively. It can be therefore concluded that the methanolic extracts of *C. ramiflora* possess potential antioxidant, antimicrobial and strong preliminary cytotoxic activity and could be further exploited for prospective scientific exploration towards bioactive principles.

Keywords: *Cynometra ramiflora*, antioxidant, antimicrobial, cytotoxic

INTRODUCTION

Medicinal plants are the imperative sources of indigenous medical systems (**Ahmed *et al.*, 2009**) and they serve the primary health care needs of more than 80% of the people in the world (**Hassan *et al.*, 2009**). Plant derived natural products have been the single most productive source of leads for the discovery of novel drugs (**Cragg and Newman, 2013**). However, the global efforts remain still invigorated by the numerous attempts to analyze bioactivity and search for bioactive compounds from plant sources. In this context, mangrove ecosystem offers a large number of relatively untapped species in an exclusive climatic condition with unique metabolic profile.

Cynometra ramiflora Linn (Family: Leguminosae) is small to medium sized tree growing in the Sundarbans mangrove forest of Bangladesh. It is also distributed in the coastal mangrove system of India, Malaysia, Sri Lanka, China, Philippines, New Guinea and Australia (**Siraj *et al.*, 2013**). The plant is locally known as Shingra (**Hasan, 2000**) and also referred to as Balitbitan, Belangkan, Gal Mendora etc. (**Siraj *et al.*, 2013**). Historically, various plant parts of *C. ramiflora* such as leaves, roots and seeds are used in folk medicine to help cure a variety of diseases such as hypertension, diabetes, gout and hypercholesterolemia (**Muhtadi *et al.*, 2014**). Leaves are used as an anti-herpetic (any of several viral diseases) and roots are purgative (an agent for removing the bowels). Seed-derived oil is used to make lotion for skin diseases (**Siraj *et al.*, 2013**). Apart from the traditional use, *C. ramiflora* has been the subject to modern experimental endeavor during the last decade, particularly in bioassay-guided activity analysis. Different extracts of *C. ramiflora* have been reported to have antioxidant (**Bunyapraphatsara *et al.*, 2003; Muhtadi *et al.*, 2014**), antihyperglycemic (**Tiwari *et al.*, 2008**), cytotoxic (**Uddin *et al.*, 2011; Muhtadi *et al.*, 2014**), antibacterial and antinociceptive (**Siraj *et al.*, 2013**) activity. However, limited research have been done on chemical constituents and systematic bioactivity screening of *C. ramiflora,* only Muhtadi et al. **(2014)** reported that this plant possessed caffeic acid, apigenin and 3-(2,3,4-trihydroxyphenyl)-7-hydroxycoumarin.

The nature of the bioactivity analysis is permutative due to the uses of various plant parts and employing different solvent systems to extract the bioactive natural products. Therefore there remains the scope to analyze the bioactivity of *C. ramiflora*. Here we report the antioxidant, antimicrobial and preliminary cytotoxic activity of methanolic and choloroformic leaf and stem extract of *C. ramiflora*.

MATERIALS AND METHODS

Drugs and chemicals

The solvents and chemicals used in the present investigation were of analytical grade from Merck (Darmstadt, Germany) unless otherwise stated. 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), and Folin-Ciocalteu's reagent were obtained from Sigma Chemical Co. Ltd. (St. Louis, MO, USA).

Collection of plant material

Fully grown *C. ramiflora* was collected from Dhangmaree, Chadpai Range of the Sundarbans East Division situated at Bagerhat district, Bangladesh on 16th December, 2011 and collected plant samples were sent to Bangladesh National Herbarium, Dhaka, Bangladesh for taxonomical identification.

Preparation and extraction of plant material

C. ramiflora leaf and stem were separated from each other and then cleaned by gentle washing with distilled water followed by air drying for several weeks. The dried material was ground into coarse powder with a motorized plant grinder

(Capacitor Start motor, Wuhu Motor Factory, China). The powder was kept in a dry, cool and dark place in a suitable airtight container until analysis commenced. About 120 gm of powdered leaf and 160 gm of powdered stem was soaked into 440 mL and 500 mL petroleum ether respectively, in a clean, flat-bottomed glass container for a period of 5 days with occasional stirring and shaking. It was then filtered and after this first filtration, the remaining residues (approx. 115 gm powdered leaf and 157 gm powdered stem) were soaked into 400 mL and 470 mL chloroform respectively, kept for a period of 6 days and then filtered; then final remaining residues (113 gm of powdered leaf and 155 gm of powdered stem) were soaked into 390 mL and 450 mL methanol respectively, kept for a period of 6 days with occasional stirring and shaking and then filtered. Coarse plant material was separated from the mixture by pouring through a clean cloth filter. These extracts were passed through filter paper, and the filtrates were evaporated, yielding the chloroformic and methanolic extracts, respectively. The extraction process yielded six extracts among which chloroform and methanol extracts of leaf and stem were used in this study.

Determination of DPPH free radical scavenging activity

In determining DPPH free radical scavenging activity, different concentrations of the extracts were prepared in the range from 1.57 to 400 $\mu g.mL^{-1}$ and then 2 mL of 0.004% DPPH solution was added in each test tube. The test tubes were allowed to stand in the dark for 30 min to complete the reaction and then absorbance was recorded at 517 nm (**Gupta *et al.*, 2003**). The decrease in absorbance with respect to the blank was also measured. Control was prepared in the same way as the sample except addition of sample or standard. Percent scavenging activity was calculated using the formula: scavenging activity = $(A_0-A_1)/A_0 \times 100\%$, where A_0 is the absorbance of control, and A_1 is the absorbance of sample or standard. The experiment was carried out in triplicate.

Determination of reducing power

The reducing power of the extract was evaluated according to the method of **Oyaizu** et al (1986). According to this method different concentrations of extracts (25-400 $\mu g.mL^{-1}$) in 1 ml of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL 0.1 %) and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Blank was prepared same as sample without addition of extract or standard. Quercetin was used as standard. Reducing power of the extract was compared with standard quercetin by drawing curve plotting absorbance against concentration.

Determination of total antioxidant activity

Total antioxidant activity in the form of the FRAP assay was carried out according to the method of **Benzie and Strain** (1996). The stock solutions included 300 mM acetate buffer (3.1 gm $C_2H_3NaO_2$ hydrate and 16 mL $C_2H_4O_2$), pH 3.6, 10 mM $FeCl_3$ hydrate solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL of 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) solution and 2.5 mL $FeCl_3$ hydrate solution and then warmed at 37°C before using. Extracts of 0.2 mL were allowed to react with 3 mL of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were then taken at 593 nm. Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve. All determinations were performed in triplicate.

Determination of total phenolic, flavonoid and tannin content

Total phenolic content of the extracts was determined by using Folin-Ciocalteau assay (**Peteros and Mylene, 2010**) where extract or standard solution (25 to 250 $\mu g.mL^{-1}$) of 1 mL was added to distilled water (9 mL), and then 1 mL of FC reagent (10 times diluted with distilled water). After 5 minutes; 10 mL 7% Na_2CO_3 was added to the mixture, kept for 30 minutes and then the absorbance was measured at 750 nm using UV spectrophotometer. The percentage of total phenolics was calculated from the calibration curve of gallic acid plotted by using the similar procedure as the extracts and expressed as mg gallic acid equivalent (GAE)/g dried plant material.

Total flavonoid content of the extracts was determined by using an aluminium chloride colorometric assay (**Peteros and Mylene, 2010**) where extract or standard solution (25 to 400 $\mu g.mL^{-1}$) of 1 mL was added to distilled water (5 mL); 0.3 mL 5% $NaNO_2$ then added to the mixture followed by addition of 0.6 mL 10% $AlCl_3$ and 2 mL 1M NaOH after 5 min. Then absorbance was measured at 510 nm; percentage of total flavonoids was calculated from the calibration curve of quercetin plotted by using the similar procedure as the extracts and expressed as mg quercetin equivalent (QE)/g dried plant material.

Total tannin content in plant extract was determined by using Folin-Denis method as described by Polshettiwar et al. 2007 (**Polshettiwar and Ganjiwale, 2007**). Extract solution (1 ml of 100 $\mu g.ml^{-1}$) was mixed with 7.5 mL distilled water and 0.5 mL FC reagent. After 5 min, 1 ml of 35% sodium carbonate was added and the final volume was adjusted to 10 mL with distilled water. The mixture was allowed at room temperature for 30 minutes and absorbance was measured at 725 nm using spectrophotometer. Gallic acid was used to prepare the standard curve.

Screening for antimicrobial activity

Ten strains of microorganisms were tested in this study. Five Gram-positive bacteria include *Bacillus cereus* (ATCC 14579), *Bacillus megaterium* (ATCC 14581), *B. subtilis* (ATCC 6059), *Micrococcus luteus* (ATCC 4698), *Staphylococcus aereus* (ATCC 25923) and five Gram-negative bacteria including *Escherichia coli* (ATCC 8739), *Klebsiella pneumoniae* (ATCC 700603), *Pseudomonas aeruginosa* (ATCC 27833), *Shigella dysenteriae* (ATCC 26131) and *Salmonella typhi* (ATCC 13311). These strains were collected from the Microbiology Laboratory, Khulna University, Bangladesh, as pure cultures were used. The bacterial isolates were cultivated in nutrient broth at 37 °C for 24 hours.

Antimicrobial activity of *C. ramiflora* extracts was tested by disc diffusion method (**Bauer *et al.*, 1966**). Bacterial strains were maintained on the nutrient agar medium. The sterile filter paper discs were prepared by adding desired concentration (250 and 500 µg/disc) of extracts on the disc with the help of a micropipette. Standard tetracycline disc (30 µg/disc), discs containing extracts and control discs were then impregnated, incubated overnight at 37°C, checked for the zone of inhibitions and then diameters of inhibition zone were measured in millimeters(mm). Each measurement was carried out in triplicate.

Determination of MIC values

The extracts that showed antimicrobial activity in disc diffusion were later tested to determine the MIC value for each bacterial sample by using broth macrodilution method (**Nascimento *et al.*, 2000**) according to the Clinical and Laboratory Standards Institute (CLSI) protocol (**CLSI, 2010**). Briefly, Bacterial samples were grown in nutrient broth for 6 hours. Approximately 100 µL of these cultures containing 10^6cells/ml was inoculated in separate tubes with nutrient broth supplemented with different concentration of the extracts ranging from 7.8 to 500 $\mu g.mL^{-1}$. Afterwards 24 hours incubation at 37°C, the MIC of each sample was determined by measuring the optical density in the spectrophotometer (620 nm), comparing the sample readout with the non inoculated nutrient broth. Tetracycline was used as standard (0.05-2 µg/ml).

Brine shrimp lethality bioassay for preliminary cytotoxic activity

The eggs of the brine shrimp, *Artemia salina*, and sea water were collected from BRAC prawn hatchery, Sreeghat, Bagerhat, Bangladesh. Followed by 24h hatching, eggs matured and these were then called nauplii. *C. ramiflora* extracts were dissolved in DMSO and was added in test tubes in such a way that each tube contained 4 mL of sea water with different concentrations of extracts ranging from 5 to 320 $\mu g.mL^{-1}$. The final volume for each test tube was adjusted to 10 mL with artificial sea water and 10 living nauplii were introduced into each tube. After observing test tubes in the subsequent 24 hours, the number of survived nauplii was recorded (**Apu *et al.*, 2010**). The percentage of dead nauplii in the test and standard group was established by linear correlation when logarithm concentration versus percentage of mortality was plotted and LC_{50} value was calculated using Graphpad Prism Version 6.01 (GraphPad Software, Inc., USA).

RESULTS AND DISCUSSION

DPPH-scavenging assay

A number of methods are available for the determination of free radical scavenging activity but the DPPH assay employing the stable 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH) has received the maximum attention owing to its ease of use and its convenience (**Concepcion *et al.*, 1998**). The method is based on the reduction of 2, 2-diphenyl-1-picrylhydrazyl (purple color) to 2, 2-diphenyl-1-picrylhydrazine (colorless compound) in the presence of antioxidant substances having hydrogen donating groups (RH) such as phenolics, flavonoids compounds due to the formation of non radical DPPH-H form (**Paixao *et al.*, 2007**). Figure 1(A) and (B) shows the dose-dependent curve of DPPH radical scavenging activity of chloroformic and methanolic leaf and stem extracts of *C. ramiflora* respectively. The leaf extracts of chloroformic and methanolic solvent system exhibited 50% inhibition (IC_{50}) at a concentration of 537.03 and 97.72 $\mu g.mL^{-1}$ while the values for stem extracts in the same solvent systems were found to be 606.7 and 31.62 $\mu g.mL^{-1}$ respectively and standard quercetin showed 8.95 $\mu g.mL^{-1}$ (Table 1). This experiment showed that the stem methanolic extract showed the higher antioxidant activity than the other extract, which could be due

to their presence of phenolic components of sample and the leaf extract of same solvent system showed moderate activity. It has been reported that free radical scavenging activity is greatly influenced by the phenolic components of samples (**Cheung *et al.*, 2003**). Chloroform extracts of both leaf and stem exhibited very poor scavenging activity that could be attributed due to absence of active antioxidant compounds (**Almey *et al.*, 2010**) and influence of the polarity of extracting solvent system (**Moure *et al.*, 2001**).

Reducing power assay

Reducing capacity is associated with antioxidant activity (**Meir *et al.*, 1995**). The reducing power of the extracts was determined by direct electron donation in the reduction of ferri cyanide $[Fe(CN)_6]^{3-}$ to ferro cyanide $[Fe(CN)_6]^{4-}$. The reducing power of the extracts increases with the increase in amount of sample. Figure 2(A) and (B) shows the reductive capabilities of the plant extract compared to standard quercetin. The data represented here (Table 1) showed that the methanolic extracts have strong reducing power than the chloroformic extract. These could be attributed due to the influence of solvent system (**Moure *et al.*, 2001**) and presence of active reductones (**Duh, 1998**). It has been published that the phenolic components in plants may act in a similar fashion as reductones and terminating free radical chain reaction (**Liu and Yao, 2007**). Lu and Foo, (2001) reported that a direct correlation exists between polar polyphenols and the reducing power observed in phosphomolybdenum method (**Lu and Foo, 2001**).

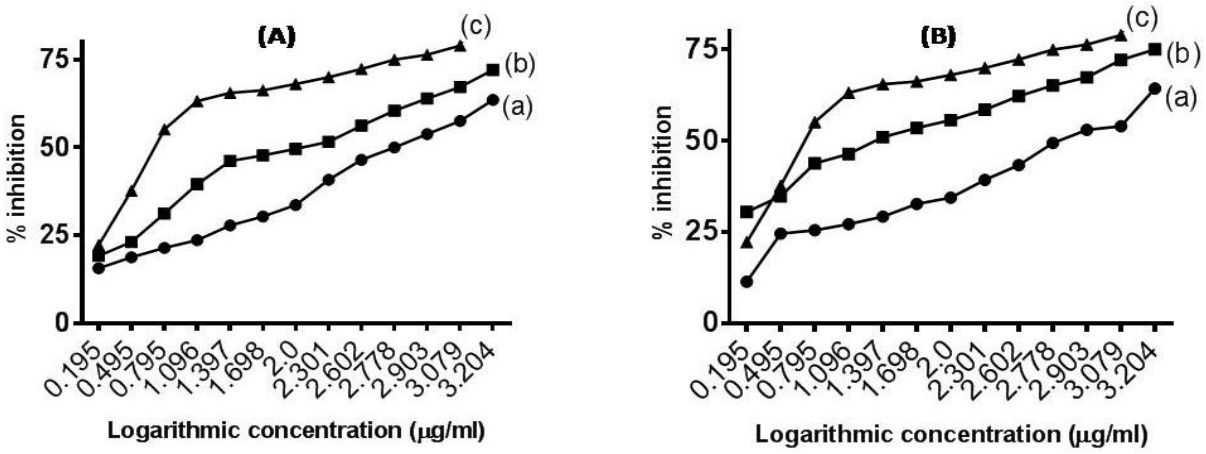

Figure 1 DPPH free radical scavenging activity of chloroform (a) and methanolic (b) extract of *C. ramiflora* leaf (A) and stem (B) in comparison with standard quercetin (c).

Results of total antioxidant capacity, total phenol, flavonoid and tannin content assay

In this study, the total antioxidant potential of sample was determined using FRAP assay. In this assay, the antioxidant efficiency of the leaf extracts under investigation was calculated with reference to the reaction signal given by an Fe^{2+} solution of known concentration, this representing a one-electron exchange reaction. A linear calibration curve of Quercetin, in the range of 6.25-100 µg.mL^{-1} with a coefficient of determination (R^2) value of 0.991 was obtained [Figure 3 (A)]. The results were expressed in µM Fe (II)/g of sample (Table 1). The results were expressed in µM Fe (II)/g of sample (Table 1). Chloroformic leaf and stem extracts of *C. ramiflora* showed the antioxidant capacity of 22.2 and 50 µM Fe (II)/g sample while leaf methanolic extract showed the moderate antioxidant capacity of 57.8 µM Fe (II)/g and stem methanolic extract showed the potential antioxidant capacity of 84.0 µM Fe (II)/g.

The results of total penolics, flavonoids and tannins content are presented in Table 1. In this study, total phenolics, flavonoids and tannins content were determined using calibration curves of standard [Figure 3(B), (C) and (D)]. The values of phenolics were found to be 6.3 and 84.8 mg in the leaf and 17.2 and 96.2 mg of GAE/g of dried plant material in the stem of chloroform and methanolic solvent system respectively (Table 1). The amount of total flavonoids content in leaf was found to be 68.4 and 86.2 mg and in stem 84.4 and 166.4 mg QE/g of dried plant material in chloroform and methanol respectively (Table 1). Total tannins content in leaf was 12.2 and 65 mg and in stem 23.2 and 80.4 mg GAE/g of dried plant material in chloroform and methanol respectively (Table 1). Analyzing the results, it has shown that the leaf and stem methanolic extracts have greater content of these compounds than the choloroformic extract. Polyphenolic compounds, like flavonoids, tannins and phenolic acids are commonly found in plants have been reported to have biological effects, including antioxidant activity (**Brown and Rice-Evan, 1998**) due to the presence of number of hydroxyl groups and their attachment with the aromatic ring, particularly in ortho-para position of phenolic structure, enhancing antioxidative capability of plant extract (**Sroka, 2005**). Phenolic compounds have the ability to donate electron that results the conversion of highly reactive free radicals to nonreactive stable molecules. Phenolic compounds not only neutralize lipid free radicals but also prevent the decomposition of highly reactive species (**Javanmardi *et al.*, 2003; Li *et al.*, 2009**). Flavonoids have a significant role in scavenging different reactive oxygen species like hydrogen peroxide, hydroxyl, peroxyl, superoxide anion etc. Tannin possesses strong antiradical and antioxidative properties especially due to large number of hydroxyl groups connected to the aromatic ring (**Sroka, 2005**). Tannins have also been reported to possess anticarcinogenic and antimutagenic potentials as well as antimicrobial properties (**Amarowicz 2007, Szollosi and Varga, 2002**). Considerable amount of total phenolic, flavonoid and tannin content was found in the methanolic extracts which are positively related with DPPH method, phosphomolybdenum method and FRAP assay, whereas chloroformic extract exhibited relatively lesser content and showed lower antioxidant activity.

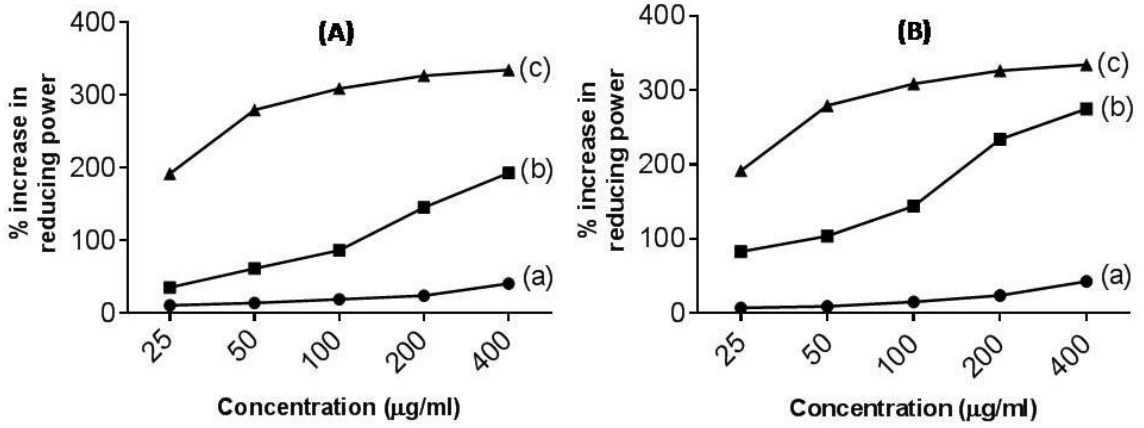

Figure 2 Reducing power of chloroform (a) and methanol (b) extract of *C. ramiflora* leaf (A) and stem (B) in comparison with standard Quercetin (c).

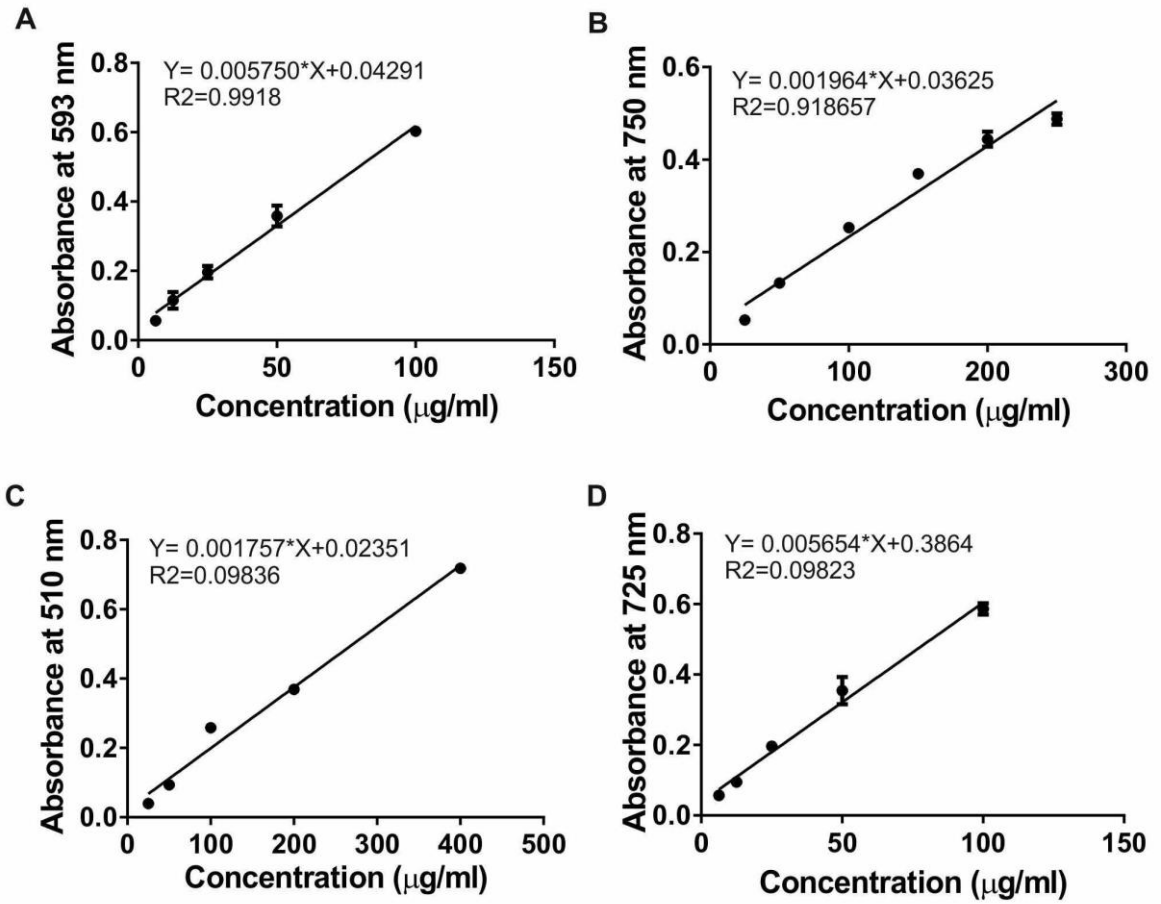

Figure 3 Standard calibration curve of, (A) Quercetin for determining total antioxidant power; (B) Gallic acid to estimate total phenolic content; (C) Quercetin to measure total flavonoid content and (D) Gallic acid to determine total tannin content of *C. ramiflora* leaf and stem, respectively.

Antimicrobial Screening

In this study, we have tested choloformic and methanolic leaf and stem extracts (250 and 500 µg/disc) of *C. ramiflora* for their antimicrobial activity against ATCC strains of some Gram-positive and Gram-negative bacteria. Standard antibiotic discs of Tetracycline (30 µg/disc) were used for comparison purpose. Among these extracts stem chloroformic extract showed better activity than the other extracts; however the leaf chloroformic extract failed to demonstrate any zone of inhibition against all the tested bacterial strains. Table 2 showed that the stem methanolic extract of *C. ramiflora* (250 µg/disc and 500 µg/disc) showed highest antimicrobial activity against *S. aereus* (17 mm and 18 mm) and *P. aeruginosa* (16 mm and 18 mm) respectively. Zone of inhibition of stem chloroformic extract of other strains includes, *B. cereus* (10 mm and 13 mm), *B. megaterium* (14 mm and 15 mm), *B. subtilis* (15 mm and 17 mm), *Micrococcus* (15 mm and 17 mm), *E. coli* (14 mm and 16 mm) and *S. dysenteriae* (9 mm and 15 mm) respectively. Stem methanolic extract of *C. ramiflora* showed moderate activity against seven bacterial strains and the zone of inhibition ranged between 6 to 12 mm and 7 to 14 mm, at the doses of 250 and 500 µg/disc, respectively (Table 3). Leaf methanolic extract showed the lowest antimicrobial activity against tested bacterial strains. Literature suggested that, the size of inhibitory zones <8 mm were considered as not active against microorganisms (**Bhalodia and Shukla, 2011**).

The MIC method was applied on extracts which showed antimicrobial activity in the disk diffusion method. The MIC values (µg.mL^{-1}) of the extracts against the experimental organisms obtained are represented in Table 2 and 3. The results revealed variability in the inhibitory concentrations of chloroformic and methanolic leaf and stem extracts of *C. ramiflora* against tested bacteria. The results revealed variability in the inhibitory concentrations of chloroformic and methanolic leaf and stem extracts of *C. ramiflora* against tested bacteria. The stem chloroformic extract showed activities in the range of MIC value 15 to 250 µg.mL^{-1} (Table 2). The lowest MIC value was found to be that of stem chloroformic extract 15 µg.mL^{-1} and 16 µg.mL^{-1} against *S. aereus* ATCC 25923 and *P. aeruginosa* ATCC 27833. The stem methanolic extract showed activities in the range of MIC value 63.1 to 482 µg.mL^{-1} (Table 2) and leaf methanolic extract showed 133 to 500 µg.mL^{-1} (Table 3).

In this study, Chloroformic stem extract exhibited significant antimicrobial activity against all Gram-positive bacterial strains and three Gram-negative bacterial strains. Particularly, *S. aureus* was found to be more sensitive than the other bacterial strains which may be due to its cell wall structure and outer membrane (**Zaika, 1988**). Our results suggest that Gram-positive bacteria are generally more sensitive to the plant extracts and this was consistent with other previous studies (**Ceylan and Fung, 2004**).

Table 1 Summary of results related to DPPH-free radical scavenging power assay, reducing power, total antioxidant capacity, total phenolic, flavonoid and tannin content of chloroformic and methanolic leaf and stem extracts of *C. ramiflora*.

Name of the tests	Plant parts	Solvent systems	Values in respective units	Regression equation	R^2value
DPPH (IC_{50} value in $\mu g.mL^{-1}$)	leaf	Ch	537.03	y = 15.18x + 8.546	0.956
		Mt	103.0	y = 15.46x + 18.89	0.935
	stem	Ch	606.7	y = 13.88x + 11.37	0.925
		Mt	32.65	y = 12.69x + 30.78	0.959
	Standard (Quercetin)				
Total antioxidant capacity (µM Fe (II)/g)	leaf	Ch	22.2 ± 0.013	Quercetin calibration curve : y = 0.005x + 0.042	0.991
		Mt	57.8 ± 0.015		
	stem	Ch	50 ± 0.007		
		Mt	84 ± 0.009		
Total phenolic content (mg GAE/g of dry plant material)	leaf	Ch	6.3 ± 0.002	Gallic acid calibration curve : y = 0.002x + 0.036	0.968
		Mt	84.8 ± 0.023		
	stem	Ch	17.2 ± 0.019		
		Mt	96.2 ± 0.01		
Total flavonoid content (mg QE/g of dry plant material)	leaf	Ch	68.4 ± 0.016	Quercetin calibration curve : y = 0.001x + 0.023	0.984
		Mt	86.2 ± 0.028		
	stem	Ch	85.4 ± 0.026		
		Mt	166.4 ± 0.019		
Total tannin content (mg GAE/g of dry plant material)	leaf	Ch	12.2 ± 0.01	Gallic acid calibration curve : 0.005x + 0.037	0.988
		Mt	65 ± 0.011		
	stem	Ch	23.2 ± 0.009		
		Mt	80.4 ± 0.009		
Reducing Power (highest value at maximum Conc.)	leaf	Ch	0.592 ± 0.006	Standard (Quercetin) : 1.673 ± 0.012	
		Mt	1.238 ± 0.089		
	stem	Ch	0.685 ± 0.005		
		Mt	1.454 ± 0.386		

Legend: Ch – Chloroform, Mt– Methanol

Brine Shrimp Lethality Bioassay

In brine shrimp lethality bioassay, LC_{50} values of chloroformic and methanolic leaf extracts of *C. ramiflora* was found to be 8.273 and 4.613 $\mu g.mL^{-1}$ respectively and stem extract at the same solvent system was found to be 5.297 and 1.596 $\mu g.mL^{-1}$, compared to positive control vincristine sulphate (VS) with a LC_{50} value of 0.128 $\mu g.mL^{-1}$ (Table 3). Percent mortality of methanolic stem extract of *C. ramiflora* was proximal to the standard indicating the strong cytotoxic activity of this extract (**Peteros and Mylene, 2010**). The crude extracts resulting in LC_{50} values less than 250 $\mu g.mL^{-1}$ are considered significantly active (**Kabir *et al.*, 2012**).

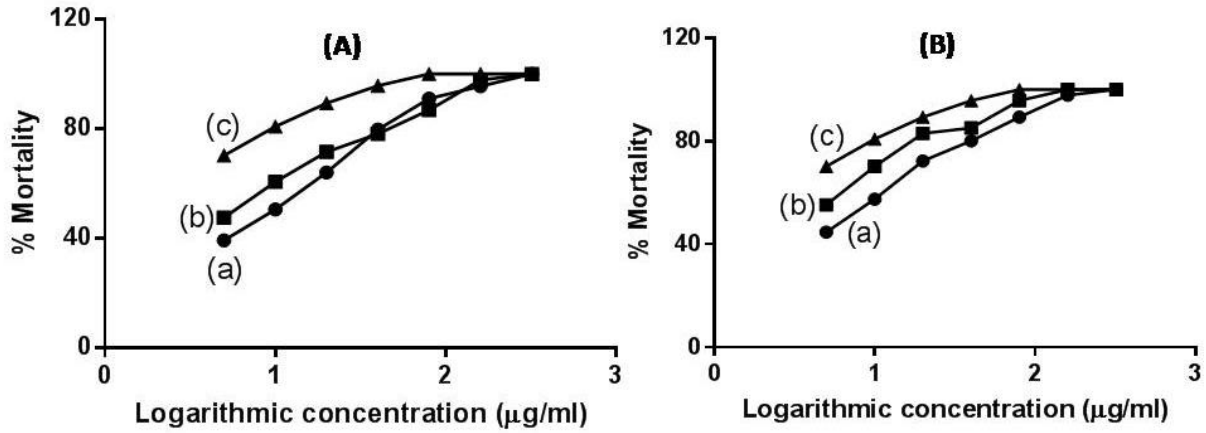

Figure 4 Brine shrimp lethality bioassay of chloroform (a) and methanolic (b) extract of *C. ramiflora* leaf (A) and stem (B) in comparison with standard vincristine sulfate (c).

Table 2 Results of disc diffusion assay of chloroformic and methanolic leaf and stem extracts of *C. ramiflora* and MIC values of the extracts against tested bacterial strains.

Tested Organisms	*C. ramiflora* leaves Mt-OH extract			*C. ramiflora* stem $CHCl_3$ extract			*C. ramiflora* stem Mt-OH extract			Positive control Tetracycline
	Zone of inhibition (mm)		MIC ($\mu g.mL^{-1}$)	Zone of inhibition (mm)		MIC ($\mu g.mL^{-1}$)	Zone of inhibition (mm)		MIC ($\mu g.mL^{-1}$)	Zone of inhibition (mm)
	250 (µg/disk)	500 (µg/disk)		250 (µg/disk)	500 (µg/disk)		250 (µg/disk)	500 (µg/disk)		30 (µg/disk)
Gram-positive bacteria										
B. cereus (ATCC 14579)	7±1	9±1	411.6	10±2.6	13±1	150	8±1.7	11±1.8	482	32
B. megaterium (ATCC 18)	8±1	9.5±2.1	350	14±1	15±1	119.7	9±1.3	10±1	251	35
B. suntilis (ATCC 6059)	6±0.5	8±1	500	15±1.7	17±0.4	27	6±0	7±0	400	30
Micrococcus	6±0	8±1.7	415	15±1	17±0	101.1	6±0	8±0	462	34
S. aereus (ATCC 25923)	9±0	12±1.1	133	17±1	18±0	15	10±1	14±0	63.1	29
Gram-negative bacteria										
E. coli (ATCC 8739)	8±1.2	9±0	369	14±1.8	16±0	82.2	12±1.7	14±0	82	28
Klebsiella(ATCC 700603)	-	-	-	-	-	-	-	-	-	-
P. aeruginosa (ATCC 27833)	7.5±1	11±0	321.6	16±1	18±0	16±0	10±1	13±0	70	32
S. dysenteriae (ATCC 26131)	-	-	-	9±1	15±0	250	-	-	-	25
S. typhi (ATCC 13311)	-	-	-	-	-	-	-	-	-	-

The brine shrimp lethality bioassay is normally conducted to draw inferences on the safety of the plant extracts and to further depict trends of their biological activities and considered as a useful tool for the preliminary assessment of toxicity (**Solis *et al.*, 1993**). The LC_{50} values of the plant extracts were obtained by a plot of percentage of the shrimp nauplii killed against the concentrations of the extracts. In this study, percent mortality of stem methanolic extract of *C. ramiflora* is nearer to the standard, indicating the extract could be a potential source of cytotoxic and/or pharmacologically active agent. This is in also agreement with the previous studies. Methanolic extracts of *Cynometra ramiflora had* selective cytotoxicity against three human cancer-cell lines (gastric: AGS; colon: HT-29; and breast: MDA-MB-435S) (**Uddin *et al.*, 2011**) and leaf ethanolic extract was most active against WiDR cell lines (**Muhtadi *et al.*, 2014**).

Table 3 Result of brine shrimp lethality bioassay of chloroformic and methanolic leaf and stem extracts of *C. ramiflora.*

Plant parts	Solvent system	LC_{50} value ($\mu g.mL^{-1}$)	Regression equation	R^2
leaf	**Ch**	**8.279**	**y = 35.50x + 17.40**	**0.963**
	Mt	**4.613**	**y = 29.32x + 30.51**	**0.979**
stem	**Ch**	**5.297**	**y = 31.26x + 27.34**	**0.963**
	Mt	**1.596**	**y = 24.45x + 45.03**	**0.979**
Vincristine sulfate (standard)	----------	**0.128**	**y = 64.64 + 16.39x**	**0.821**

CONCLUSION

Findings from the present study indicate potential antioxidant and strong cytotoxic activity of *C. ramiflora* stem. Confirmation of the bioactivity, particularly cytotoxic activity, in vitro and in vivo set up is the immediate step ahead. The presence of antioxidant and cytotoxic activity thus implicates *C. ramiflora* for potential neutral and pharmaceutical applications, respectively. Thus, bioactivity guided appropriate separation scheme might be employed for the identification of bioactive compound(s) in pure form.

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REFERENCES

AHMED, Z. U., HASAN, M. A., BEGUM, Z. N. T., KHONDKER, M., KABIR, S. M. H., AHMED M., AHMED, A. T. A., RAHMAN, A. K. A., HAQUE, E. U. (2009). Encyclopedia of Flora and Fauna of Bangladesh: Index Volume-Flora. Asiatic Society of Bangladesh, 1st ed. Dhaka, Bangladesh.

ALMEY, A. A. A., KHAN, A. J. C., ZAHIR, S. I., SULEIMAN, M. K., AISYAH, M. R., RAHIM, K. K. (2010). Total phenolic content and primary antioxidant activity of methanolic and ethanolic extracts of aromatic plants' leaves. *International Food Research Journal*, 17, 1077-1084.

AMAROWICZ, R. (2007). Tannins: the new natural antioxidants. *European Journal of Lipid Science and Technology*, 109(6): 549-551. http://dx.doi.org/10.1002/ejlt.200700145

APU, A. S., MUHIT, M. A., TAREQ, S. M., PATHAN, A. H., JAMALUDDIN, A. T. M. (2010). Antimicrobial activity and Brine Shrimp Lethality Bioassay of the leaves extract of *Dillenia indica* linn. *Journal of Young Pharmacists*, 2(1), 50-53. http://dx.doi.org/10.4103/0975-1483.62213

BAUER, A. W., KIRBY, W. M., SHERRIS, J. C., TRUCK, M. (1966). Antibiotic susceptibility testing by a standardized single disc method. *American Journal of Clinical Pathology*, 45(4), 493-496.

BENZIE, I. F. F., STRAIN, J. J. (1996). Ferric reducing ability of plasma (FRAP) a measure of antioxidant power: The FRAP assay. *Analytical Biochemistry*, 239(1): 70-6.

BHALODIA, N. R., SHUKLA, V. J. (2011). Antibacterial and antifungal activities from leaf extracts of *Cassia fistula* l.: An ethnomedicinal plant. *Journal of Advanced Pharmaceutical Technology & Research*, 2(2), 104–109. http://dx.doi.org/10.4103/2231-4040.82956

BROWN, J. E., RICE-EVANS, C. A. (1998). Luteolin-Rich Artichoke Extract Protects Low Density Lipoprotein from Oxidation in vitro. *Free Radical Research*, 29(3): 247-255. http://dx.doi.org/10.1080/10715769800300281

BUNYAPRAPHATSARA, N., JUTIVIBOONSUK, A., SORNLEK, P., THERATHANATHORN, W., FONG, H. H. S., PEZZUTO, J. M., KOSMEDER, J. (2003). Pharmachological studies of plants in the mangrove forest. *Thai Journal of Phytopharmacy*, 10(2), 1-12.

CEYLAN, E., FUNG, D. Y. C. (2004). Antimicrobial activity of spices. *Journal of Rapid Methods & Automation in Microbiology*, 12(1), 1-55. http://dx.doi.org/10.1111/j.1745-4581.2004.tb00046.x

CHEUNG, L. M., CHEUNG, P. C. K., OOI, V. E. C. (2003). Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chemistry*, 81(7), 249-255. http://dx.doi.org/10.1016/S0308-8146(02)00419-3

Clinical and Laboratory Standards Institute (CLSI) (2012). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. Wayne, PA, USA.

CONCEPCION, S.-M., LARRAURI, J.A., SAURA-CALIXTO, F. 1998. A procedure to measure the antiradical efficiency of polyphenols. *Journal of the Science of Food and Agriculture*, 76 (2), 270-276. http://dx.doi.org/10.1002/(SICI)1097-0010(199802)76:2<270::AID-JSFA945>3.0.CO;2-9

CRAGG, G.M., NEWMAN, D.J. (2013) Natural products: a continuing source of novel drug leads. *Biochimica et Biophysica Acta*, 1830(6), 3670-3695. doi: http://dx.doi.org/10.1016/j.bbagen.2013.02.008

DUH, P. D. (1998). Antioxidant activity of Budrock (*Arctium lappa*, L.): its scavenging effect on free radical and and active oxygen. *Journal of American Oil and Chemistry*, 75(4), 455-461. http://dx.doi.org/10.1007/s11746-998-0248-8

GUPTA, M., MAZUMDAR, U. K., SIVAHKUMAR, T., VAMSI, M. L. M., KARKI, K., SAMBATHKUMAR, R., MANIKANDAN, L. (2003). Antioxidant and anti-inflammatory activities of *Acalypha fruticosa*. *Nigerian Journal of Natural Products and Medicine*, 7, 25-29. http://dx.doi.org/10.4314/njnpm.v7i1.11700

HASAN, A., RAHMAN, S., DEEBA, F., MAHMUD, S. (2009). Antimicrobial activity of some plant extracts having hepatoprotective effects. *Journal of Medicinal Plants Research*, 3(1), 020-023

HASAN, D. Z. (2000). *Some plants of Sundarbans* (1st ed.). Techtona computing, Chittagong, Bangladesh.

JAVANMARDI, J., STUSHNOFF, C., LOCKE, E., VIVANCO, J. M. (2003). Antioxidant activity and total phenolic content of Iranian *Ocimum* accessions. *Food Chemistry*, 83(4): 547-550. http://dx.doi.org/10.1016/S0308-8146(03)00151-1

KABIR, S. M. T., SABRIN, F., HASAN, M. N., ISLAM, K. D., BILLAH, M. M. (2012). Investigation of preliminary cytotoxic activity of ethanolic bark and leaf extracts of Amoora cucullata. *Journal of Innovation & Development Strategy*, 6(2): 24-28.

LI, H. Y., HAO, Z. B., WANG, X. L., HUANG, L., LI, J. P. (2009). Antioxidant activities of extracts and fractions from *Lysimachia foenumgraecum* Hance. *Bioresource Technology*, 100(2): 970-974. http://dx.doi.org/10.1016/j.biortech.2008.07.021

LIU, Q., YAO, H. (2007). Antioxidant activities of barley seeds extracts. *Food Chemistry*, 102(3), 732-737. http://dx.doi.org/10.1016/j.foodchem.2006.06.051

LU, Y., FOO, L. Y. (2001). Antioxidant activity of polyphenols from sage (Salvia officinalis). *Food Chemistry*, 75(2), 197-202. http://dx.doi.org/10.1016/S0308-8146(01)00198-4

MEIR, S., KANNER, J., AKIN, B., HADAS, S. P. (1995). Determination and involvement of aqueous reducing compounds in oxidative defense system of various senescing leaves. *Journal of Agricultural and Food Chemistry*, 43(7), 1813–1819. http://dx.doi.org/10.1021/jf00055a012

MOURE, A., CRUZ, J. M., FRANCO, D., DOMINGUEZ, J. M., SINEIRO, J., DOMINGUEZ, H., JOSE-NUNEZ, M., PARAJO, J. C. (2001). Natural antioxidants from residual sources. *Food Chemistry*, 72(2), 145-171. http://dx.doi.org/10.1016/S0308-8146(00)00223-5

MUHTADI, HARYOTO, AZIZAH, T., INDRAYDHA, P., SUHENDI, A., YEN, K., H. (2014). Antioxidant, cytotoxic activities and chemical constituents of *Cynometra ramiflora* linn. *International Journal of Indigenous Medicinal Plants*, 47(1), 2051-4263.

NASCIMENTO, G. G. F., LOCATELLI, J., FREITAS, P. C., SILVA, G. L. (2000). Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. Brazilian Journal of Microbiology, 31(4), 1517-8382. http://dx.doi.org/10.1590/S1517-83822010000300007

OYAZU, M. (1986). Studies on product of browning reaction. Antioxidative activities of products of browning reaction prepared from glucosamine. *The Japanese Journal of Nutrition and Dietetics*, 44(7), 307-315.

PAIXAO, N., PERESTRELO, R., MARQUES, J. C., CAMARA, J. S. (2007). Relationship between antioxidant capacity and total phenolic content of red, rose and white wines. *Food Chemistry*, 105, 204–214. http://dx.doi.org/10.1016/j.foodchem.2007.04.017

PETEROS, N. P., UY, M. M. (2010). Antioxidant and cytotoxic activities and phytochemical screening of four Philippine medicinal plants. *Journal of Medicinal Plants Research*, 4(5): 407-414.

POLSHETTIWAR, S. A., GANJIWALE, R. O. (2007). Spectrophotometric estimation of Total tannins in some ayurvadic eye drops. *Indian Journal of Pharmaceutical Science*, 69(4): 574-576. http://dx.doi.org/10.4103/0250-474X.36949

SIRAJ, A.M., MALIK, S., EMRUL, H., SANJANA, S., FARJANA, Y. (2013). Evaluation of neuropharmachological, antibacterial and antinociceptive activity of methanolic extract of the bark of *Cynometra ramiflora* linn. (leguminosae). *International Journal of Research in Ayurveda and Pharmacy*, 4(2), 192-197. http://dx.doi.org/10.7879/2277-434304220

SOLIS, P. N., WRIGHT, C. W., ANDERSON, M. M., GUPTA, M. P., PHILLIPSON, J. D. (1993). A microwell cytotoxicity assay using Artemia salina. *Planta Medica*, 59(3): 250-252.

SROKA Z. A. (2005). Antioxidative and antiradical properties of plant phenolics. Department of Pharmacognosy, Wroclaw University of Medicine, pl. Nankiera 1, 50-140 Wrocław, Poland.

SZOLLOSI, R., VARGA, I. S. (2002). Total antioxidant power in some species of Labiatae Adaptation of FRAP method. *Acta Biological Szegediensis*, 46(3-4): 125-127.

TIWARI, P., RAHUJA, N., KUMAR, R., LAKSHMI, V., SRIVASTAVA, M. N., AGARWAL, S., C., RAGHUBIR, R., SRIVASTAVA, A. K. (2008). Search for antihyperglycemic activity in few marine flora and funa. *Indian Journal of Science and Technology*, 1(5), 1-5. http://dx.doi.org/10.17485/ijst/2008/v1i5/29349

UDDIN, S. J., GRICE, I. D., TIRALONGO, E. (2009). Cytotoxic effects of Bangladeshi medicinal plant extracts. *Evidence-Based Complementary and Alternative Medicine*, 2011(2011), 7. http://dx.doi.org/10.1093/ecam/nep111

ZAKIA, L. L., (1988). Spices and herbs - their antimicrobial activity and its determination. *Journal of Food Safety*, 9(2), 97-118. http://dx.doi.org/10.1111/j.1745-4565.1988.tb00511.x

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PHENOLIC COMPOUDNS IN TRADITIONAL BULGARIAN MEDICAL PLANTS

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ABSTRACT

A number of plants and plant products have medicinal properties that have been validated by recent scientific developments throughout the world, owing to their potent pharmacological activity, low toxicity and economic viability. In recent years, the use of natural antioxidants present in traditional medicinal plants has become of special interest in the scientific world due to their presumed safety and nutritional and therapeutic value. In this present study, comparative phenolic compounds in traditional Bulgarian medical plants. The medical plants were analyzed for their tannins content by titrimetric method; rutin was determined spectrophotometrically by using ammonium molybdate; the total phenolics content was determined by using Folin-Ciocalteu assay and the total flavonoids were used the colorimetric reaction with aluminum (III) chloride. The present paper shown by the results of total phenolic and total flavonoid contents, and rutin and tannins in medical plants that they must be relatively safe for the patient.

Keywords: chicory(Cichoriumintybus L.),white birch (Betula pendula),Cotinuscoggygria, Geranium sanguineumL. and rose hip fruits (Rosa caninaL.), total phenolics, total flavonoids, rutin and tannins

INTRODUCTION

The World Health Organization (WHO) has estimated that almost 80% of the earth's inhabitants believe in traditional medicine for their primary health care needs, and that most of this therapy involves the use of plant extracts and their active components (**Kishore Dubey *et al*., 2015; Winston, 1999**). A number of plants and plant products have medicinal properties that have been validated by recent scientific developments throughout the world, owing to their potent pharmacological activity, low toxicity and economic viability (**Kishore Dubey *et al*., 2015**) .In recent years, the use of natural antioxidants present in traditional medicinal plants has become of special interest in the scientific world due to their presumed safety and nutritional and therapeutic value (**Kishore Dubey *et al*., 2015; Ajila *et al*., 2007**). The majority of the antioxidant activity of plants is due to the presence of phenolic compounds (flavonoids, phenolic acids and alcohols, stilbenes, tocopherols, tocotrienols), ascorbic acid and carotenoids (**Kishore Dubey *et al*., 2015**).

Bulgaria is situated in the Balkan Peninsula, South-East Europe, Mediterranean and continental climates. The relief of the country is quite diverse ranging from plains to low hills and high mountains. The climate is moderate continental to modified continental, but in southern regions reflects rather a strong Mediterranean influence. As a result of this climatic condition, the Bulgarian flora is remarkable for its diversity (3500 plant species including 600 known medicinal plants) (**Ivancheva and Stancheva, 2000 ;Ivancheva *et al*., 2006**).

Traditional Bulgarian medicinal plants have been used to treat human diseases in Bulgaria for thousands of years, and people are becoming increasingly interested in them because of their good health effects and low toxicity. In recent years, studies on the antioxidant activities of Traditional Bulgarian medicinal plants have increased remarkably in light of the increased interest in their potential as a rich source of natural antioxidants. Several studies have indicated that Traditional Bulgarian medicinal plants possess more potent antioxidant activities than common dietary plants, and contain a wide variety of natural antioxidants, such as total phenolics, flavonoidsand tannins (**Atanassova *et al*., 2011**).

In recent years, interest in plant-derived food additives has grown. Furthermore plant extracts of Bulgarian white birch (*Betula pendula*L.) leaves have been shown to possess health-promoting properties. The white birch leaves extract were strong diuretic and have effect at nephrolithiasis and urinary bladder lythiasos, sedative effect on spasms of smooth muscle. It might be used in following conditions: kidney diseases, ischia nerve inflammation and podagra and atherosclerosis and also it has an antimicrobial effect (**Christova-Bagdassrian *et al*., 2014; Harbone, 1993**).

The extracts from the leaves of the white birch significantly increase diuresis, and with this and the emission of sodium and chloride ions, ie act as salidiuretik. Until recently it was assumed that the diuretic action is due to the presence of resinous substances. Therefore birch buds were preferred because they are rich in resins. However, it is clear that flavonoids have a greater role in the diuretic effect. They are contained mainly in the leaves. In addition, the leaves contain potassium nitrate, which enhances the diuretic effect of the flavonoids. This effect was related to total flavonoids (**Christova-Bagdassrian *et al*., 2014; Neoretal , 2006**).

*Cotinus coggygria*is one of two species constituting a minor genus of the family *Anacardiaceae*, viz., *Cotinus coggygria*Scop. (syn.: *Rhus cotinus* L.) Itself and *Cotinusobovatus* Raf., the American smoketree. Its wide distribution extends from southern Europe, the Mediterranean, Moldova and the Cauca-sus, to central China and the Himalayas (**Christova-Bagdassarian *et al*., 2016; Novakovic *et al*., 2007; *Matic et al*., 2011**). *C. coggygria*is a common medicinal plant (well known as 'smradlika' or 'tetra') in the Bulgarian folk medicine for outer use predominantly (**Christova-Bagdassarian *et al*., 2016; Landzhev Chemical, I., 2010; Ivanova *et al*., 2013).**

Plants of the family *Anacardiaceae* have a long history of use by various peoples for medicinal and other purposes. *Rhus glabra*is traditionally used in the treatment of bacterial diseases such as syphilis, gonorrhea, dysentery and gangrene, while *R. coriaria*, besides its common use as a spice consisting of ground dried fruits with salt, is also widely used as a medicinal herb, particularly for wound healing (**Christova-Bagdassarian *et al*., 2016; Matic et al., 2011; Rayne and Mazza, 2007**)

In folk medicine, *Cotinus coggygria*is routinely used as an antiseptic, anti-inflamatory, antimicrobial and antihaemorragic agent in wound-healing (**Christova-Bagdassarian *et al*., 2016; Rayne and Mazza, 2007; Demirci *et al*.,**

2003), as well as for countering diarrhea, paradontosis, and gastric and duodenal ulcers (**Christova-Bagdassarian *et al*., 2016; Rayne and Mazza, 2007; Ivanova *et al*., 2007**). However, these by-products are still a good and cheap source of high-quality polyphenolic compounds which can be used in different therapeutic procedures with the purpose of free radical neutralisation in biological systems (**Christova-Bagdassarian *et al*., 2016; Bucić-Kojić *et al*., 2007; Heim *et al*., 2002; Yilmaz and Toledo, 2004**)

Cichorium intybus L., commonly known as chicory, belongs to family *Asteraceae* and widely distributed in Asia and Europe(**ZahidKhorshid *et al*., 2015; Bais *et al*., 2001**). All parts of this plant possess great medicinal importance due to the presence of a number of medicinally important compounds such as alkaloids, inulin, sesquiterpene lactones, coumarins, vitamins, chlorophyll pigments, unsaturated sterols, flavonoids, saponins and tannins (**ZahidKhorshid *et al*., 2015; Bais *et al*., 2001, Atta *et al*., 2010; Molan *et al*., 2003; Muthusamy *et al*., 2008; Nandagopal *et al*., 2007**). The whole plant has numerous applications in food industry and medicine (**Denev *et* al., 2014; Ilaiyaraja *et al*., 2010**). Its dried roots were used as a substitute or adulterant in coffee powder (**Denev *et* al., 2014; Jung *et al*., 1994**). The young leaves can be added tosalads and vegetable dishes, while chicory extracts are used for the production of invigorating beverages (**Denev *et* al., 2014**).Leaves of chicory are good sources of phenols, vitamins A and C as well as potassium, calcium, and phosphorus (**ZahidKhorshid *et al*., 2015; Muthusamy *et al*.,2008**) *C. intybus* has been traditionally used for the treatment off ever, diarrhea, jaundice and gallstones **(ZahidKhorshid *et al*., 2015; Abbasi *et al*., 2009; Afzal *et al*., 2009**).During the past decade, there is a growing interest in natural plant extracts with potential antioxidant activity, because of their improved healthy effect (**Denev *et al*., 2014; Alexieva *et al*., 2013; Mihaylova *et al*., 2013**). The expanded application is due to their protective properties against oxidative stress disorders, as well asoxidative damage in food products (**Denev *et al*., 2014; Ivanov et al., 2014**).It is well known that polyphenols from plant extracts possessed strong antioxidant activities. Their presence in medicinal plant that are natural source of inulin-type fructans prebiotics additionally increase the biological activity of the obtained extracts (**Denev *et al*., 2014; Petkova *et al*., 2012; Vrancheva *et al*., 2012**).

Wild growing rose hip fruits (*Rosa canina* L.) are widespread plant in Bulgaria with great importance in herbal medicine. The *Rosa canina* fruits are a valuable source for food and pharmaceutical industry. They contain a wide variety of biologically and physiologically active ingredients, such as vitamins (C, B, P, PP, E, K), flavonoids, carotenes, carbohydrates (mono- and oligosaccharides), organic acids (tartaric, citric), trace elements and others (**Taneva *et al*., 2016; Ognyanov *et al*., 2014; Mihaylova *et al*., 2015**).

These compounds play an important role in maintaining fruit quality and determining nutritive value. Rose hips are also well known to have the highest vitamin C content (300–4000 mg/100 g) among fruits and vegetables (**Taneva *et al*., 2016; Demir *et al*., 2001**). In Bulgaria rose hip fruits are typically consumed as infusion. It was found that juice and aqueous extracts from rose hip possessed exceptional antioxidant activity (**Taneva *et al*., 2016; Demir *et al*., 2001)**. This makes them suitable for use both in the fresh or dry state, or in the form of extracts in food products and cosmetics (**Taneva *et al*., 2016; Ognyanov *et al*., 2014; Mihaylova *et al*., 2015**). According to some authors, the higher values of antioxidant activity of rose hip extracts due to synergism between polysaccharides and organic acids (gallic, cinnamic, ellagic), with phenolic antioxidants: flavonoids (rutin, kaempferol, quercetin) (**Taneva *et al*., 2016; Ognyanov *et al*., 2014; Mihaylova *et al*., 2015**).

The most common antioxidants contained in fruits are ascorbic acid, carotenoids and polyphenol substances with proven antioxidant capacity (**Taneva *et al*., 2016; Mihaylova *et al*., 2015**).

Geranium macrorrhizum L.is a perennial herb native from the Balkans, occurring occasionally also in the Carpathian Mountains and in the Alps. It is known as "Zdravets" which means "healthy" in Bulgarian folk medicine. A methanol extract from leaves possesses strong hypotensive activity, cardiotonic, capillary anticomplementary and sedative action as well (**Ivancheva *et al*., 2006; Genova *et al*., 1989; Ivancheva and Wollenweber, 1989; Ivancheva *et al*., 1992**). Central depressive action of methanol extracts has also been demonstrated. The whole plant is rich in tannins with more in the stems than in the green foliage. No alkaloids and cardiological glycosides have been found. The presence of six flavonol aglycones in aerial parts of *G. macrorrhizum* has been established (kaempferol 3-methylether (*isokaempferide*), kae-3,7-dimethylether (*kumataketin*), kae-3,4'- dimethylether (*ermanin*), quercetin, qu-7,3'-dimethylether and qu-3,7,3',4'-tetramethylether (*retusin*)); two of these, namely ermanin and retusin were said to be present in the roots too (**Ivancheva *et al*., 2006**). The focus in the present study is a comparative evaluation of the total phenolic and total flavonoid contents, rutin and tannins in traditional Bulgarian medical plants as sources for human health.

MATERIAL AND METHODS

Plant material

The leaves from chicory (*Cichorium intybus* L.), white birch (*Betula pendula*), zdravec (*Geranium macrorrhizum* L.), smradlika (*Cotinus coggygria,* syn.: *Rhus cotinus* L.) and rose hip fruits (*Rosa canina* L.) were harvested from different regions of Bulgaria. All sample data are stated in the sampling protocol. The dried leaves and rose hip fruit were kept in a dry place until further use.

Sample preparation

A dry simple of 0.5 g was weighted and phenolic and flavonoid compounds were extracted with 50 mL80% aqueous methanol on an ultrasonic bath for 20 min. An aliquot (2 mL) of the extracts was ultracentrifugated for 5 min at 14 000 rpm. The extract prepared in this way was used for further spectrophotometric determination of polyphenols.

Determination of total phenolics assay

The total phenolic contents of medicinal plants were determined by using the Folin-Ciocalteu assay.An aliquot (1 mL) of extracts or standard solution of gallic acid (10, 20, 40, 60, 80, 100 and 120 mg/L) was added to 25 mL volumetric flask, containing 9mL of distilled deionised water (dd H_2O). A reagent blank using dd H_2O was prepared. One milliliter of Folin-Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 min, 10 mL of 7%Na_2CO_3 solution was added to the mixture. To the solution the dd H_2O was added up to volume of 25mL and mixed. After incubation for 90 min at room temperature, the absorbance against prepared reagent blank was determined at 750 nm with an UV-Vis Spectrophotometer BOECO – Germany. All samples were analyzed in duplicates (**Marinova *et al*., 2005**).

Determination of total flavonoids assay

The total flavonoid contents were measured by aluminum chloride colorimetric assay. An aliquot (1 mL) of extracts or standard solution of catechin (10, 20, 40, 60, 80, 100 and 120 mg/L) wasadded to 10 mL volumetric flask, containing 4 mL of distilled deionised water (dd H_2O). To the flask was added 0.3 mL 5% $NaNO_2$. After 5 min, 0.3 mL of 10% $AlCl_3$ was added. At 6thmin, 2mL 1 M NaOH was added and the total volume was made up to 10 mL with dd H_2O. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510nm. UV-Vis Spectrophotometer BOECO – Germany. All samples were analyzed in duplicates (**Marinova *et al*., 2005**).

Rutin assay

The analyses of rutin content in Bulgarian medicinal plants were performed according to The International Pharmacopoeia and AOAC method, after modified methods with using 80% aqueous methanol. Pipet 2 mL aliquots solution into 50 mL volumetric flask was added to 2 mL deionized water (dd H2O) and 5 mL ammoniummolybdat. The solution was added volume (50mL) with dd H2O and mixed. Was prepared standard solution of rutin (0.0200 g dissolved into 2 mL methanol) was added volume (50 mL) with 80% aqueous methanol. An aliquot (1 mL) of standard solution into 50 mL volumetric flask and dilute to volume with distilled deionized water (dd H_2O). A reagent blank using dd H_2O was prepared. The absorbance against prepared reagent blank was determined at 360 nm with an UV-Vis Spectrophotometer BOECO – Germany. All samples were analyzed in duplicates (**Atanassova *et al*., 2009a**).

Calculations

Calculations are based on averaging results from analyses of duplicate samples. Calculate content (%) of rutin (R) in sample as follows:

$$R(\%) = \frac{A_{sample} \times C \times 50 \times 100}{A_{stand} \times W \times 2}$$

Where:
A_{sample} - Absorbance of sample was determined at 360 nm;
A_{stand}- Absorbance of standard solution was determined at 360 nm;
C – Concentration of standard solution of rutin (g/mL);
W – weight (g) of sample for analyses;
2 – Volume (mL) of sample for analyses;
100 – Percent, %.

Tannins assay

The analyses of tannins content in traditional Bulgarian medicinal plantswere performed according to The International Pharmacopoeia and AOAC method, after modified methods. Measured 25 mL of this infusion into 1 L conical flask and add 25 mL indigo solution and 750mL distilled deionized water (dd H_2O). Titred with 0.1 N water solution of $KMnO_4$ until blue solution changes to green, then add a few drops at time until solution becomes golden yellow. Was prepared standard solution of Indigo carmine (dissolve 6 g indigo carmine in 500mL distilled deionized water (dd H_2O) by heating, cool add 50 mL 96% - 98% H_2SO_4, diluted to 1 L and then filtered. For the blank similarly titred mixture of

25 mL indigocarmine solution and 750mL ddH_2O. All samples were analyzed in duplicates (**Atanassova *et al*., 2009b**).

Calculations

Calculations are based on averaging results from analyses of duplicate samples. Calculate content (%) of tannins (T) in sample as follows:

$$T(\%) = \frac{(V - V_0) \times 0.004157 \times 250 \times 100}{g \times 25}$$

Where:
V – Volume of 0.1 N water solution of $KMnO_4$ for titration of sample, mL;
V_0 – Volume of 0.1 N water solution of $KMnO_4$ for titration of blank sample, mL;
0.004157 – Tannins equivalent in 1 mL of 0.1 N water solution of $KMnO_4$;
g – Mass of the sample for analyses, g;
250 - Volume of volumetricflask, mL;
100 – Percent, %.

Statistical analysis

All experiments were performed in triplicates. Analysis at every time point from each experiment was carried out induplicate or triplicate. The statistical parameters are calculated in terms of the reproducibility of the experimental data using a statistical package universal ANOVA.

RESULTS AND DISCUSSION

Different phytochemicals have various protective and therapeutic effects which are essential to prevent diseases and maintain a state of well being. Methanolic extract of rose hip fruits (*Rosa canina* L.), chicory (*Cichorium intybus* L.), zdravec (*Geranium macrorrhizum* L.), smradlika (*Cotinus coggygria,* syn.: *Rhus cotinus* L.) and white birch (*Betula pendula* L.) were analyzed for phytoconstituents. The quantitative estimation of phenolic compounds of white birch (*Betula pendula* L.), smradlika (*Cotinus coggygria,* syn.: *Rhus cotinus* L.), zdravec (*Geranium macrorrhizum* L.), chicory (*Cichorium intybus* L.) and rose hip fruits (*Rosa canina* L.) show that the traditional Bulgarian medical plants are rich in total phenolics, total flavonoids according to the data shown in the Table1 and Figure 1. It is well that plant flavonoids and phenols in general, are highly effective free radical scavenging and antioxidants. The phenolic constituents found in herbs have attracted considerable attention as being the main agents of antioxidant activity, although they are not the only ones. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygenquenchers. In addition, they have a metal chelation potential. Hence, the antioxidantactivity of phenolics plays an important role in the adsorption or neutralization of free radicals (**Dutra *et al*., 2008; Laguerre *et al*., 2007**).
The major compounds that are related to the antioxidant potential of a plant (or plant part) are the total phenolics and, more specifically, the flavonoids (**Dutra *et al*., 2008; Kumar Ashok *et al*., 2012**), though there is no consensus as to whether these are the sole substances in plants that act in scavenging free radicals. It is well known that plant phenolics, in general are highly effective in free radical scavenging and they are antioxidants. The presence of these phytochemicals in medical plants is thus a significant finding of the present study. The content of total phenolics and total flavonoids in white birch (*Betula pendula* L.), varying between 5256,30 mg GAE/100g dw to 2245,70 mg CE/100g dw, was found to be much higher than and in rose hip fruits (*Rosa canina* L.) - 406,79 mg GAE/100g dw to 290,13 mg CE/100g dw, respectively, as shown in Table 1 and Figure 1 with gallic acid and catechin as standards. These results indicate that the higher antioxidant activity of the white birch (*Betula pendula* L.) methanol extract, compared to the rose hip fruits (*Rosa canina* L.) methanol extract, may be correlated to the phenolic and flavonoid content of respective medical plant extract.

Table 1 Total phenolics and total flavonoids in studied Traditional Bulgarian medical plants

Bulgarian medical plants	Total phenolics, (mg GAE/100g dw)	Total flavonoids, (mg CE/100g dw)
Rose hip fruits (*Rosa canina* L.)	406,79÷0,02 (RDS 0,03; n=3)	290,13÷0,01 (RDS 0,03; n=3)
Chicory (*Cichorium intybus* L.)	635,87÷0,05 (RDS 0,07; n=3)	315,15÷0,04 (RDS 0,01; n=3)
Zdravec (*Geranium macrorrhizum* L.)	1530,70÷0,02 (RDS 0,01; n=3)	110,20÷0,06 (RDS 0,05; n=3)
Smradlika (*Cotinus coggygria,* syn.: *Rhus cotinus* L.)	2581,60÷0,02 (RDS 0,08; n=3)	810,40÷0,04 (RDS 0,04; n=3)
White birch (*Betula pendula* L.)	5256,30÷0,04 (RDS 0,08; n=3)	2245,70÷0,04 (RDS 0,01; n=3)

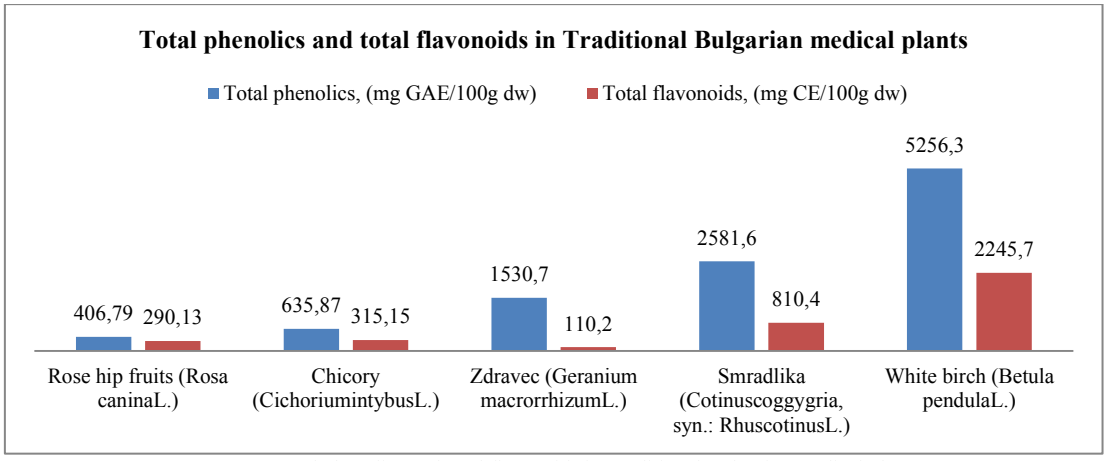

Figure 1 Total phenolics and total flavonoids in Traditional Bulgarian medical plants

The presence of rutin and tannins in chicory (*Cichorium intybus* L.), rose hip fruits (*Rosa canina* L.), zdravec (*Geranium macrorrhizum* L.), white birch (*Betula pendula* L.) and smradlika (*Cotinus coggygria,* syn.: *Rhus cotinus* L.) show that the traditional Bulgarian medical herbs are rich in tannins, rutin according to the data shown in the Table 2 and Figure 2. The phytochemical screening and quantitative estimation of the percentage of chemical constituents of the plants studied showed that the dry herbs were rich in rutin and tannins.
Tannins are distributed all over the plant kingdom (**Bate-Smith, 1962**). The term tannin refers to the use of tannins in tanning animal hides into leather; however, the term is widely applied to any large polyphenolic compound containing sufficient hydroxyls and other suitable groups (such as carboxyls) to form strong complexes with proteins and other macromolecules. Tannins have molecular weights ranging from 500 to over 3000 (**Bate-Smith, 1962; Cheng *et al*., 2003**). Tannins may be employed medicinally in antidiarrheal, haemostatic, and antihemorrhoidal compounds (**Bate-Smith, 1962; Lin et al., 2004**). Tannins can also be effective in protecting the kidneys (52, 55). Tannins are also beneficial when applied to the mucosal lining of the mouth (**Bate-Smith, 1962; Habtemariam and Varghese, 2015**). Rutin is a common dietary flavonoid widely distributed in the plant kingdom. It is also present in plant-derived beverages and foods as well as numerous medicinal and nutritional preparations (**Sando and Lloyd, 1924; Habtemariam and Varghese, 2015**). Today, rutin is among the most popular natural flavonoids known for its multifunctional nutritional and therapeutic uses (**Sando and Lloyd, 1924; Habtemariam and Lenti, 2015**). As far as commercial exploitation of rutin is concerned, however, very few plants store it in large amounts to merit the cost of its extraction from natural sources (**Sando and Lloyd, 1924**). They were known to show medicinal activity as well as exhibiting physiological activity. The presence of these phenolic compounds in traditional Bulgarian medical herbs is a significant finding in this present study. The content for rutin of white birch (*Betula pendula* L.) varied between 6,24 % was found to be much higher than and chicory (*Cichorium intybus* L.) - 2,09 %, respectively as shown in Table 2 and Figure 2 with rutin as
standards. The content for tannins of smradlika (*Cotinus coggygria,* syn.: *Rhus cotinus* L.) varied between 11,15 % was found to be much higher than and chicory (*Cichorium intybus* L.) - 2,26 %, respectively as shown in Table 2 and Figure 2 and KMnO4 as titrate. It is important to notice that the comparison of

the results for rutin and tannin contents in the medical herbs will be not correct because of the different methods of analysis.

Table 2 Tannins and rutin in studied Traditional Bulgarian medical plants

Bulgarian medical plants	Tannins, (%)	Rutin, (%)
Chicory (*Cichorium intybus* L.)	2,26÷0,04 (RDS 1,8; n=3)	2,09±0,01 (RDS 0,6; n=3)
Rose hip fruits (*Rosa canina* L.)	4,11±0,09 (RDS 2,3; n=3)	2,16±0,04 (RDS 1,9; n=3)
Zdravec (*Geranium macrorrhizum* L.)	6,08±0,07 (RDS 1,2; n=3)	3,20±0,08 (RDS 2,3; n=3)
White birch (*Betula pendula* L.)	9,04±0,03 (RDS 0,4; n=3)	6,24±0,02 (RDS 0,3; n=3)
Smradlika (*Cotinus coggygria,* syn.: *Rhus cotinus* L.)	11,15±0,06 (RDS 0,5; n=3)	3,06±0,06 (RDS 1,9; n=3)

CONCLUSION

In conclusion, on the basis of the results of this research showed that total phenolic, total flavonoid, rutin and tannin contents are important components in traditional Bulgarian medical plants grown in the country. The use of medical plants as the first choice in self0treatment of minor conditions continues to expand rapidly across the world. This makes the safety of medical plants an important public health issue. The results can be used in public health campaigns to stimulate the consumption of traditional Bulgarian plants as chicory (*Cichorium intybus* L.), rose hip fruits (*Rosa canina* L.), zdravec (*Geranium macrorrhizum* L.), white birch (*Betula pendula* L.) and smradlika (*Cotinus coggygria,* syn.: *Rhus cotinus* L.) which are able to provide significant health protection in order to prevent chronic diseases.

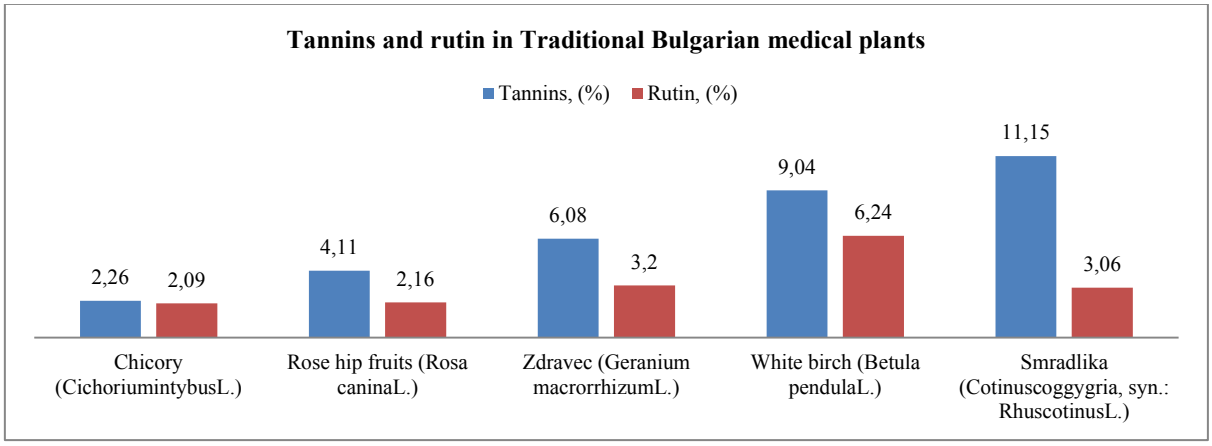

Figure 2 Tannins and rutin in Traditional Bulgarian medical plants

REFERENCES

Abbasi, A.M., Khan, M.A., Ahmad, M., Zafar, M., Khan, H.,Muhammad, N., Sultana, S., 2009. Medicinalplants used for thetreatment of jaundice and hepatitisbasedonsocio-economicdocumentation. *African Journal of Biotechnology*, *8*(8), 1643–1650.

Afzal, S., Afzal, N., Awan, M.R., Khan, T.S., Gilani, A., Khanum, R., &Tariq, S. (2009). Ethno-botanicalstudies from NorthernPakistan .*Journal of Ayub Medical College Abbottabad*, 21)l), 52–57.

Ajila, C.M., Naidu, K.A., Bhat, U.J.S, & Rao, P. (2007) Bioactive compounds and antioxidant potential of mango peel extract. *Food Chemistry*,*105*, 982–988. http://dx.doi.org/10.1016/j.foodchem.2007.04.052 .

Alexieva, I., Mihaylova, D., & Popova, A. (2013). Evaluation of theantioxidantcapacity of aqueous extracts of freshsamardala (*Alliumbulgaricum*L.) leaves. *Scientific works*, 826-831.

Atanassova, M., & Christova-Bagdassarian, V. (2009). Determination of tanninscontent by titrimetricmethod for comparison of different plantspecies. *Journal of the University of Chemical TechnologyandMetallurgy*, *44*(44), 413-415.

Atanassova M., & Bagdassarian ,V. (2009). Rutin content in plantproducts. *Journal of the University of Chemical Technology and M Marinova, D., Ribarovam F., Atanassova, M.(2005). Total phenolics and total flavonoids in Bulgarian fruits and vegetables. Journal of the University of Chemical Technology and Metallurgy, 40(3), 255−260.*

Atanassova, M., Georgieva, S., & Ivancheva, K. (2011). Total phenolic and total flavonoid contents, antioxidant capacity and biological contaminants in medicinal herbs. *Journal of the University of Chemical Technology and Metallurgy*, *46*(1), 81-88.

Atta, A.H., Elkoly T.A., Mouneir, S.M., Kamel G., Alwabel, N.A., &Zaher, S. (2010). Hepatoprotective effect of methanol extracts of Zingiber officinale and Cichorium intybus . *Indian Journal of pharmacy science*, *72*(5), 564–570. http://dx.doi.org/10.4103/0250-474x.78521

Bate-Smith, S. (1962). *Flavonoid compounds. In: Comparative biochemistry*. Florkin M Mason HS (Eds), Vol III, Academic Press, New-York, 75-809. http://dx.doi.org/10.1016/b978-0-12-395544-9.50025-x

Bais, H.P., Ravishankar, G.A., &Cichoriumintybus, L. (2001). cultivation,processing, utility, valueaddition and biotechnology, with anemphasison current status and futureprospects. *Ournal of the science of food and agriculture*. *81*,467–484. http://dx.doi.org/10.1002/jsfa.817.abs

Bucić-Kojić, A., Planinić, M., Tomas, S., Bilić, M., & Velić, D. (2007). Study of solid–liquid extraction kinetics of total polyphenols from grape seeds. *Journal of Food Engineering*, *81*, 236–242. http://dx.doi.org/10.1016/j.jfoodeng.2006.10.027

Cheng, H.Y., Lin, C.C., & Lin, T.C. (2002). Antiherpes simplex virus type 2 activity of casuarinin from the bark of *Terminalia arjuna* Linn. *Antiviral Researc*, *55*, 447-455. http://dx.doi.org/10.1016/s0166-3542(02)00077-3

Christova-Bagdassrian, V.L., Chohadjieva, D., & Atanassova, M. (2014). Total Phenolics and Total Flavonoids, Nitrate Contents and Microbiological Tests in Dry Extract of Bulgarian White Birch Leaves (*Betula pendula*). *International Journal of Advanced Research, 2* (6), 668-674.

Christova-Bagdassarian, V.L., Atanassova, M.S., Hristova, V.K., & Ahmad, M.A. (2016). Soild-Liquid Extraction Kinetics of Total Phenolic, Total Flavonoid, Rutin and Tannin Contents in 50% Ethanol Extract of *Cotinuscoggygria. International Journal of Scientific & Engineering Research*, *7*(2).

Demir ,F., & Ozcan, M. (2001). Chemical and technological propertiesof rose (*Rosa canina*L.) fruits grown wild in Turkey. *Journal of Food Engineering*, *47,* 333-336. http://dx.doi.org/10.1016/s0260-8774(00)00129-1

Demirci, B., Demirci, F., & Baser, K.H. (2003). Composition of the essen-tial oil of *Cotinuscoggygria*(Scop.) from Turkey. *Flavor and Fragrance Journal, 18*, 43-44. http://dx.doi.org/10.1002/ffj.1149 .

Denev, P., Petkova, N., Ivanov, I., Sirakov, B., Vrancheva, R., & Pavlov, A. (2014). Determination of biologically active substances in taproot of common chicory (*Cichoriumintybus L.*), ScientificBulletin. *Series F. Biotechnologies*, 124-129.

Dutra, R.C., Leite , M.N., & Barbosa N.R. (2008). Quantification of phenolic constituents and antioxidant activity of *Pterodon emarginatus* Vogel seeds. *International Journal of Molecular Sciences*, 9, 606–614. http://dx.doi.org/10.3390/ijms9040606

Genova, E., Hristova, J. and Beeva J. 1998, Pharmacia, 45, 10.

Habtemariam, S., &Varghese, G.K. (2014). The antidiabetic therapeutic potential of dietary polyphenols. *Current Pharmaceutical Biotechnology*, *15*, 391–400. http://dx.doi.org/10.2174/1389201015666140617104643 .

Habtemariam, S., & Lenti, G. (2015).The therapeutic potential of rutin for diabetes: An update. Mini Review. *Medical Chemistry*, *15*, 524–528. http://dx.doi.org/10.2174/1389557515071504241037 21

Habtemariam, S., & Varghese, G. K. (2015). Extractability of Rutin in Herbal Tea Preparations of *Moringa stenopetala* Leaves. *Beverages*, *1*, 169-182. http://dx.doi.org/10.3390/beverages1030169 .

Harbone ,J.B .(1993). *In: The flavonoids. Advances in Research since* 1986. Chapmann and Hall, London, 121-129.

Heim, K. E., Tagliaferro, A. R., &Bobilya, D. J. (2002). Flavonoid antioxidants: chemistry, metabolism and structure-activity relation-ships. *The Journal of*

Nutritional Biochemistry, *13*, 572–584 . http://dx.doi.org/10.1016/s0955-2863(02)00208-5
Ilaiyaraja, N., & Khanum, F. (2010). Evaluation of Antioxidant and Toxicological properties of Chicoryleaves. *International Journal of Pharmacology and biological archives*, *1*(2), 155 –163.
Ivancheva, S. & Wollenweber E. (1989). *Indian Drugs*, *27*, 167.
Ivancheva, S., Manolova, N., Serkedjieva, J., Dimov, V., & Ivanovska, N. (1992). *Plant Polyphenols, W. Hemingway and Laks*, P. (Eds.), Premium Press, New York and London, 717. http://dx.doi.org/10.1007/978-1-4615-3476-1_43
Ivancheva S., & Stancheva, B. (2000). Ethnobotanical inventory of medicinal plants in Bulgaria .*Journal of Ethnopharmacology*, *69*, 165. http://dx.doi.org/10.1016/s0378-8741(99)00129-4 .
Ivancheva, S., Nikolova, M., & Tsvetkova, R. (2006). *Pharmacological activities andbiologically active compounds of Bulgarian medicinal plants*, pp. 87-103, book Phytochemistry: Advances in Research, Editor: Filippo Imperato, Research Signpost, Kerala, IndiaKishore Dubey, N., Kedia, A., Prakash, B., & Kishore, N. (2015). *Plants of Indian Traditional Medicine with Antioxidant Activity*, pp. 1-14, book Plants as a Source of Natural Antioxidants, Edited by Nawal Kishore Dubey, Banaras Hindu University, India. http://dx.doi.org/10.1079/9781780642666.0001 .
Ivanova, D., Pavlov, D., Eftimov, M., Kalchev , K., Nashar, M., Tzaneva, M., & Valchev S. (2013). Subchronic Toxicity Study of Ethanol Infusion from *CotinusCoggygria* Wood in Rats. *Bulgarian Journal of Agricultural Science, 19* (2), 182–185.http://dx.doi.org/10.1016/j.jep.2004.08.033.
IvanovI., V, Rancheva, R., Marchev, A., Petkova, N., Aneva, I., Denev, P., Georgiev, G., & Pavlov A.(2014). Antioxidantactivities and phenoliccompounds in Bulgarian Fumariaspecies. *International Journal of Current Microbiology and Applied Science*, *3*(2), 296-306.
Jung,G., Shaffer, J., Varga., & Everhart G. (1996). Performance of 'Grasslands Puna' Chicory at Different Management Levels *Journal of Agronomy*,*88*, 104 – 111. http://dx.doi.org/10.2134/agronj1996.00021962008800010022x
Kumar Ashok, P., & Upadhyaya, K. (2012).Tannins are Astringent. *Journal of Pharmacognosy and Phytochemistry*, *1*(3), 45-50.
Laguerre, M., Lecomte, J.& Villeneuve, P. (2007) Evaluation of the ability of antioxidants to counteract lipid oxidation: existing methods, new trends and challenges. *Progress in Lipid Research*, *46*, 244–282. http://dx.doi.org/10.1016/j.plipres.2007.05.002
Landzhev Chemical, I. (2010). *Encyclopedia of Medicinal Plants in Bulgaria*. Trud,Sofia, 551 (Bg).
Lin, L.U., Shu-wen, L., Shi-bo, J., & Shu-guang ,W. (2004).Tannin inhibits HIV-1 entry by targeting gp 41. *Acta Pharmacologica Sinica*, *25*(2):213-218. http://dx.doi.org/10.1038/aps.2009.31
Matic, S., Stanic, S., Bogojevic, D., Solujic, S., Grdovi, N., Vidakovic ,M., & Mihailovic M.(2011). Genotoxic potential of *Cotinuscoggygria*Scop. (*Anacardiaceae*) stem extract *in vivo*. *Genetics and Molecular Biology, 34*(2), 298-303.http://dx.doi.org/10.1590/s1415-47572011005000001.
Mihaylova, D., Georgieva, L., & Pavlov, A. (2013)**.** *Invitro*antioxidantactivity and phenoliccomposition of *Nepetacataria*L Extracts. *International Journal of Agriculture Science and Technology*, *1*(4), 74-79.
Mihaylova, D., Georgieva, L., Pavlov, A. (2015). Antioxidantactivity and bioactive compounds of *Rosa canina* L.herbal preparations. Scientific Bulletin. Series F.*Biotechnologies*, 160-165.
Molan, A.L., Duncan, A.J., Barryand, T.N., & McNabb, W.C. (2003). Effects of condensed tannins and crude sesquiterpene lactones extracted from chicory on the motility of larvae of deer lungworm and gastrointestinal nematodes. *Parasitology International*, 52, 209–218. http://dx.doi.org/10.1016/s1383-5769(03)00011-4
Muthusamy, V.S., Anand, S., Sangeetha, K.N., Sujatha, S., Arun, B., & Lakshami, B.S. (2008). Tannins present in Cichoriumintybusenhanceglucoseuptake and inhibitadipogenesis in 3T3-L1adipocytesthrough PTP1B inhibition. *Chemico-Biological Interactions*, *174* (1), 69–78. http://dx.doi.org/10.1016/j.cbi.2008.04.016
Nádia, R., Barbosa, R., Farah Silva, A., & Caetano Polonini, H. (2015). *Antioxidant Plants from Brazil*. 97-108, book Plants as a Source of Natural Antioxidants, Edited by Nawal Kishore Dubey, Banaras Hindu University, India–2015. http://dx.doi.org/10.1079/9781780642666.0097 .
Nandagopal, S., & Ranjithakumari, B.D. (2007). Phytochemical andantibacterialstudies of chicory (*Cichoriumintybus L.*) – a multipurposemedicinalplant. *Advance in biological research*, *1*(1-2), 17–21.
Neoretal .(2006). Monography. Available from: http://neopharm.ro/data/ufiles/files/Neorenal-Monography.pdf
Novakovic, M., Vučkovic, I., Janackovic, P., Sokovic, M., Tesevic, V., & Milosavljevic, S. (2007). composition, antibac-terial and antifungal activity of the essential oils of *Cotinuscoggygria*from Serbia. *Journal of Serbian Chemical Society, 72*, 1045-1051. http://dx.doi.org/10.2298/jsc0711045n.
Petkova, N., Vrancheva, R., Ivanov, I., Denev, P. , Pavlov, A., & Aleksieva J.(2012). Analysis of biologicallyactivesubstances in tubers of Jerusalemartichoke(*Helianthustuberosus L.*). 50 years FoodRDIInternational Scientific-PracticalConference" Food,Technologies& Health" Proceedings Book.
Rayne, S. & Mazza, G. (2007). Biological activities of extracts from Sumac (*Rhus spp*.). *Plant Foods for Human Nutrition, 62*,165-175. http://dx.doi.org/10.1007/s11130-007-0058-4
Sando, C.E., & Lloyd, J.R. (1924). The isolation and identification of rutin from the flowers of elder (*Sambucus canadebsis* L.). *Journal of Biological Chemistry*, *58*, 737–745.
Stéphane, Q., Tatiana, V., Diana, K., Michael, J., Patrick, P., & Christian, B. (2004). Main structural and stereochemical aspects of the antiherpetic activity of nonahydroxyterphenoyl containing Cglycosidic ellagitannins. *Chemistry & biodiversity*, *1*(2):247-58.*http://dx.doi.org/10.1002/cbdv.200490021.*
Taneva, I., Petkova, N., Dimov, I., Ivanov, I., & Denev P. (2015). Characterization of Rose Hip (*Rosa canina*L.) FruitsExtracts and Evaluation of Their *in vitro* AntioxidantActivity, *Journal of Pharmacognosy and Phytochemistry*, *5*(2): 35-38.
Vrancheva, R., Petkova, N., Ivanov, I., Denev, P., Pavlov, A., & Aleksieva, J. (2012). Carbohydratecomposition andantioxidantactivity of rootextracts of *InulaHelenium* L.Youth Scientific conference "Klimentovidni", 3,62.
Winston, J.C. (1999). Health-promoting properties of common herbs. *American Journal of Clinical Nutrition,70*, 491–499.
Yilmaz, Y., & Toledo, R. T. (2004). Major flavonoids in grape seeds and skins: antioxidant capacity of catechin, epicatechin, and gallic Acid. *Journal of Agricultural and Food Chemistry*, *52*, 255–260. http://dx.doi.org/10.1021/jf030117h .
ZahidKhorshid, A., , Shalini, S., Sakeran, I.,ZidanNahla, M., Hasibur, R., & Ansari A. A. (2015). Phytochemical, antioxidant and mineral composition of hydroalcoholic extract of chicory (Cichorium intybus L.) leaves . *Saudi Journal of Biological Science,22*, 322–326. http://dx.doi.org/10.1016/j.sjbs.2014.11.015 .