JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

COMPARATIVE CHARACTERIZATION OF POSSIBILITIES OF USING LOW-ESTERIFIED AND AMIDATED PECTIN IN FERMENTED DAIRY PRODUCTS

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doi: 10.15414/jmbfs.2016.6.1.649-651

ARTICLE INFO

Received 4. 2. 2016
Revised 2. 3. 2016
Accepted 6. 3. 2016
Published 1. 8. 2016

Regular article

OPEN ACCESS

ABSTRACT

The possibilities of using low-esterified amidated pectin isolated from Bulgarian plant cultures have been studied. The comparison between pectin extracted from celery and commercial low-esterified amidated pectin showed that celery pectin can be used successfully for preparation of functional fermented dairy products enriched with soluble dietary fibers. It was found that the addition of amidated pectin extracted from celery in an amount exceeding 1 g.kg^{-1} deteriorates the organoleptic characteristics of the product, but viscosity of the produced experimental dairy product remained unchanged in comparison with that of the control sample. Therefore, it is recommended that the concentration of low-esterified amidated pectin used in preparation of dairy products do not exceed the limits established in this study.

Keywords: Yoghurt, amidated pectin, rheology, celery

INTRODUCTION

Yogurts are characterized by a high biological and health potential associated with their composition and the presence of selected active microorganisms (**Beena, 2000**).
It is well known that dietary fibers are a physiologically important component of food and have a positive impact on the digestive tract and various functional processes in the human body (**Ramaswamy and Basak, 1992**). Dairy products do not contain fibers and it is appropriate to be enriched with these polysaccharides to enhance their biological value (**Khurana and Kanawjia, 2007**).
Pectins are the most commonly used fibers in the manufacture of food products. It is known that these natural polymers are characterized by certain technological properties and possess distinct functional and health benefits (**Combo *et al*., 2011**). The addition of pectins in the manufacture of fermented dairy products is based on their ability to interact with the casein micelles in milk in the presence of calcium ions, forming stable gel structures in the pH range of 2.5 to 4.5 (**Maroziene and Kruif , 2000**).
Amidated pectin E 440 is a modified form of pectin, highly sensitive to the concentration of calcium ions in regard to its stabilizing properties. The use of low-esterified amidated pectin to form protein gel structures in the manufacture of foods requires a high concentration of calcium ions in the medium **(Uresti *et al*., 2003).**
The aim of this study is to make a comparative evaluation of technological possibilities to obtain fermented dairy products with the addition of low-esterified amidated pectin isolated from Bulgarian crop plants.

MATERIAL AND METHODS

Materials

The experiments were carried out with raw cow's milk, whose organoleptic, physicochemical and microbiological properties were evaluated according to the requirements of Regulation (EC) No 853/2004.
The milk fat content was standardized to 36±1 g.kg^{-1}.
A starter culture for yogurt containing specific strains (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) was used in a quantity of 20 g.kg^{-1}.
To perform the experiment, the following materials were used: low-esterified amidated pectin isolated from celery with degree of esterification (DE) – 28%, anhydrouronic content – 68% and degree of amidation – 28% and commercial pectin Pe Amid CM 020 (Herbstreith & Fox, Germany), degree of esterification - 27%, anhydrouronic content – 70%, degree of amidation - 25%.

Methods

The classical technology for production of yogurt was applied in laboratory conditions (**Kondratenko and Simov, 2003**).
Pectin can be incorporated in the milk as a solution or in a dry state, in combination with a certain amount of sugar (**Vlaseva, 2014**), before pasteurization or as pre-pasteurized solution before inoculation with the yoghurt starter cultures. In the present study pectin was incorporated before the pasteurization process.
The dispersion of pectin with 60 g.kg^{-1} sugar was conducted at 80 °C for 5 min at speed 9900 g, in a dry state. The amounts of added pectin were 1.2 and 3 g.kg^{-1} by Polytron® PT45-80 (Kinematika, Switzerland) with technical characteristics – 220 V; 50 Hz; 1600 W;
The change in the amount of lactose and its biochemical transformation to lactic acid during fermentation was defined by:
- Active acidity (pH), potentiometrically measured with a 7110 WTW (Germany) pH-meter
- Titratable acidity, measured according to ISO 6091:2010 (IDF 86:2010);

The structural characteristics of the coagulum after manufacture were determined by:
- the amount of separated serum, which characterizes the stabilizing capacity of the added pectins, determined by centrifugal test: 10 cm^3 of the prepared sample was centrifuged at 2400 g, immediately after coagulation (**Kuncheva *et al.*, 2007**) by Centrifuge - CH90 – 2A;.
- the viscosity of yoghurt was determined by a rheoviscometer, at a temperature of 25 °C by a Rheotest 2 (Germany) rotational viscometer;
- the organoleptic characterization was made according to the Bulgarian State Standard (BSS) 12:2010. In all analyzed samples the packaging was graded 10 points (the maximum score for this indicator).

Statistical analysis

The statistical analysis was performed using Microsoft Excel 2010 (ANOVA). Multiple comparisons were made by the LSD method. The results are presented as means value ± SD (n=3).

RESULTS AND DISCUSSION

The dynamics of acid formation during the coagulation of milk with the addition of low-esterified amidated pectin – commercial, or isolated from celery, in a quantity of 1-3 $g.kg^{-1}$ was researched. The results obtained on the dynamics of the indicators active acidity (pH) and titratable acidity (TA), expressed as % lactic acid (% l.a.) during coagulation (min), are presented in Table 1.

Table 1 Dynamics of acid formation, depending of the time of coagulation of milk with the addition of low-esterified amidated pectin

Time, min	Control sample		Concentration of low-esterified amidated pectin - commercial, $g.kg^{-1}$						Concentration of low-esterified amidated pectin from celery $g.kg^{-1}$					
	0		1		2		3		1		2		3	
	TA, % l.a.	pH	TA, % l.a.	pH	TA, % l.a.	pH	TA, % l.a.	pH	TA, % l.a.	pH	TA, % l.a.	pH	TA, % l.a.	pH
0±1	0.198 ± 0.009^a	6.6 ± 0.1^a	0.189 ± 0.009^a	6.6 ± 0.1^a	0.198 ± 0.009^a	6.6 ± 0.1^a	0.189 ± 0.009^a	6.5 ± 0.1^a	0.225 ± 0.009^b	6.4 ± 0.1^a	0.225 ± 0.009^b	6.3 ± 0.1^b	0.234 ± 0.009^c	6.3 ± 0.1^b
30±2	0.225 ± 0.009^a	6.5 ± 1.0^a	0.198 ± 0.009^b	6.5 ± 0.1^a	0.225 ± 0.009^a	6.4 ± 0.1^a	0.225 ± 0.009^a	6.5 ± 0.1^a	0.252 ± 0.009^c	6.4 ± 0.1^a	0.261 ± 0.009^d	6.3 ± 0.1^b	0.270 ± 0.009^d	6.3 ± 0.1^b
60±3	0.225 ± 0.009^a	6.5 ± 0.1^a	0.216 ± 0.009^a	6.4 ± 0.1^a	0.261 ± 0.009^b	6.4 ± 0.1^a	0.252 ± 0.009^b	6.4 ± 0.1^a	0.270 ± 0.009^b	6.3 ± 0.1^b	0.261 ± 0.009^b	6.2 ± 0.1^b	0.288 ± 0.009^c	6.2 ± 0.1^b
90±2	0.279 ± 0.018^a	6.3 ± 0.1^a	0.243 ± 0.009^b	6.2 ± 0.1^b	0.288 ± 0.009^a	6.2 ± 0.1^b	0.315 ± 0.009^c	6.2 ± 0.1^b	0.387 ± 0.009^d	6.1 ± 0.1^b	0.333 ± 0.009^e	6.1 ± 0.1^b	0.333 ± 0.009^e	6.1 ± 0.1^b
120±3	0.315 ± 0.009^a	6.3 ± 0.1^a	0.351 ± 0.009^b	6.1 ± 0.1^b	0.450 ± 0.009^c	5.8 ± 0.1^c	0.423 ± 0.009^d	6.0 ± 0.1^c	0.486 ± 0.009^e	5.8 ± 0.1^c	0.378 ± 0.009^e	5.8 ± 0.1^c	0.432 ± 0.009^d	5.8 ± 0.1^c
150±3	0.459 ± 0.018^a	6.0 ± 0.1^a	0.441 ± 0.009^a	5.8 ± 0.1^a	0.504 ± 0.009^b	5.4 ± 0.1^b	0.513 ± 0.009^b	5.6 ± 0.1^b	0.567 ± 0.009^c	5.3 ± 0.1^b	0.576 ± 0.009^c	5.5 ± 0.1^b	0.540 ± 0.009^d	5.6 ± 0.1^b
180±4	0.513 ± 0.009^a	5.4 ± 0.1^a	0.594 ± 0,009^b	5.4 ± 0.1^a	0.567 ± 0.009^c	5.3 ± 0.1^a	0.585 ± 0.009^c	5.2 ± 0.1^a	0.585± 0.009^c	5.1 ± 0.1^a	0.612 ± 0.009^d	5.2 ± 0.1^a	0.585 ± 0.009^c	5.2 ± 0.1^a
210±5	0.603 ± 0.009^a	5.1 ± 0.1^a	0.639 ± 0.009^b	5.3 ± 0.1^a	0.621 ± 0.009^b	5.1 ± 0.1^a	0.639 ± 0.009^b	5.0 ± 0.1^a	0.612 ± 0.009^a	5.0 ± 0.1^a	0.639 ± 0.009^b	5.0 ± 0.1^a	0.621 ± 0.009^b	5.1 ± 0.1^a

^{a,b,c,d,e} Means with different letters within a row are significantly different ($p<0.05$)

A marked prolongation of the milk coagulation time from 180 to 210 min was established in the experimental samples. The reported extension of the coagulation process is in direct relation with the increase in osmotic pressure of the culture medium as a result of presence of sucrose at a concentration of 60 $g.kg^{-1}$, which affects the vital activity of the lactic acid microorganisms. The dynamics of the lactic acid process expressed by the indicators active acidity, titratable acidity and coagulation time of the experimental samples with addition of low-esterified and amidated pectin are close to that of the control sample. The obtained results fully correspond with the studies of other researchers, which indicates that the inhibitory effect of amidated pectin on the acid coagulation of casein in the manufacture of fermented dairy products is observed in values above 2 $g.kg^{-1}$ (**Lara *et al.*, 2004**).

An important indicators determining the quality of fermented dairy products are the stability of coagulum and the tendency to syneresis. For this reason, the syneresis of coagulums observed in the control samples and experimental samples, expressed as amount of separated serum was determined by a centrifugal test.

Table 2 presents the results on the amount of serum in $cm^3.dm^{-3}$, determined by centrifugal test.

Table 2 Quantity of serum from the coagulum of samples produced by the addition of low-esterified and amidated pectin

Concentration of low-esterified amidated pectin, $g.kg^{-1}$	Control Sample	Low-esterified amidated pectin – commercial			Low-esterified amidated pectin from celery		
	0	1	2	3	1	2	3
Quantity of serum, $cm^3.dm^{-3}$	300 ± 10^a	300 ± 10^a	320 ± 5^b	340 ± 10^c	300 ± 10^a	330 ± 10^b	340 ± 10^c

^{a,b,c} Means with different letters within a row are significantly different ($p<0.05$)

The results of the centrifugal test on the quantity of serum indicate that the values of this parameter increase with the increase in the concentration of low-esterified amidated pectin. The analysis confirmed the data obtained by **Lara *et al.* (2004)**, which found that when the amidation pectin values are in the range of 2-6 $g.kg^{-1}$, the quantity of the serum increases. This tendency is explained by the gradual release of calcium ions from the casein aggregates during coagulation, due to a decrease in the pH values and the electrostatic adsorption of pectin molecules on casein particles. These processes upset the balance of casein-casein, pectin-pectin and casein-pectin bonds in the structure of the protein gel. The obtained results show that the addition of amidated pectin in fermented dairy products leads to an increase in the amount of serum, and it is recommended to use it in practice in a concentration not higher than 1 $g.kg^{-1}$. The viscosity (eta) of fermented dairy products depending on the amount and type of used amidated pectin in the range of variation of speed gradient (D) of 0.17 to 72.9 s^{-1} was determined. The obtained results are presented in Figure 1.

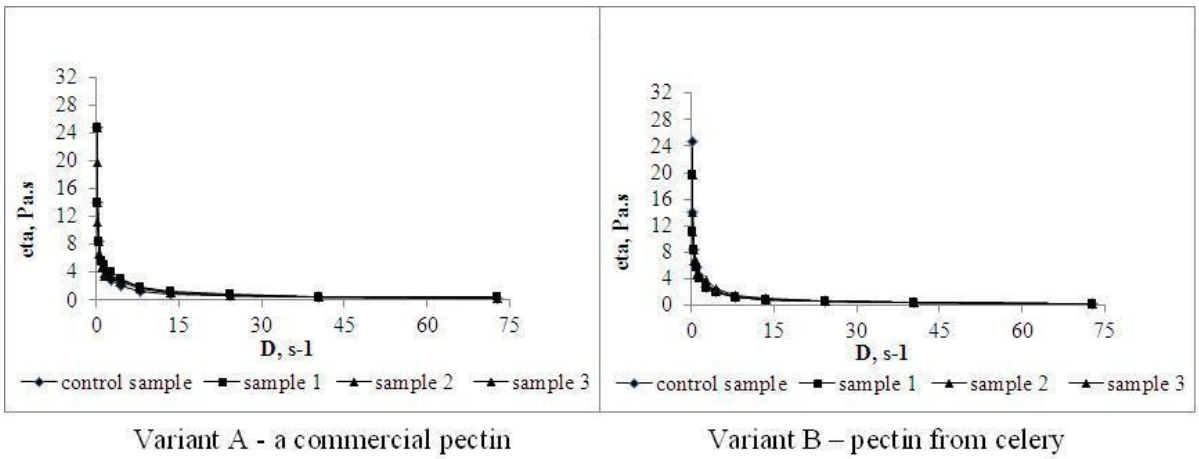

Figure 1 Viscosity of the resulting fermented dairy products with the addition of low-esterified amidated pectin

The graphically expressed dependencies show that the viscosities of the fermented dairy products with added commercial low-esterified amidated pectin and pectin extracted from celery have values close to those of the control sample. The organoleptic profile (package, appearance, texture, density of coagulum, taste and aroma) of fermented dairy products with added low-esterified amidated pectin from celery and commercial pectin in an amount of 1 to 3 g.kg^{-1} was also investigated (Figure 2).

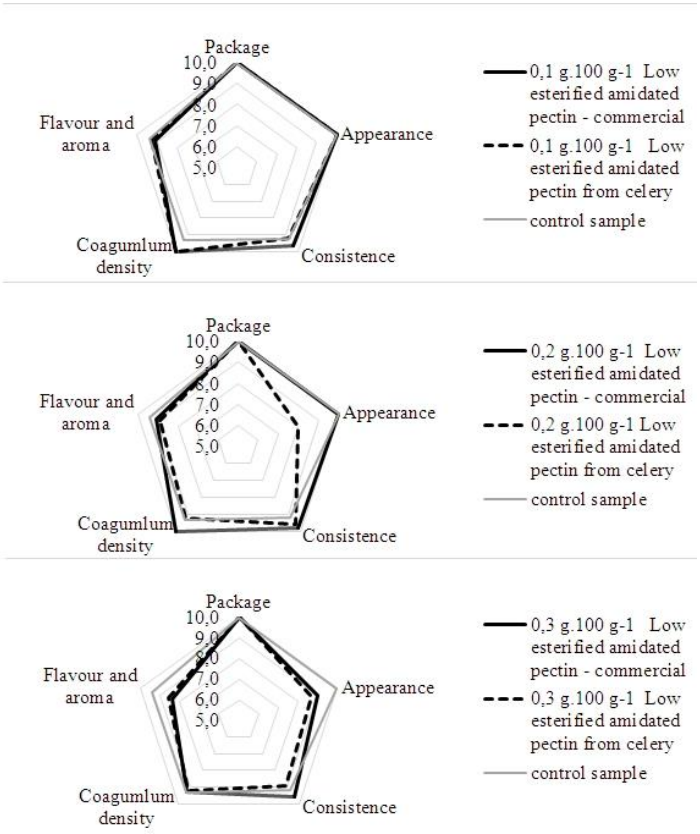

Figure 2 Organoleptic characteristic of lactic acid products with set coagulum and the addition of low-esterified amidated pectin

The obtained results reveal that the highest total score has been awarded to the variant with the addition of amidated pectin in an amount of 1 g.kg^{-1}. In all evaluated indicators - density of coagulum, appearance, texture and flavor, this variant has characteristics similar to those of the control sample. It was found that the organoleptic characteristics of fermented dairy products with low-esterified amidated pectin extracted from celery are similar to those of commercial pectin. Given these properties, low-esterified amidated pectin isolated from Bulgarian celery may be used as an alternative to commercial pectins.

CONCLUSION

Low-esterified amidated pectin isolated from celery can be used successfully for the preparation of fermented dairy products enriched with soluble dietary fibers. It was found that the addition of amidated pectin from celery in an amount exceeding 1 g.kg^{-1} deteriorates the organoleptic characteristics of the product, while the indicator viscosity of the finished product remains analogous to that of the control sample. Therefore, the recommended low-esterified amidated pectin concentrations for use in fermented dairy products should not exceed the established limits.

Acknowledgements: This research was financially supported by The Scientific Investigation Section Project 3/13-H, fund "Science", University of Food Technologies, Plovdiv, Bulgaria. We gratefully acknowledge material support from Herbstreith & Fox, Germany.

REFERENCES

BEENA, A. 2000. Healthbenefits of fermented milks, *In: ICAR. Winter school on recent developments in fermentation of milk, Kerela Agricultural University*, 88-95.

BULGARIAN NATIONAL STADARD 12:2010 – Bulgarian yogurt, [in Bulgarian].

COMBO, A., AGUEDO, M., PAQUOT, M. 2011. Les oligosaccharides pectiques: production et applications possible [Pectin oligosaccharides: production and possible applications], 15(1): 153-164, [in French].

ISO 6091:2010 (IDF 86:2010). Determination of titratable acidity (Reference method).

KHURANA, H., KANAWJIA, S. 2007. Recent Trends in Development of Fermented Milks. *Current Nutrition & Food Science*, 3:91-108. http://dx.doi.org/10.2174/1573401310703010091

KONDRATENKO, M., SIMOV, Z. 2003. Bulgarian yoghurt. *Association of milk producers in Bulgaria*, 149 – 176, [in Bulgarian].

KUNCHEVA, M., PAVLOVA, K., PANCHEV, I., DOBREVA, S. 2007. Emulsifying power of mannan and glucomannan produced by yeasts. *International Journal of Cosmetic Science*, 29(5):377-384. http://dx.doi.org/10.1111/j.1468-2494.2007.00393.x

LARA, M., LAU, K., DICKINSON, E. 2004. Effects of low-methoxyl amidated pectin and ionic calcium on rheology and microstructure of acid-induced sodium caseinate gels. *Food Hydrocolloids*, 18(2):271-281. http://dx.doi.org/10.1016/s0268-005x(03)00083-3

MAROZIENE, C., KRUIF, G. 2000. Interaction of pectin and casein micelles. *Food Hydrocolloids*, 14(4):391-394. http://dx.doi.org/10.1016/s0268-005x(00)00019-9

RAMASWAMY, H., BASAK, S. 1992. Pectin and raspberry concentrate effects on the rheology of stirred commercial yoghurt. *Journal of Food Science*, 57:357-360. http://dx.doi.org/10.1111/j.1365-2621.1992.tb05494.x

URESTI, R., ARIAS, N., CABRIALES, J., RAMIREZ, J., VAZQUEZ, M. 2003. Use of amidated low methoxyl pectin to produce fish restructured products. *Food Hydrocolloids*, 17:171-176. http://dx.doi.org/10.1016/s0268-005x(02)00049-8

VLASEVA, R. 2014. Functional dairy products. *Academic Publishing of University of Food Technologies*, 89-97, [in Bulgarian].

JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

DETECTION OF MATERNAL COLONIZATION OF GROUP B STREPTOCOCCUS BY PCR TARGETING *Cfb* AND *ScpB* GENES

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doi: 10.15414/jmbfs.2016.6.1.713-716

ARTICLE INFO

Received 22. 10. 2015
Revised 22. 3. 2016
Accepted 14. 4. 2016
Published 1. 8. 2016

Regular article

OPEN ACCESS

ABSTRACT

Group B Streptococcus (GBS) is a leading cause of neonatal morbidity and mortality. Molecular based tests, such as polymerase chain reaction (PCR), can detect GBS within hours and can be used intrapartum allowing for selective intrapartum antibiotic prophylaxis (IAP) in women carrying GBS. The aim of this work was to evaluate PCR as a rapid screening method for detection of maternal colonization of GBS compared to culture. Vaginal/rectal swabs were collected from 120 pregnant women at 35-37 weeks of gestation and cultured on CNA medium. GBS was identified by gram staining and catalase, hippurate and CAMP tests and confirmed by latex agglutination for GBS antigens. PCR was done using two assays; one targeting the *cfb* gene and the other targeting the *scp*B gene. Results revealed thatGBS colonization was detected in 15%, 23.3% and 21.7% of pregnant women by culture, *cfb* PCR assay and *scpB* PCR assay respectively. *cfb* PCR assay showed 100% sensitivity and 90.2% specificity whereas *scpB* PCR assay showed 94.4% sensitivity and 91.2% specificity. PCR could detect GBS genome at a concentration of as low as 10^{-2} for *cfb* PCR and 10^{-3} for *scpB* PCR. In conclusion, PCR is a rapid, specific and sensitive tool for detection of maternal colonization of GBS. PCR assay targeting *scpB* gene is more sensitive than that targeting *cfb* gene.

Keywords: Group B Streptococcus, polymerase chain reaction, pregnancy

INTRODUCTION

Vertical transmission of group B streptococcus (GBS) from a vagina colonized mother to her infant during labor can cause life-threatening infections in newborn. Maternal GBS colonization is associated with increased risk of transfer to the neonatal intensive care unit in term infants **(Brigtsen *et al.*, 2015).** Neonatal sepsis and pneumonia are the most important GBS-related neonatal infections, followed by meningitis, celullitis, osteomyelitis and septic arthritis **(Schrag *et al.*, 2002).** GBS is also associated with invasive and noninvasive infections in pregnant women and non-pregnant adults, especially the elderly or those with underlying medical conditions **(Dutra *et al.*, 2014).**
The centers of disease control and prevention (CDC) recommended antenatal screening for all pregnant women at 35-37 weeks of gestation for the prevention of early onset GBS disease with vaginal/rectal cultures and selective IAP administration to GBS-positive women **(CDC, 2010).** Prenatal screening by culture is currently the gold standard method for detection of anogenital GBS colonization. However, cultures require several days (24–72 h) to yield results, thus precluding their use for intrapartum screening and these are only performed at 35–37 weeks gestation **(Emonet *et al.*, 2013).** For this reason, there is a requirement for a rapid diagnostic test to detect GBS colonization status of women in labour, those in preterm labour or women who have not had prenatal care **(Gavino and Wang, 2007).**
An ideal screening test for GBS colonization is which could accurately identify pregnant women who carry the bacteria (even low-count bacteria carriers) and presenting a short turnaround time **(de-Paris *et al.*, 2011).** New rapid molecular-based tests, such as polymerase chain reaction (PCR), can detect GBS within hours. They have the potential to be used intrapartum and to allow for selective IAP in women carrying GBS **(Emonet *et al.*, 2013).** PCR assays have been developed to detect a variety of genetic targets, including genes encoding C protein, the 16S rRNA, and the 16S-23S spacer region **(Bergeron and Ke, 2004).** The objective of our study was to evaluate PCR targeting *cfb* and *scpB* genes as a screening method for detection of maternal colonization of GBS compared to culture.

MATERIAL AND METHODS

Detection of GBS by culture

The study included 120 consenting pregnant women, between 35 and 37 weeks of gestation, attending the antenatal clinic of Suez Canal University Hospital in Ismailia, Egypt. Vaginal/rectal swabs were collected from each patient and inoculated into Lim broth; a selective medium consisting of Todd-Hewitt broth supplemented with 10 µg/ml colistin and 15 µg/ml nalidixic acid. Cultures are incubated at 37°C for 24 hours and then subcultured onto CNA medium (Colombia blood agar supplemented with 10 µg/ml colistin and 15 µg/ml nalidixic acid) and incubated at 37°C for 24 hours. Colonies with a narrow zone of beta hemolysis were suggestive of GBS and were further identified by being gram positive cocci, catalase negative, CAMP (Christie, Atkins, and Munch-Peterson)positive and hippurate hydrolysis positive. Confirmation was done by a streptococcus latex agglutination test (BIOTEC Laboratories).

Detection of GBS by PCR targeting *cfb* and *scpB* genes

All the 120 specimens were tested by PCR for detection of GBS using two sets of primers; one targeting the *cfb* gene which encodes the CAMP factor and the other one targeting the *scpB* gene which encodes C5a peptidase.
After DNA extraction, PCR reaction for each assay was performed in a 25 µl volume containing 2.4 µl DNA template, 12.5 µl of 2X power Taq PCR master mix (QIAGEN, Germany), 0.7 µM of each primer. The volume for each PCR reaction was completed to 25 µl with nuclease free water. For the *cfb* PCR, primers published by **Ke *et al.* (2000)** were used. For the *scpB* PCR, the primers described by **Dmitriev *et al.* (2004)** were used. The primer sequence and amplicon size for each target gene are shown in Table (1). A negative control consisting of the reaction mixture and nuclease-free water was added in each run. In addition, a reference *S. agalactiae* (ATCC 12386) strain was used as positive control.

Table 1 Primer sequence and amplicon size for each target gene

Target gene	Primer Sequences	Amplicon Size (bp)
***cfb* gene**	5'-TTTCACCAGCTGTATTAGAAGTA-3' 5'- GTTCCCTGAACATTATCTTTGAT-3'	153
***scpB* gene**	5'-ACAATGGAAGGCTCTACTGTTC-3' 5'-ACCTGGTGTTTGACCTGAACTA-3'	255

Amplifications were carried out in a Thermocycler (Eppendorf, USA) and consisted of initial denaturation step at 94˚C for 3 minutes followed by 33 cycles of 45 seconds at 94˚C, 45 seconds at 57˚C, and one minute at 72˚C. These are followed by an extension step at 72˚C for 7 minutes. The amplicons obtained were run on 2% agarose gel with 0.5 µg/ml ethidium bromide in a Tris-borate-EDTA buffer. The gels were run in an electrophoresis gel tank at 100V for 30 minutes. After electrophoresis the sizes of DNA fragments were calculated using 100 bp ladder as DNA molecular size standards. Each gel run contained a negative control and a positive control. Finally, the DNA was visualized and photographed using a Gel Documentation System (BioSpectrum 310 Imaging System, USA).

Evaluation of PCR assay

The specificity and sensitivity of PCR assay using the previously mentioned primers were evaluated compared to culture. The analytical sensitivity (i.e. the detection limit or the minimal number of genome copies that canbe detected) of each assay was determined by testing serial 10-fold dilutions of purified genomic DNA from a reference GBS strain (ATCC 12386), containing from 10^{-1} to 10^{-7} CFU/ml and starting with a concentration of 38.1 ng/µl. The stock DNA concentration was measured using the nanodrop technique (NanoDrop ND-1000 spectrophotometer). Each 10-fold dilution was added directly to the PCR reaction mixture before PCR amplification for each of the *cfb* and *scpB* genes. Amplifications were carried out using the same conditions used in the two PCR assays carried out before. A negative control was included in the reaction for both genes. After gel electrophoresis, the DNA was visualized and photographed. The analytical sensitivity of each of the two PCR assays was determined as the least concentration of genomic DNA at which the PCR gave a detectable band.

RESULTS AND DISCUSSION

Eighteen samples out of 120 were identified as GBS by culture. The rate of maternal colonization of GBS by the culture method was 15%. Twenty eight specimens (23.3%) were positive for GBS using the *cfb* PCR assay. Positive specimens showed specific bands of approximately 153bp in size (Figure 1). Twenty six specimens (21.7%) were positive for GBS using the *scpB* PCR assay. Positive specimens showed specific bands of approximately 255bp in size (Figure 2).

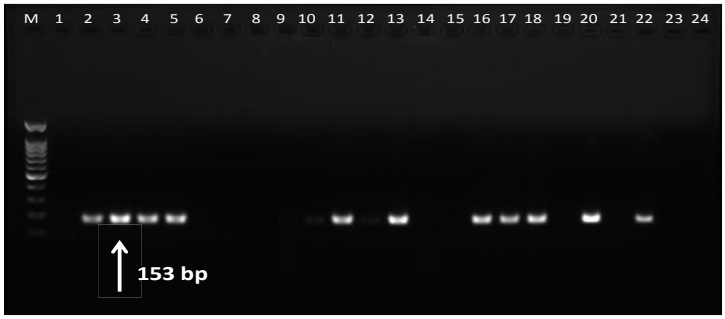

Figure (1) *cfb* PCR assay (153 bp amplicon**)**
M is a 100 bp DNA ladder, Lane 1 is a negative control, Lane 2 is a positive control; Lanes 3-24 are tested specimens

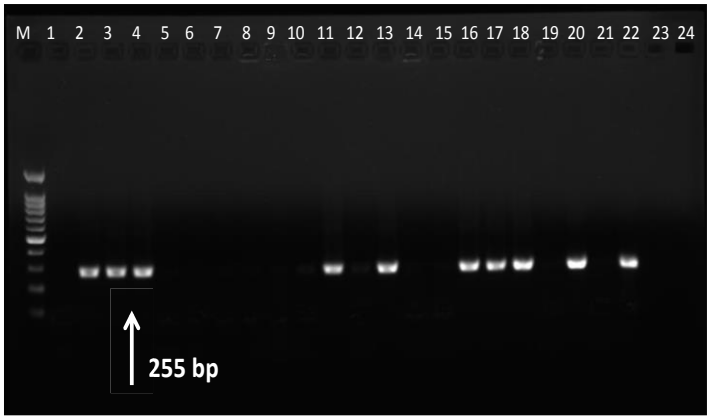

Figure (2) *scpB* PCR assay (255 bp amplicon)
M is a 100 bp DNA ladder, Lane 1 is a negative control, Lane 2 is a positive control, Lanes 3-24 are tested specimens

All the eighteen culture positive specimens were also positive by the *cfb* PCR assay while only 17 of them were positive by *scpB* PCR assay. Among the 102 culture negative specimens, 10 were positive by *cfb* PCR assay while 9 were positive by *scpB* PCR assay. In comparison to the culture method, the *cfb* PCR assay exhibited 100% sensitivity and 90.2% specificity with a positive predictive value of 64.3% and a negative predictive value of 100% whereas the *scpB* PCR assay revealed 94.4% sensitivity and 91.2% specificity with a positive predictive value of 65.4% and a negative predictive value of 98.9%. On determining the detection limit of PCR, it was found that both *cfb* and *scpB* PCR assays were able to detect GBS DNA at a concentration of $0.01(10^{-2})$, but the *cfb* PCR assay was slightly more sensitive being able to detect GBS DNA at a lower concentration (one log 10 difference;10^{-3}) (Figure 3).

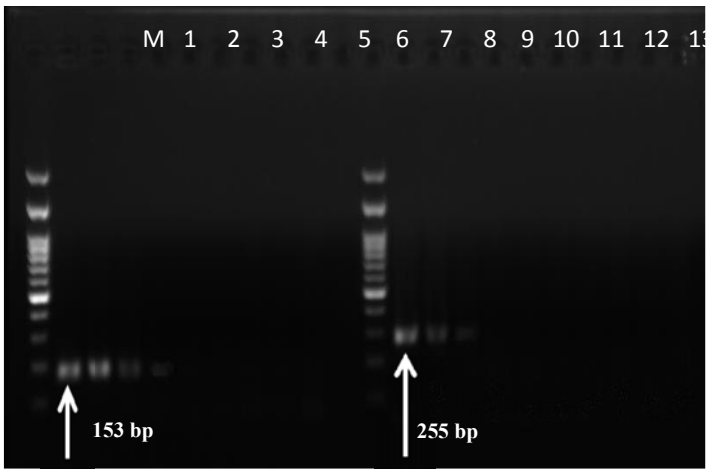

Figure (3) Detection limits of *cfb* PCR assay (153 bp amplicon)and *scp*B PCR assay (255 bp amplicon)
M & lane 11 are 100 bp DNA ladder, Lanes 1& 12 are neat DNA, Lanes 2-9 & 13-20 are serial 10-fold dilutions of DNA extract, Lanes 10 & 21 are negative control

Although culture methods are the current standard for prenatal GBS screening, the implementation of more sensitive molecular diagnostic tests may be able to further reduce the risk of early-onset GBS infection **(Buchan *et al.*, 2015).** Using the culture method, this study reported that the rate of GBS colonization in pregnant women was 15%. This rate varies greatly among countries. It was reported to be 7.98% in Italy **(Puccio *et al.*, 2014)**, 7.2% in Ethiopia **(Woldu *et al.*, 2014),** 14% in Denmark **(Peterson *et al.*, 2014),** 20% in USA **(Page-Ramsey *et al.*, 2013)**, 21.8% in Taiwan **(Lee and Lai, 2014)**, 20.7% in Kuwait and 18.4% in Lebanon **(Ghaddar *et al.*, 2014).**These variations in colonization rates relate to intrinsic differences in populations and to lack of standardization in culture methods employed for ascertainment, Also, a change in the prevalence over time, or real population differences account for some of the disparity in these reported prevalence rates**.** In spite of the great variation of prevalence rates, **Le and Heath (2013)** reported that the serotype distribution of GBS isolates is similar in Africa, Western Pacific, Europe, the Americas and the Eastern Mediterranean regions and has not changed over the past 30 years.
This study showed that PCR using *cfb* and *scpB* genes was more sensitive for detection of GSB than the culture method as the rate of detection was 15% by the culture method compared to 23.3 % by *cfb* PCR assay and 21.7% by *scpB* PCR

assay. A previous study done in Egypt by **Shabayek *et al*. (2009)** reported that GBS was detected in 25.3% of isolates by culture, 30.6% by *cfb* PCR assay and 30% by *scpB* PCR assay. **Bakhtiari *et al.* (2012)** found out that the frequencies of GBS carriage were 9.3% by the culture method and 11.2 % by a PCR assay targeting *cfb* gene.

The increased sensitivities of GBS-specific PCR assays over the culture method could have many explanations; a possible explanation may be the presence of nonviable GBS that could be detected by PCR but not by culture, as well as inability of culture to detect low bacterial numbers. Suppression of GBS growth by *Enterococcus fecalis* that exist in the vaginal flora (antagonistic phenomenon) has also been documented **(Park *et al.*, 2001).** Antibiotics and feminine hygiene products have also shown to interfere with the detection of GBS by culture but have no detrimental effect on PCR **(Ostroff and Steaffens, 1995).** Inadequate specimen collection and transport from obstetrical clinics to the laboratory may have some effect especially in case of light colonization **(Rosa-Fraile *et al.*, 2005).**

Great sensitivity, high negative predictive value and rapid results are desirable parameters of a screening test. In our study, the sensitivities of *cfb* and *scpB* PCR assays were 100% and 94.4% respectively. In the study of **Rallu *et al.* (2006)**, their sensitivities were 75.3% and 99.6% respectively. **Goudarzi *et al.* (2015)** reported PCR sensitivity 72.2%. The high sensitivity in our study is probably attributed to the use of selective and enriched broth media previous to performing the PCR.

The negative predictive values were 100% and 98.9% for *cfb* and *scpB* PCR assays respectively, which were similar to the findings of **de-Paris *et al.* (2011)**who reported a negative predictive value of 100%. This finding is important because it indicates that all samples with negative results are truly negative, which affords to safely with hold treatment from women presenting PCR negative samples. This is so important in clinical routine because false negative results in a screening test may lead to serious consequences for the patient, considering that this test is used to take a decision about antibiotic prophylaxis.

The specificities were found to be 90.2% and 91.2% for *cfb* and *scpB* PCR assays respectively. These were less than the 95.65% reported by **Mulleur *et al.* (2014)** and the 100% reported by **Daher *et al.* (2014).** However, even being considered the gold standard, culture results can be false negative. It is known that culture may not be absolutely effective in the detection of GBS, since other bacteria of the genital tract can inhibit the growth of GBS even when using the selective broth. So, the supposedly false positive results in PCR may actually indicate the presence of GBS in the studied material, since this is an analytical technique whose sensitivity could be greater than the bacteriological examination. The gold standard performance affects the positive predictive value parameter. In this study the positive predictive value was 64.3% and 65.4% for *cfb* and *scpB* PCR assays respectively compared to the 59% found by **de-Paris *et al.* (2011).** Regarding the analytical sensitivity assessment of PCR assays, it was evident that the *cfb* PCR assay was slightly more sensitive being able to detect GBS DNA at a lower concentration (10^{-3}) than that detected by the *scpB* PCR assay (10^{-2}).

The PCR assays in this study required about 100 minutes for sample processing, PCR amplification, and gel electrophoresis and even with using a previous incubation in selective broth, it required 24 hours to give the final result. This offers an advantage over the culture method which is a time-consuming method requiring at least 48 hours for full GBS identification.

CONCLUSION

Although more expensive than the standard culture method, the PCR technique targeting *cfb* and *scpB* genes is rapid, specific and has a higher sensitivity in detecting GBS carriers during pregnancy with the *scpB* PCR assay being more sensitive than the *sfb* PCR assay. PCR allows for accurate diagnosis of GBS which will be translated into more rational use of antibiotics and more effective treatment of carrier females leading to reduction of newborn morbidity and mortality. Yet, the cost-effectiveness of such PCR tests need to be more elucidated by further studies to see if it can be used as a routine screening method in centers with maternity wards.

REFERENCES

BAKHTIARI, R., DALLAL, MS., MEHRABADI, J., HEIDARZADEH, S., AND POURMAND, M. 2012. Evaluation of culture and PCR methods for diagnosis of groupB streptococcus carriage in Iranian pregnant women. Iran J Public Health, 41(3), 65-70.

BERGERON, MG., KE, D. 2004. New DNA-Based PCR approaches for rapid real-time detection and prevention of Group B Streptococcal infections in newborns and pregnant women. Reprod Med Rev, 11, 25-41.http://dx.doi.org/10.1017/s1462399401003805

BRIGTSEN, AK., JACOBSEN, AF., DEDI, L., MELBY, KK., FUGELSETH, D., WHITELAW A. 2015**.**Maternal Colonization with Group B Streptococcus Is Associated with an Increased Rate of Infants Transferred to the Neonatal Intensive Care Unit. Neonatology. 108(3), 157-163.http://dx.doi.org/10.1159/000434716

BUCHAN, BW., FARON, ML., FULLER, D., DAVIS, TE., MAYNE, D., LEDEBOER, NA. 2015. Multicenter clinical evaluation of the Xpert GBS LB assay for detection of group B Streptococcus in prenatalscreening specimens. J Clin Microbiol, 53(2), 443-8. http://dx.doi.org/10.1128/jcm.02598-14

(Centers for Disease Control and Prevention). 2010. Prevention of Perinatal Group B Streptococcal Disease. MMWR Morb Mortal Wkly Rep, 59:(RR-10), 1-32.

DAHER, RK., STEWART, G., BOISSINOT, M.,BERGERON, MG.2014**.** Isothermal recombinase polymerase amplification assay applied to the detection of groupBstreptococci in vaginal/anal samples. Clin Chem, 60(4), 660-6. http://dx.doi.org/10.1373/clinchem.2013.213504

de-PARIS, F., MACHADO, AB., GHENO, TC., ASCOLI, BM., OLIVEIRA, KRPD., BARTH, AL. 2011. Group B *Streptococcus* detection: comparison of PCR assay and culture as a screening method for pregnant women. Braz J Infect Dis, 15(4), 323-327.http://dx.doi.org/10.1590/s1413-86702011000400004

DMITRIEV, A., SUVOROV, A., SHEN, AD., YANG, YH. 2004. Clinical diagnosis of group B streptococci by scpB gene based PCR. Indian J Med Res, 119 (Suppl):233–6.

DUTRA, VG., ALVES, VMN., OLENDZKI, AN., DIAS, CAG., DE BASTOS, AFA., SANTOS, GO., DE AMORIN, ELT., SOUSA, MÂB., SANTOS, R., RIBEIRO, PCS., FONTES, CF., ANDREY, M., MAGALHÃES, K., ARAUJO, AA., PAFFADORE, LF., MARCONI, C., MURTA, EFC., FERNANDES, JPC., RADDI, MSG., MARINHO, PS., BORNIA, RBG., PALMEIRO, JK., DALLA-COSTA, LM., PINTO, TCA., BOTELHO, AN., TEIXEIRA, LM., FRACALANZZA, SEL. 2014. *Streptococcus agalactiae* in Brazil: serotype distribution, virulence determinants and antimicrobial susceptibility. BMC Infect Dis, 14, 323.http://dx.doi.org/10.1186/1471-2334-14-323

EMONET, S., SCHRENZEL, J., MARTINEZ DE TEJADA, B. 2013. Molecular-based Screening for Perinatal Group B Streptococcal Infection: Implications for Prevention and Therapy. Mol Diagn Ther, 17, 355–361. http://dx.doi.org/10.1007/s40291-013-0047-2

GAVINO, M., WANG, E. 2007. A comparison of a new rapid real-time polymerase chain reaction system to traditional culture in determining group B Streptococcus colonization. Am J Obstet Gynecol, 197, 388e1-388e4. http://dx.doi.org/10.1016/j.ajog.2007.06.016

GHADDAR, N., ALFOUZAN, W., ANASTASIADIS, E., AL JISER, T., ITANI, SE., DERNAIKA, R., EID, T., GHADDAR, A., CHARAFEDDINE, A., DHAR, R., EL HAJJ, H. 2014.Evaluation of chromogenic medium and direct latex agglutination test for detection of group B streptococcus in vaginal specimens from pregnant women in Lebanon and Kuwait. J Med Microbiol, 63(Pt 10), 1395-9. http://dx.doi.org/10.1099/jmm.0.066738-0

GOUDARZI, G., GHAFARZADEH, M., SHAKIB, P., ANBARI, K. **2015.**Culture and Real-Time PCR Based Maternal Screening and Antibiotic Susceptibility for Group B Streptococcus: An Iranian Experience. Glob J Health Sci, 7(6), 45075. http://dx.doi.org/10.5539/gjhs.v7n6p233

KE, D., MENARD, C., PICARD, FJ., BOISSINOT, M., OUELLETTE, M., ROY,PH., BERGERON, MG. 2000. Development of conventional and real-time PCR assays for the rapid detection of group B streptococci. Clin Chem, 46(3), 324–31.

LE DOARE, K., HEATH, PT. 2013.An overview of global GBSepidemiology. Vaccine,31 Suppl 4, D7-12. http://dx.doi.org/10.1016/j.vaccine.2013.01.009

LEE, WT., LAI, MC. 2014. High prevalence of *Streptococcus agalactiae* from vaginas of women in Taiwan and its mechanisms of macrolide and quinolone resistance. J Microbiol Immunol Infect, (14)00055-3.http://dx.doi.org/10.1016/j.jmii.2014.03.002

MUELLER, M., HENLE, A., DROZ, S., KIND, AB., ROHNER, S., BAUMANN, M., SURBEK, D. 2014.Intrapartum detection of GroupBstreptococci colonization by rapid PCR-test on labor ward.Eur J Obstet Gynecol Reprod Biol**,**176, 137-41.http://dx.doi.org/10.1016/j.ejogrb.2014.02.039

OSTROFF, RM., STEAFFENS, JW. 1995. Effect of specimen storage, antibiotics, and feminine hygiene products on the detection of Group B Streptococcus by culture and the STREP B OIA test. Diagn Microbiol Infect Dis, 22, 253—9.http://dx.doi.org/10.1016/0732-8893(95)00046-d

PAGE-RAMSEY, SM., JOHNSTONE, SK., KIM, D., RAMSEY, PS. 2013. Prevalence of group B Streptococcus colonization in subsequent pregnancies of group B Streptococcus-colonized versus noncolonized women.Am J Perinatol, 5, 383-8. http://dx.doi.org/10.1055/s-0032-1326981

PARK, CH., VANDEL, NM., RUPRAI, DK., MARTIN, EA., GATES, KM., COKE, D. 2001. Detection of Group B Streptococcal colonization in pregnant women using direct latex agglutination testing of selective broth. J Clin Microbiol, 39, 408—9.http://dx.doi.org/10.1128/jcm.39.1.408-409.2001

PETERSEN, KB., JOHANSEN, HK., ROSTHØJ, S., KREBS, L., PINBORG, A., HEDEGAARD, M. 2014.Increasing prevalence of group B streptococcal infection among pregnant women. Dan Med J, 61(9), A4908.

PUCCIO, G., CAJOZZO, C., CANDUSCIO, LA., CINO, L., ROMANO, A., SCHIMMENTI, MG., GIUFFRÈ, M., CORSELLO, G. 2014**.** Epidemiology of Toxoplasma and CMV serology and of GBS colonization in pregnancy and neonatal outcome in a Sicilian population. Ital J Pediatr, 40, 23. http://dx.doi.org/10.1186/1824-7288-40-23

RALLU F, BARRIGA P, SCRIVO C, MARTEL-LAFERRIÈRE V, LAFERRIÈRE C.2006. Sensitivities of antigen detection and PCR assays greatly increased compared to that of the standard culture method for screening for group B Streptococcus carriage in pregnant women. J Clin Microbiol, 44(3), 725–728.http://dx.doi.org/10.1128/jcm.44.3.725-728.2006

ROSA-FRAILE, M., CAMACHO-MUNOZ, E., RODRIGUEZ-GRANGER, J., LIÉBANA-MARTOS, C. 2005.Specimen storage in transport medium and detection of Group B Streptococci by culture. J ClinMicrobiol, 43, 928–930.http://dx.doi.org/10.1128/jcm.43.2.928-930.2005

SCHRAG, S., GORWITZ, R., FULTZ-BUTTS, K., SCHUCHAT, A. 2002. Prevention of perinatal group B streptococcal disease. Revised guidelines from CDC. Morb Mortal Wkly Rep RecommRep, 51, 1-22.

SHABAYEK, S., ABDALLA, S., ABOUZEID, A. 2009. Vaginal carriage and antibiotic susceptibility profile of Group B Streptococcus during late pregnancy in Ismailia, Egypt. J Infec Public Health, 2, 86-90.http://dx.doi.org/10.1016/j.jiph.2009.03.004

WOLDU, ZL., TEKLEHAIMANOT, TG., WAJI, ST., GEBREMARIAM, MY. 2014.The prevalence of Group B Streptococus recto-vaginal colonization and antimicrobial susceptibility pattern in pregnant mothers at two hospitals of Addis Ababa, Ethiopia.Reprod Health, 11, 80. http://dx.doi.org/10.1186/1742-4755-11-80

JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

MODULATION OF DIGESTIVE ENZYMES AND LIPOPROTEIN METABOLISM BY ALPHA MANGOSTEEN EXTRACTED FROM MANGOSTEEN (*GARCINIA MANGOSTANA*) FRUIT PEELS

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doi: 10.15414/jmbfs.2016.6.1.717-721

ARTICLE INFO

Received 16. 7. 2015
Revised 20. 4. 2016
Accepted 21. 4. 2016
Published 1. 8. 2016

Regular article

OPEN ACCESS

ABSTRACT

The present work deals with the potential of *Garcinia mangostana* L. (Mangosteen, Clusiaceae) for modulation of digestive and plasma lipid transfer protein as an approach to discover novel inhibitors. *Garcinia mangostana* (in methanol, dicholromethane and hexane) extracts were screened for alpha amylase, alpha glucosidase and Cholesteryl ester transfer protein (CETP) inhibition assays. TLC, HPLC, LC-MS analysis were performed and was compared with reference standard. Alpha amylase results obtained were 39.4 µg/ml, 11.87 µg/ml and 9.048 µg/ml respectively. For CETP inhibition assay the dose response was done only for the hexane extract as others were not showing potent inhibition. Thus an IC_{50} of 10.89 µg/ml was obtained and the hexane extract was taken for further analysis to discover the compound responsible for the activity. Alpha mangosteen was found to be the active compound in *Garcinia mangostana* responsible for the potent inhibitor activity of alpha amylase and CETP enzyme in plant raw material.

Keywords: α-mangosteen, α-amylase, α-glucosidase, CETP inhibition, LC-MS

INTRODUCTION

Naturally available plant materials including herbs, food-processing by-products, contain active compounds, which have numerous health benefits. The identification of active ingredients and the mechanism of their action are very important to understand their health benefits. Some of these phytochemicals have been found to inhibit particular enzymes to treat specific disease. Bioactive molecules from plant sources having ability to inhibit the activity of digestive enzymes are targeted as preventative measures. Not only digestive enzymes but also metabolic enzymes, which catalyze the biochemical reactions taking place in body, are now the new and emerging trends to be evaluated for the specific diseases. For example, cholesteryl ester transfer protein is a metabolic enzyme, which facilitates the transfer of cholesteryl esters (CE) from high-density lipoprotein (HDL) to low-density lipoprotein (LDL) in exchange of triglycerides. Some plant-based food compounds, such as berry polyphenols are reported as having amylase inhibition activity (**McDougall *et al*., 2005**). There is still lack of research and report to prove the efficiency of compounds directly by cholesteryl ester transfer protein assay. Thus, there is need of exploring the novel inhibitors of such enzymes would definitely provide consumers a wide range of options to consume as food product and also for its disease preventing health benefits.

The present study deals with the exploration of such novel enzyme modulators, which are from natural plant sources. The phytochemicals which are responsible for the health benefits coming from plant sources are of considerable interest due to their wide range of physiological properties like antiallergenic, antiartherogenic, anti-inflammatory, antimicrobial, antioxidant, antithrombotic and cardio protective effects. Phytochemicals especially phenolic compounds have been reported to have many benefits on human health due to their presence in fruits and vegetables in high levels. **Zadernowski *et al*. (2009)** determined the composition of phenolic acids in various parts of mangosteen fruit (*Garcinia mangostana*) by GC-MS. Based on the traditional uses, certain plant extracts were selected which have already been reported for antioxidant, antidiabetic, antihyperlipidemic activities such as leaves of *Camellia sinensis*, *Azadirachta indica,* pericarp of *Garcinia mangostana* and rhizome of *Zingiber cassumunar* (**Ghasemzadeh *et al*. 2010, Mary *et al*. 2012**). These plant extracts were selected due to their physiological properties and were hypothesized to be potential inhibitors of digestive and metabolic enzyme. The overall objective of the present study was to discover novel inhibitors of metabolic and digestive enzymes with the help of certain biochemical assays.

Garcinia mangostana, known as "Queen of fruits" is mostly found in India, Sri Lanka, Myanmar, Malaysia, Philippines and Thailand. The pericarp of the fruit contains 5.5 % (w/w) tannin resin and a yellow crystalline bitter principle compound, later named as mangosteen and chemical formula is $C_{20}H_{22}O_5$ (Nadkarni & Nadkarni, 1999). Xanthones from mangosteen fruit have been found to have significant biological activities such as antibacterial, anti-inflammatory, antioxidants, antitumoral, antifungal, antiallergic, and antiviral etc. (**Suksamrarn *et al*., 2006**). Garcinone E, Alpha mangosteen, Beta mangosteen, Gamma mangosteen and gartanin are the important xanthones present in mangoteen fruit. The bioactive compounds from the fruit also positively affect lipid profile of plasma and also antioxidant activity in rats which are fed with cholesterol-containing diets (**Leontowicz *et al*., 2007**). Garcinone E, mangostinone, alpha, beta and gamma mangosteens, gartanin, 1,7-dihydroxy-2-(3-methylbut-2-enyl)-3-methoxyxanthone) and 1,5-dihydroxy-2-(3-methylbut-2-enyl)-3-methoxyxanthone were isolated from pericarps of mangosteen fruit (**Asai *et al*. 1995**). A new polyoxygenated xanthone, mangostanol and other known xanthones gartanin, α-mangostin, 8 deoxygartanin, γ-mangostin, 5,9-dihydroxy-2,2-dimethyl-8-methoxy-7-(3-methylbut-2-enyl)-2H,6H-pyrano[3,2-b]xanthen-6-one,2-(γ,γ-dimethylallyl)-1,7-dihydroxy-3- ethoxyxanthone, garcinone E, and epicatechin were also isolated from mangosteen fruit hulls (**Chairungsrilerd *et al*., 1996**). Mangostenol, mangostenone and mangostenone B, three new xanthones along with the known xanthones α -mangosteen, trapezifolixanthone, garcinone B, tovophyllin B, beta mangosteen, mangostinone, mangostanol and flavonoid epicatechin were also isolated from mangosteen fruit hulls (**Suksamrarn *et al*., 2002**). Four new compounds garcimangosone A, garcimangosone B and garcimangosone C, also a benzophenone glucoside, garcimangosone D were also isolated (**Huang *et al*., 2001**).

MATERIALS AND METHODS

Materials

Garcinia mangostana (Mangosteen fruit) was procured from local market at Pathumthani, Thailand. Other plant materials were collected from G7 Synergon Ltd, Bangalore, India. Raw materials were cleaned; pericarp was separated from

edible pulp for Mangosteen fruit. Pericarp was dried at 50°C in hot air oven for 10-12 hrs. Dried samples (< 10 % M.C.) were ground in Tefal food processor (Hong Kong) until fine powder, stored in air tight containers in dark at room temperature (25 ± 2°C) for further analysis. Alpha amylase enzyme (Type VI B from porcine pancreas from Sigma), 2-chloro-4-nitrophenol-α-D-maltotrioside, acarbose, sodium dihydrogen orthophosphate and disodium hydrogen phosphate dihydrate (from Hi Media). Dimethyl sulphoxide was obtained from Sigma Aldrich. CETP Inhibitor Drug Screening Kit (Bio Vision) was procured from Gila Laboratories, Bangalore, India.

Preparation of crude extract

Hexane, dicholromethane and methanol were used to extract the bioactives from *Garcinia mangostana*, 20g of the powdered sample was extracted in 100mL of each of the mentioned solvents. Methanol and hexane extraction was carried out at 55°C and with dichloromethane at 37°C for 5 hours using an orbital shaker at 60 rpm. Mixture was further centrifuged at 10000 rpm, for 10 mins. Supernatant was collected and filtered using 0.45 µm membrane filters and evaporated to near dryness. Residue then collected was weighed and stored at 4 °C for further use in assay.

Preparation of rat intestinal mucosa

The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Govt. of India. Sprague Dawley rats (N=2) were sacrificed and their intestine was separated and chilled with ice cold phosphate buffer (80 mM, pH 7.0). Rat's intestinal mucosa was homogenized with four parts of ice-cold phosphate buffer (80 mM, pH 7.0). Tube was chilled with ice and kept for homogenization for 10 min. Large cell debris and nuclei like materials were separated by centrifugation In an eppendorf centrifuge at 10,000 rpm for 10 min maintained at 4°C and finally the supernatant was collected and stored at -20 °C until further use (**Leontowicz *et al*., 2007**).

Digestive and metabolic enzymes assay

α-amylase assay

Extract was dissolved in methanol and was diluted with phosphate buffer (pH 6.9) to obtain various concentrations ranging from 1-200µg/ml. The inhibitory activity of sample was assayed using procine pancreatic α-amylase, Type VI-B enzyme and 2-chloro-4-nitrophenol-α-D-maltotrioside (CNPG-3 reagent) as a substrate. Phosphate buffer (40 µl, 40 mM, pH 6.9) along with the test sample (1-200µg/ml), reference standard (0.1-10µg/ml) and 20 µl enzyme was pre incubated at 37°C for 10 mins. 80 µl substrate reagent (CNPG3) was added and incubated at 37°C for 8 mins in a 96 well plate and absorbance was read at 405 nm. A control reaction was also carried out without the test sample. The following formula was used to calculate the Inhibition %

Inhibition (%) = {Control absorbance - Test absorbance} x 100

Plot of percentage inhibition against the sample concentration was plotted and a logarithmic regression curve was established to calculate the IC_{50} value using Graph Pad Prism software. Half maximal inhibitory concentration (IC_{50}), represents the concentration of sample (µg/ml) necessary to decrease the activity of the enzyme by 50%.

α- Glucosidase activity of Mangosteen Extracts

Extract was dissolved in methanol and further diluted with phosphate buffer (pH 7.0) to obtain various concentrations ranging from 1-200µg/ml. The inhibitory activity of sample was assayed using α-glucosidase enzyme, isolated from rat intestinal mucosa and sucrose as a substrate. Phosphate buffer (40 µl, 80 mM, pH 7.0) along with the test sample (1-200µg/ml), reference standard (0.1-10 µg/ml) and 20 µl enzyme was pre incubated at 37°C for 30 min. The mixtures were incubated at 37°C for 20 mins after addition of sucrose solution (100 µl). The reaction was arrested by heating in a boiling water bath and cooled. Sample (50 µl) was added with 100 µl of glucose reagent from glucose reagent kit and incubated for 10 min at room temperature (25 °C), followed by measuring the absorbance at 510 nm (**Tadera *et al*., 2006**). A control reaction was carried out without the test sample. The following formula was used to calculate the Inhibition %

Inhibition (%) = {Control absorbance - Test absorbance} x 100

The IC_{50} values were calculated using Graph Pad Prism software as mentioned in

α-amylase assay

CETP inhibition activity of Mangosteen extracts

The assay uses a donor molecule containing a fluorescent self-quenched neutral lipid that is transferred to an acceptor molecule In the presence of CETP (rabbit serum). CETP-mediated transfer of the fluorescent neutral lipid to the acceptor molecule results in an increase in fluorescence. Inhibitor of CETP will inhibit the lipid transfer and therefore decreases the fluorescence Intensity. Varying concentrations of the plant extracts ranging from (10-200 µg/mL) were used for the assay. Rabbit serum (1.5 µl) was added as enzyme source to the test sample and kept for pre-incubation at 37 °C for 10 min. Master mix (20 µl) containing 5 µl donor molecule, 5 µl acceptor molecule and 10 µl CETP assay buffer was added and remaining volume was made up with 20 µl of buffer and kept for incubation at 37 °C for 30 min. Blank without rabbit serum and positive control containing rabbit serum, only without test inhibitors were also conducted. The samples were analyzed by Fluorescence spectrometer (excitation wavelength 465 nm and emission wavelength of 535 nm) using a Tecan reader in a 96 well flat black microtiter plate.

Identification of active compound

Thin Layer Chromatography (TLC)

Screening of herbal products and their quality evaluations can be done easily with the help of standardized thin layer chromatographic techniques (**Mohammad *et al*., 2010**). According to **Mishra *et al*. (2009)**, quantitative estimation of alpha mangosteen can be done in fruit pericarp of *Garcinia mangostana* by using high performance thin layer chromatographic (HPTLC) method where methanol and chloroform was found to give highest and second highest recovery of alpha mangosteen, the bioactive xanthone. For our work, several solvent combinations were tried and optimized for separation of compounds in the extracts.

High Performance liquid chromatography (HPLC) and Liquid Chromatography Mass Spectrometry (LC-MS)

The separation of compounds was performed using HPLC 1200 (Agilent Technologies) with UV detector system. The reverse phase C18 Column (Phenomenex) was used, wavelength at 250 mm. The mobile phase (80 acetonitrile: 20 water) was filtered through fine membranes (0.4-0.5 micron) and samples to be injected were dissolved in HPLC grade solvent DMSO and further diluted with methanol. The injection volume for all samples was 10 µl. The flow rate was 0.6 ml/min, Total run time given was 45 min and detection was done at 210, 254, 310, 320 and 455 nm. Mass analysis was done in Agilent Triple Quad 6410 LC MS-MS instrument from Agilent Technologies. In this case, mobile phase used was Acetonitrile and 0.1% acetic acid in the ratio of 70:30 (v/v).

RESULTS AND DISCUSSION

α amylase inhibition assay

Acarbose was taken as standard drug which is a prescribed α amylase inhibitor for the treatment in Type 2 diabetes (**Loo *et al*., 2007**). Inhibition percentage for different conc. (0.1-10 µg/ml) of Acarbose was plotted on a nonlinear regression graph using graph pad prism software. Fig 1 illustrates the dose response curve, which has log of concentration of Acarbose on X axis and % inhibition (dose response) on Y axis.

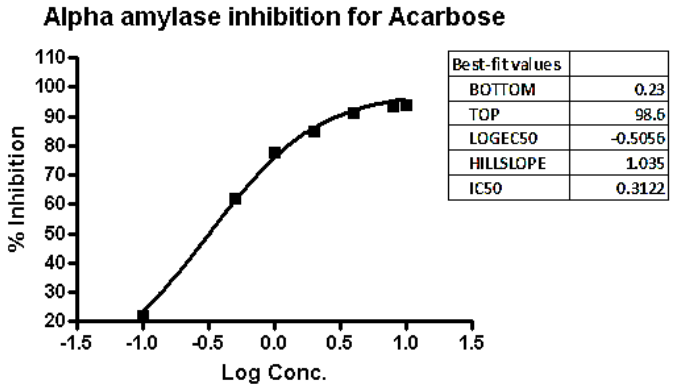

Figure 1 Dose response curve (Alpha Amylase Inhibition assay) for Reference standard Acarbose

The graph signifies the behavior of the curve at different concentrations of reference standard. An IC_{50} of 0.31 µg/ml was obtained. Acarbose has been proven as a very safe drug and a prescribed medication to treat Type 2 diabetes (**Mertes, 2001**). Thus the natural sources of alpha amylase inhibitors can be compared with this reference to prove their inhibitory activities.

***Garcinia mangostana* extracts**

The methanolic, dichloromethane and hexane extract of *Garcinia mangostana* were analyzed for the alpha amylase inhibition assay at concentrations of (10-100) µg/ml. Methanol (1% v/v) was used as vehicle control. Percentage of inhibition was calculated using the equation:

$$\% \text{ Inhibition} = \{(\text{Control OD} - \text{Test OD})/\text{Control OD}\} * 100$$

The percentage inhibition plotted against log of concentration of the dose gave an IC_{50} of 39.4 µg/ml, 11.87 µg/ml and 9.05 µg/ml for methanol, dicholromethane and hexane extract respectively of *Garcinia mangostana*. Fig 2 illustrates the dose response curves for all the three extracts of *Garcinia mangostana.* The percentage inhibition was found increasing with the increased concentration of extracts. The three extracts are showing different results with the most potent activity found in the hexane extract having IC_{50} of 9.05 µg/ml which is slightly more potent than IC_{50} of dichloromethane extract which is 11.87 µg/ml. The results reflect that the active compound is of non-polar nature.

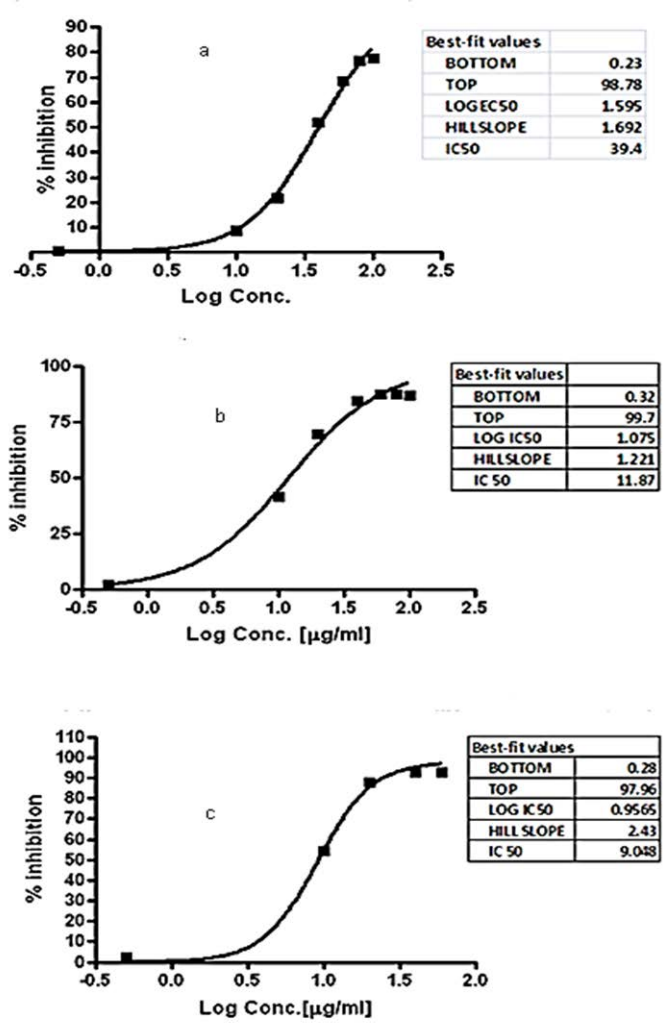

Figure 2 Dose response curve (α amylase inhibition assay) for a) methanolic, b) dichloromethane and c) hexane extract of *Garcinia mangostana*

In a study where different fractions of *Garcinia mangostana* plant were tested, polyphenols rich fraction was found to have highest inhibitory activity with IC_{50} of 5.4 µg/mL but total phenolic content was not having any correlation with the alpha amylase inhibitory activity. Oligomeric proanthocyanidins were the main compounds present in the fraction having high inhibitory activity (**Loo *et al*., 2007**). In another study, the ethanol extract of the aerial parts of *Salvia Virgata* showed an Inhibitory activity on pancreatic α - amylase. Further the study led to Identification of the active flavone, chrysoeriol. (**Nickavar *et al*., 2013**). Tea polyphenols (0.05 mg/ml) have also inhibited α amylase with 61%, which also suggested that tea polyphenol might possess antinutritional properties (**He *et al*., 2006**). In the present study *Garcinia mangostana* has been found to have a very significant activity against alpha amylase enzyme and has already been reported to have a very good antioxidant activity. Thus the correlation between the two activities can be a good area to explore.

Alpha glucosidase inhibition assay

Methanol, dichloromethane and hexane extracts of *Garcinia mangostana* were assayed for alpha glucosidase inhibition assay with Acarbose as standard and did not show any significant inhibition. Berry extracts rich in polyphenols inhibited alpha amylase and alpha glucosidase (**Ashley *et al*., 2012**). Anthocyanins inhibit alpha-glucosidase activity and thus, is helpful in reducing the blood glucose levels. Simultaneously, ellagitannins are found to inhibit alpha amylase enzyme. Thus intake of berries which are rich both in anthocyanins and ellagitannins can cause a cumulative effects in digestion of carbohydrates (**McDougall *et al*., 2005**). Potent inhibitory compounds have also been investigated by quantifying the polyphenolic composition in effective and less effective extracts. Modulation of their glycemic response has been studied in humans also to prove the authenticity of their effectiveness. Polyphenols and phlorotannins (PHT) from brown seaweeds have also been found to inhibit α amylase and α glucosidase enzyme. PHT extracted from *Ascophyllum nodosum* and *Fucus vesiculosus* inhibited α amylase and α glycosidase with very low IC_{50} values which is comparable to other plant polyphenols (**Roy et al., 2011**). Orlistat induced weight loss mechanism behind which is lipid inhibition has generated interest of scientists in finding nutraceuticals that inhibit the breakdown of macromolecules such as carbohydrates and fats. Acarbose and phaseolamine have been reported to have some promising results relating to weight loss (**Tucci *et al*., 2010**). Thus, the pericarp of *Garcinia mangostana,* which is a good source of alpha amylase inhibitors as well as have a very significant antioxidant activity, can be targeted as therapeutic drug in future. Many diseases related to macromolecules digestion can be solved by deciphering such compounds which can delay nutrient digestion and thus does not allow them to get absorbed fast in the body, helping in curing diseases associated with them.

CETP inhibition assay

Methanol, dichloromethane and hexane extracts of *Garcinia mangostana* were analyzed for CETP inhibition assay at various concentrations (10-100 µg/ml) by using CETP inhibition drug screening kit. Similar to α amylase inhibition results, it was found that hexane extract gave a remarkable inhibition of 85.67%. Due to this significant result, the extract was taken for dose response study and an IC_{50} of 10.89 µg/ml was obtained (Fig 3).

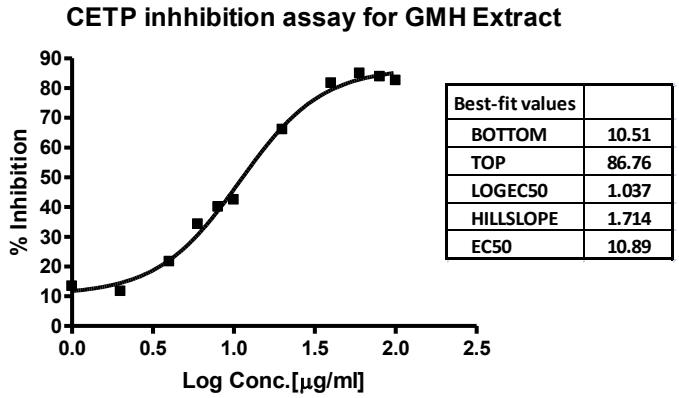

Figure 3 *Dose response curve (CETP Inhibition assay) for hexane extract of* Garcinia mangostana

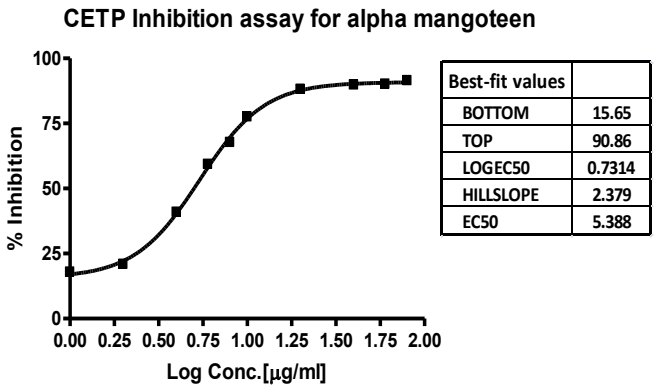

Figure 4 Dose response curve (CETP Inhibition assay) for reference standard α mangosteen

In order to confirm that α mangosteen is the potent compound, CETP inhibition assay was again performed with the reference standard and below is the dose

response curve (Fig 4) obtained for the different concentrations of α mangosteen. A significant IC_{50} of 5.388 µg/ml was obtained which confirmed that the potent active compound responsible for CETP inhibition is α mangosteen. The IC_{50} for hexane extract is 10.89 µg/ml which is in the range of two fold and thus acceptable. Till date, the most active natural CETP inhibitor, and [10] - dehydrogingerdione, has been found from the rhizomes of *Zingiber officinale* Roscoe. This active compound inhibited cholesteryl ester transfer protein with IC_{50} values of 35 µM and it was compared against a positive control D-Sphingosine that gave an IC_{50} of 11.5 µM. Very few compounds isolated from the natural sources have been identified as CETP inhibitors among which dehydrogingeridione was found to have the strongest inhibitory activity against cholesteryl ester transfer protein (**Choi *et al*., 2011**). Four ceramides which have been isolated from the gorgonian *Acabaria undulata* and N, N-dimethylsphingosine found to inhibit cholesteryl transfer protein with IC_{50} values of 46.8, 57.3, 86.3, 65.6 and 6.3 µm (**Jeong *et al*., 1997**). Xanthohumol, a prenylated chalcone also showed the highest inhibition against cholesteryl ester transfer protein. Naringenin chalcone showed weak cholesteryl ester transfer protein inhibition compared with xanthohumol. Isoxanthohumol and Naringenin significantly decreased the inhibitory activity. According to these results, it was concluded that the prenyl group and chalcone structure of xanthohumol were responsible for the cholesteryl ester transfer protein inhibitory activity. Xanthohumol had a potent cholesteryl ester transfer protein inhibitory property with an IC_{50} of 31.2 µg/ml (88.0 µm). The mechanism behind was noncompetitive and chalcone structure and prenyl group were found to be essential for its inhibitory action (**Hirata *et al*., 2012**). The observation from Japanese populations with CETP deficiency has led to the conclusion that cholesteryl ester transfer protein inhibiting drugs may elevate concentration of high density lipoprotein and thus decrease cardiovascular risk and atherosclerosis (**Barter *et al*., 2006 and 2007**).Thus from the present study, where alpha mangosteen has been found as a novel inhibitor of cholesteryl ester transfer protein. As the screening was performed by biochemical assay, further if taken up for analysis in human plasma, the results then can be translated into the possibility of a drug as cholesteryl ester transfer protein inhibitor derived from a natural source and can be a promising cure for certain diseases.

Thin layer chromatography

Chloroform and ethyl acetate (80:20) solvent system gave the best result in silica plates. It showed one major band with the separation of one particular compound which made it easier for further analysis of HPLC. One prominent band in Chloroform: ethyl acetate (80:20) solvent system indicated one major compound in the hexane extract of *Garcinia mangostana* pericarp. Along with this majorly present compound some other compounds were also suspected to be present in small quantity.

High performance Liquid Chromatography (HPLC)

HPLC was further used for analyzing the compounds of the hexane extract of *Garcinia mangostana* pericarp. Result showed one major peak prominent at RT 16.28 min along with some other minor compounds (Fig 5).

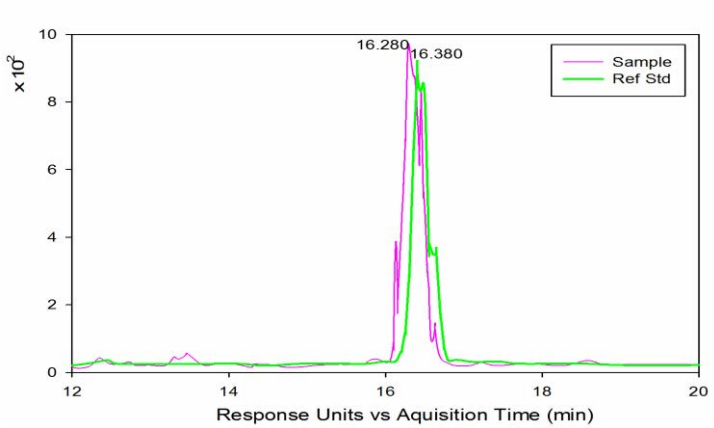

Figure 5 Comparison between HPLC results *of Garcinia mangostana* hexane extract and α mangosteen

Method for detection and quantification of α mangosteen in plasma consists of reversed phase high performance liquid chromatography (C18 reversed phase column) with ultraviolet detection. The mobile phase consisted methanol and water (95:5 % v/v) at a flow rate of 1.0 ml/min. Under these conditions, the retention time for α mangosteen was 10.84 min (**Syamsudin *et al*., 2010**). In another study, chromatographic separation was done on a Hypersil BDS C-18 column using a gradient mobile phase (70-80 % acetonitrile in 0.1 % v/v ortho phosphoric acid). The flow rate used was 1 ml/min with UV detection at 320 nm where two extracts showed similar pattern and a major peak of α mangosteen was obtained for which retention time was 16.32 min (**Pothitirat and Gritsanapan, 2009**). It has been found that acetone and water (80:20) is an efficient solvent system to extract a wide variety of xanthones. Subsequent to which HPLC analysis was done using C-18 reverse phase column and a 30 min gradient of 65–90% methanol in 0.1% formic acid solvent system was found effective to separate different xanthones with UV detection at 254 nm. Various xanthones and 9 hydroxycalabaxanthone, α mangosteen, β mangosteen, gartanin, 8 desoxygartanin and 3 mangosteen were identified (**Edward, 2007**). Thus this method proved to be a good method for analysis of xanthones in the pericarp of *Garcinia mangostana*. Fig 5 illustrates the comparative HPLC results of hexane extract of *Garcinia mangostana* and α mangosteen where the peak in pink colour represents the hexane extract and green color represents alpha mangosteen. At 310 nm, the retention time of α mangosteen was found to be at 16.38 min which is very much near to the result obtained for the sample i.e. 16.28 min. In this case, under the same chromatographic conditions similar retention time has been achieved.

Liquid Chromatography Mass Spectrometry (LC-MS)

Fig 6 shows the LC-MS results for *Garcinia mangostana* hexane extract in negative mode. The chromatogram clearly indicated the presence of α mangosteen in the sample along with other xanthones such as Garcinone E etc. Result illustrated green color peak for hexane extract of *Garcinia mangostana* and pink color for standard α mangosteen. LC-MS results for the sample were found comparable with reference standard results. *M/z* value for sample was found to be 409.2 compared to α mangosteen M.W. 409.3 (**Zarena *et al*., 2009**). Thus it can be concluded that α mangostin was present as major compound in the sample and might be responsible for potent activity.

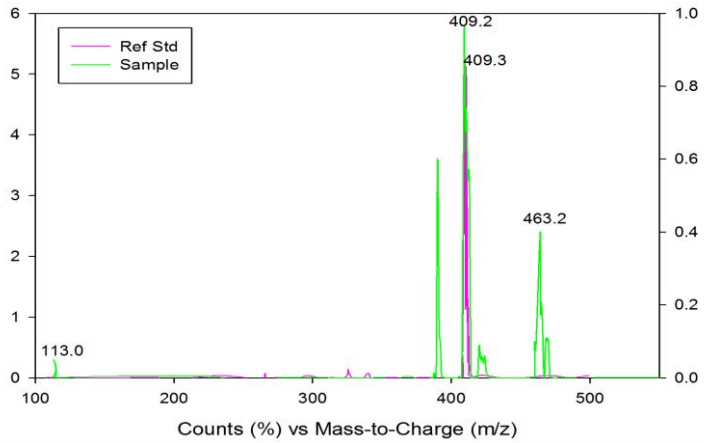

Figure 6 Comparison between LC-MS results of *Garcinia mangostana* hexane extract and α **mangosteen**

To determine the concentration of alpha mangosteen for toxicological investigation of a nutraceutical formulation, the LC separation was developed on a Phenomenex Gemini C-18 column using a 12 min. gradient consisting of 1% formic acid and methanol: acetonitrile (20:80, v/v). In a single 35 min chromatographic run, ethyl acetate, acetone and hexane extracts of mangostin peel were analysed by HPLC (**Xiuhong *et al*., 2007**). The polarity of the solvents played a major role in the extraction and purification of a compound from a complex mixture. This requires a comprehensive study about the type of compounds present in the raw material. Seven xanthones 3-isomangostin-*m/z* 427, 8-desoxygartanin- *m/z* 379, gartanin-*m/z* 395, α-mangostin- *m/z* 409, garcinone E - *m/z* 463, 9-hydroxycalabaxanthone- *m/z* 407 and β-mangostin - *m/z* 423 were detected with α mangostin being the predominant compound in all the extracts. α mangostin has *m/z* value of 409 in the negative mode and it shows m/z value of 411 in the positive mode which shows that it is a kind of neutral compound which gives reading in both the modes (**Zarena *et al*., 2009**).

CONCLUSION

The focus of the study was to discover novel natural inhibitors of digestive and metabolic enzymes which can be taken up for further clinical trials and can be targeted as therapeutic drugs in future. Among many plant extracts tested for the inhibitory activity, not many have been found to show the potent activity. In the current study, the methanolic, dichloromethane and hexane extracts of *Garcinia mangostana* was found to be effective against alpha amylase enzyme and thus can be targeted as a therapeutic drug for Diabetes. The hexane extract of *Garcinia mangostana* pericarp has shown potent activity against cholesteryl ester transfer protein. The findings of the study conclude that the pericarp of *Garcinia mangostana* is a remarkable inhibitor of α amylase enzyme with the most effective hexane extract of IC_{50} 9.048 µg/ml. The pericarp of *Garcinia mangostana* is also a remarkable inhibitor of cholesteryl ester transfer protein

with the hexane extract of IC_{50} 10.89 µg/ml. The most bioactive compound present in the pericarp of *Garcinia mangostana* is responsible for its potent activity to inhibit cholesteryl ester transfer protein with an IC_{50} of 5.388 µg/ml which is also comparable to the hexane extract of *Garcinia mangostana* pericarp. The pericarp of *Garcinia mangostana* is a rich source of xanthones having antidiabetic and anticholesterol activity. The pericarp of *Garcinia mangostana* can be further purified to identify the active compound responsible for its inhibitory activity against alpha amylase enzyme. α mangosteen can be taken for further clinical trials to prove the results for CETP inhibition in animal studies. Application of alpha mangosteen in food products and beverages can be done to develop functional foods.

Acknowledgments: This research was financially supported by the Thai Government Scholarship (Queens Scholarship) at Asian Institute of Technology, Thailand. Lab facilities and technical assistance was provided for animal experiments at G7 Synergon, India and animals were borrowed from Aacharya Institute of Technology, India

REFERENCES

ASAI, F., TOSA, H., TANAKA, T., IINUMA, M.A. 1995. Xanthone from pericarps of *Garcinia mangostana*. *Photochemistry*, 39(4), 943-944. http://dx.doi.org/10.1016/0031-9422(95)00042-6

BARTER, P.J., CAULFIELD, M., & ERIKSSON, M. 2007. Effects of Torcetrapib in patients at high risk for coronary events. *The New England Journal of Medicine,* 357, 2109–22. http://dx.doi.org/10.1016/j.jvs.2008.02.014

BARTER, P.J., & KASTELEIN, J.J. 2006. Targeting Cholesteryl Ester Transfer Protein for the Prevention and Management of Cardiovascular Disease. *Journal of the American College of Cardiology*, 47,492-499. http://dx.doi.org/10.1016/j.jacc.2005.09.042

CHAIRUNGSRILERD, N., TAKEUCHI, K., OHIZUMI, Y., NOZOE, S., & OHTA, T. 1996. Mangostanol, a prenyl xanthone from Garcinia mangostana. Phytochemistry, 43(5), 1099-1102. http://dx.doi.org/10.1016/s0031-9422(96)00410-4

CHOI, S.Y., PARK, G.S., LEE S.Y., KIM, J.Y., KIM, Y.K. 2011. The conformation and CETP inhibitory activity of [10]-Dehydrogingerdione isolated from *Zingiber officinale*. *Archives of Pharmacol Research,* 34(5), 727-731. http://dx.doi.org/10.1007/s12272-011-0505-5

GHASEMZADEH, A., JAAFAR, H.Z.E., RAHMAT, A. 2010. Antioxidant activities, total phenolics and flavonoids content in two varieties of Malaysia young ginger (*Zingiber officinal* Roscoe). *Molecules,* 15, 4324-4333. http://dx.doi.org/10.3390/molecules15064324

HE, Q., LV, Y., YAO, K. 2006. Effects of tea polyphenols on the activities of a-amylase, pepsin, trypsin and lipase. *Food Chemistry*, 101, 1178–1182. http://dx.doi.org/10.1016/j.foodchem.2006.03.020

HIRATA, H., TAKAZUMI, K., SEGAWA, S., OKADA, Y., KOBAYASHI, N., SHIGYO, T., CHIBA, H. 2012. Xanthohumol, a prenylated chalcone from Humulus lupulus L., inhibits cholesteryl ester transfer protein. *Food Chemistry*, 134(3), 1432-1437. http://dx.doi.org/10.1016/j.foodchem.2012.03.043

HUANG, Y.L., CHEN, C.C., CHEN, Y.J., HUANG, R.L., SHIEH, B.J. 2001. Three Xanthones and a Benzophenone from *Garcinia Mangostana*. *Journal of Natural Products*, 64(7), 903-6. http://dx.doi.org/10.1021/np000583q

JEONG, T.S., AHN, J.A. , KIM, Y.K., BOK, S.H., KWON, B.M. 1997. CETP inhibitory activity of ceramides, isolated from the gorgonian *Acabaria Undulata.* *Biorganic and Medicinal Chemistry*, 7, 1481-1482. http://dx.doi.org/10.1016/s0960-894x(97)00248-5

LEONTOWICZ, **M.,** LEONTOWICZ, H., DRZEWIECKI, **J.,** JASTRZEBSKI, Z., HARUENKIT, R., POOVARODOM, **S.,** YONG-SEO, **P.,** JUNG, **S.T.,** KANG, **S.K.,** TRAKHTENBERG, S., GORINSTEIN, S. 2007. Two exotic fruits positively affect rat's plasma composition. *Food Chemistry*, 102(1), 192-200. http://dx.doi.org/10.1016/j.foodchem.2006.05.046

LOO, A.E.K., HUANG, D. 2007. Assay-guided fractionation study of α-amylase inhibitors from *Garcinia mangostana* pericarp. *Journal of Agricultural and Food Chemistry, 55,* 9805–9810. http://dx.doi.org/10.1021/jf071500f

MCDOUGALL, G.J., STEWART, D. 2005. The inhibitory effects of berry polyphenols on digestive enzymes. *Bio factors,* 23, 189-95. http://dx.doi.org/10.1002/biof.5520230403

MERTES, G. 2001. Efficacy and safety of Acarbose in the treatment of Type 2 diabetes: data from a 5-year surveillance study. *Diabetes Research and Clinical Practice,* 52, 193-204. http://dx.doi.org/10.1016/s0168-8227(98)00045-x

MISHRA, H., DWIVEDI, B.K., MEHTA, D., MEHTA, B.K., JAIN, D.C. (2009). Development and validation of high performance thin-layer chromatographic method for determination of α mangostin in fruit pericarp of mangosteen plant (*Garcinia mangostana* l.) using ultraviolet visible detection. *Records of Natural Products,* 4178-186.

MOHAMMAD, A., BHAWANI, S.A., SHARMA, S. 2010. Analysis of Herbal Products by Thin-layer Chromatography: A Review. *International Journal of Pharma and Bio Sciences, 1(*2), 1-50.

NADKARNI, K.M., NADKARNI, A.K. 1999. *Indian Materia Medica - with Ayurvedic, Unani-Tibbi, Siddha, Allopathic, Homeopathic, Naturopathic and Home remedies*. Vol.1. Popular Prakashan Private Ltd., Bombay, India. ISBN No. 81-7154-142-9.

NICKAVAR, B., ABOLHASANI, L., IZADPANAH, H. 2013. Bioactivity-guided Separation of an α-Amylase Inhibitor Flavonoid from *Salvia virgata*. *Iran Journal of Pharmaceutical Research*, 12(1), 57-61.

POTHITIRAT, W., GRITSANAPAN, W. 2009. HPLC Quantitative analysis method for the determination of α-Mangostin in Mangosteen Fruit rind extract. *Thai Journal of Agricultural Science, 42, 7-12.*

ROY, MARIE-CLAUDE, ANGUENOT, R., FILLION, C., BEAULIEU, M., BERUBE, J., RICHARD, D. 2011. Effect of a commercially-available algal phlorotannins extract on digestive enzymes and carbohydrate absorption *in vivo*. *Food Research International, 44(9), 3026-29.* http://dx.doi.org/10.1016/j.foodres.2011.07.023

SUKSAMRARN, S., SUWANNAPOCH, N., RATANANUKUL, P., AROONLERK, N., SUKSAMRARN, A. 2002. Xanthones from the green fruit hulls of Garcinia mangostana. Journal of Natural Products, 65(5), 761-3.

SUKSAMRARN, S., KOMUTIBAN, O., RATANANUKUL, P., CHIMNOI, N., LARTPORNMATULEE, N., SUKSAMRARN, A. 2006. Cytotoxic prenylated xanthones from the young fruit of Garcinia mangostana. *Chemical and Pharmaceutical Bulletin*, 54(3), 301–305. http://dx.doi.org/10.1002/chin.200634209

SYAMSUDIN, FAIZATUN, LESTARI, R. 2010. HPLC Analysis and pharmacokinetic study of Mangostin after orally administration in rats. *International Journal of Pharma and Bio Sciences,* 1(1), 1-7.

TADERA, K., MINAMI, Y., TAKAMATSU, K., MATSUOKA, T. 2006. Inhibition of α-glucosidase and a-amylase by flavonoids. *Journal of Nutritional Science and Vitaminology*, 52, 149-153. http://dx.doi.org/10.3177/jnsv.52.149

TUCCI, S.A., BOYLAND, E.J., HALFORD, J.C. 2010. The role of lipid and carbohydrate digestive enzyme inhibitors in the management of obesity: a review of current and emerging therapeutic agents. *Diabetes Metabolic Syndrome and Obesity: Targets and Therapy,* 3, 125–143. http://dx.doi.org/10.2147/dmsott.s7005

WALKER, E.B. 2007. HPLC analysis of selected xanthones in mangosteen fruit**.** *Journal of Separation Science,* 30, 1229–1234. http://dx.doi.org/10.1002/jssc.200700024

XIUHONG, J., A., BHARATHI, A.A., IKHLAS, A.K. 2007. Quantitative and qualitative determination of six xanthones in *Garcinia mangostana* L. by LC PDA and LC ESI-MS. *Journal of Pharmaceutical and Biomedical Analysis*, 43, 1270-1276. http://dx.doi.org/10.1016/j.jpba.2006.10.018

ZADERNOWSKI, R., CZAPLICKIA, S., NACZK, M. 2009. Phenolic acid profiles of mangosteen fruits (*Garcinia mangostana*). *Food Chemistry,* 112, 685–689. http://dx.doi.org/10.1016/j.foodchem.2008.06.030

ZARENA, A.S., SANKAR, U. 2009. Screening of xanthone from mangosteen (*Garcinia mangostana* L.) peels and their effect on cytochrome c reductase and phosphomolybdenum activity. *Journal of Natural Products*, 2, 23-30.

JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

CYANOGENIC GLYCOSIDES AS A POTENTIAL BIOREGULATOR

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doi: 10.15414/jmbfs.2016.6.1.743-746

ARTICLE INFO

Received 3. 3. 2016
Revised 5. 5. 2016
Accepted 8. 6. 2016
Published 1. 8. 2016

Review

OPEN ACCESS

ABSTRACT

Natural substances which are considered to be a food that provides medical and health benefits are called bioregulator. Bioregulators can be used in medicine for preventing and treating migraine, hypertension, chronic inflammation, and other reaction source diseases. Amygdalin is considered for one of the most important bioregulator. It is a controversial nature cyanogenic glycoside abundant in the seeds of *Rosaceae* family. The family includes herbs, shrubs, and trees and most species are deciduous, but some are evergreen. In the past few years, has been a renewed interest about distribution of *Rosaceae* fruits because amygdalin has been used for many years in traditional and alternative medicine. Therefore, it is included in regulatory pathways and processes. Recent data indicate potential regulatory activity of amygdalin in signaling pathways of highly metastatic cells, suggesting that amygdalin might not only be an innovative tool to neutralize metastatic dissemination but also to complement mTOR-inhibitor based regimens.

Keywords: Amygdalin, cyanogenic glycosides, apoptosis, mTOR pathway

INTRODUCTION

In recent years considerable effort has been made to identify the metabolic factors linking nutrition with partial physiological processes **(Monget and Martin, 1997; Kolesárová *et al*., 2011)**. Numerous data from various mammalian species have shown that natural substances may influence the physiological functions **(Prunier and Quesnel, 2000)** or on the other side tumorigenesis, carcinogenesis, angiogenesis **(Subash *et al*., 2010; Aggarwal *et al*., 2009)**. The most widely plant constituents, which are biologically active and provide medical and health benefits are called bioregulators **(Brower, 1998; Zeisel, 1999)**. The function of bioregulators is also important to examine from viewpoint of prevention of many reproductive alterations **(Medveďová *et al*., 2010).** Neuroendocrine regulation manages reproductive system through axis hypothalamic, pituitary and gonads **(Javorka *et al*., 2012)**. Sex hormones (female-estrogens, progesterone, male-androgens: testosterone) are steroids (fat soluble compounds) that control sexual maturity and reproduction. The endocrine glands ovaries, testes, or adrenal cortex regulate sexual development of an organism and affects the growth or function of the reproductive organs. The endocrine and extracellular signalling systems provide a means of communication between distant organs via the circulatory system, specific cell populations, neighbouring cell populations, and the external and internal environments **(Chedrese, 2009).** Fruits and vegetables contain many different natural components, some essential nutrients and also contain a variety of bioactive substances, which have other beneficial health effects **(Kris-Etherton *et al*., 2002)**. In the past few years, there has been a renewed interest in evaluating the bioregulator content and distribution in patterns of fruits and vegetables **(Flood *et al*.,2002; Ruiz *et al*., 2006)**.

Rosaceae family

Family *Rosaceae*, comprised of over 100 genera and 3000 species, is the third most economically important plant family in temperate regions **(Dirlewanger *et al*., 2002)**. *Rosaceae* contain edible members such as almonds, apples, plums, peaches, pears, raspberries, sour cherries, sweet cherries, and strawberries. Other non-edible species with almost exclusively ornamental value include rose, hawthorn, potentilla, cotoneaster, and pyracantha. The products of this family are in high demand for their nutritional and esthetic values edible **(Vavilov, 1951)**. *Rosaceae* fruits are also a major human dietary source of phytochemicals, such as flavonoids, cyanogenic glycosides, phytoestrogens **(Mazur *et al*., 2000)**, and phenols that could potentially yield health and disease - fighting advantages. L-Ascorbic acid, quercetin, kaempferol, myricetin, p-coumaric acid, gallic acid, and ellagic acid are well known antioxidants and/or cancer-inhibiting compounds that have been identified in these fruits **(Macheix *et al*., 1991, 1998; Selmar, 1999)**. The family *Rosaceae* has found for own rich generic representation of an application in the prevention and treatment of many pathological conditions. In the past few years, has been a renewed interest about distribution of these fruits **(Fakuta *et al*., 2003; Chang *et al*., 2005)**. *In vitro* and *in vivo* studies on animal models provide evidence that fruit and leaf extracts from many *Rosaceae* species inhibit some cancers or have strong antioxidant activities **(Yau *et al*., 2002)**.
Seeds of *Rosaceae* fruits contain a substantial amount of dietary protein **(Nout et al., 1995)** along with significant amounts of oil and fiber but this part of apricot also depending on the variety, contain the toxic cyanogenic glycoside - amygdalin **(Gomes *et al*., 1998). Islamiyat *et al*. (2014)** have developed and applied a high performance liquid chromatographic procedure for amygdalin quantification to investigate extraction efficiency and to determine levels in a range of commercially-available foods. Their results showed that seeds from *Rosaceae* species contained relatively high amounts (range 0.1–17.5 mg/g) of amygdalin compared to seeds from *non-Rosaceae* species (range 0.01–0.2 mg/g) **(Yildirim *et al*., 2010).** The apricot (*Prunus armeniaca L.*) is a member of the *Rosaceae* family. Apricot fruit, being a rich source of vitamins and minerals and is one of the most familiar crops worldwide. Their trees are not ubiquitous since they can only grow in certain regions where the environmental conditions are favourable **(Baytop *et al*., 1999)**. The fresh apricot fruit contains carbohydrates, vitamins C and K, β-carotene, niacin, and thiamine. Organic acids, phenols, volatile compounds, esters, and terpenoids have also been isolated **(Ruiz *et al*., 2006; Riu-Aumatell *et al*., 2005; Safer *et al*., 2006)**.

Bioactive substance - Amygdalin

Natural plant origin products like amygdalin are still a major part of traditional medicine **(Hwang *et al*., 2008)**. Amygdalin (vitamin B17; previously called laetrile) is one of many nitrilosides, which are natural cyanide-containing substances abundant in the seeds of prunasin family and other *Rosaceous* plants **(Chang *et al*., 2005; Pak *et al*., 1999)**. The distribution of the cyanogenic glycosides in the plant kingdom is relatively wide and they are present mainly in more than 2650 plant species **(Franciscu and Pinotti, 2000; Haque and Bradbury, 2002)**. There are approximately 25 cyanogenic glycosides which have found in the edible parts of plants being: amygdalin (almonds); dhurrin (sorghum); linamarin (cassava, lima beans); lotaustralin (cassava, lima beans); prunasin (stone fruit); and taxiphyllin (bamboo shoots) **(Gonzales and Sabatini,**

1989). Cyanide is a toxic substance, mainly due to its affinity for the terminal cytochrome oxidase in the mitochondrial respiratory pathway **(Brattsten *et al*., 1983)**. The lethal dose of cyanide for vertebrates lies in the range of 35–150 μmol/kg, if applied in a single dose. Much higher amounts of HCN can be tolerated if consumed or administered over a longer period **(Davis and Nahrstedt, 1985).** Biosynthesis and degradation of cyanogenic glycosides (CNGs) are well documented in many plants **(Jones *et al*., 2000; Lechtenberg and Nahrstedt, 1999).**

But the genetic control of cyanogenesis has no unique mechanism, the plants show variation in the amount of the produced HCN. The production of HCN depends on both the biosynthesis of CNGs and on the existence (or absence) of its degrading enzymes. The biosynthetic precursors of the CNGs are different L-amino acids, these are hydroxylated then the *N*-hydroxylamino acids are converted to aldoximes, these are turned into nitriles. The last ones are hydroxylated to α-hydroxynitriles and then they are glycosilated to CNGs. The generation of HCN from CNGs is a two steps process involving a deglycosilation and a cleavage of the molecule (regulated by β-glucosidase and α-hydroxynitrilase). The tissue level compartmentalisation of CNGs and their hydrolysing enzymes prevents large-scale hydrolysis in intact plant tissue. The actual level of CNGs is determined by various factors both developmental and ecological ones, which are reviewed too **(Vetter, 2000)**.

Amygdalin is composed of two molecules of glucose, one of benzaldehyde, which induces an analgesic action, and one of hydrocyanic acid, which is an anti-neoplastic compound **(Zhou *et al*., 2012**).

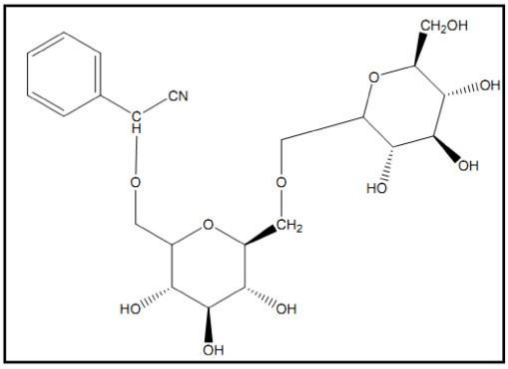

Figure 1 Chemical structure of amygdalin **(Abdel-Rahman, 2011)**.

Action of endogenous plant enzymes can release hydrogen cyanide causing potential toxicity issues for animals and humans, including cell death by blocking cytochrome oxidase and the arrest of the ATP production **(Bolarinwa *et al*., 2014)**. Amygdalin has been used to treat cancers and relieve pain **(Ellison *et al*., 1978; Shim *et al*., 2000)**. Amygdalin was reported to selectively kill cancer cells at the tumor site without systemic toxicity and to effectively relieve pain in cancer patients **(Zhou *et al*., 2012)**.The acute toxicity experiments of amygdalin have proved that the toxicity of oral administration route is far greater than the intravenous route **(Adewusi and Oke, 1985; Park *et al*., 2013)**. The maximum tolerance dose of intravenous and intramuscular injection of amygdalin in mice, rabbits, dogs are 3g/kg, 0.075 g/kg orally respectively **(Zhang and Jin, 1986; Rauws *et al*., 1982)** and human intravenous injection are 5g (approximately 0.07 g/kg). Previous studies on amygdalin have focused on its purification, toxicity related to the release of cyanide, anti-tumor mechanism, and identification of its metabolites in plasma or herbs, and its pharmacological effect on cancers **(Rauws *et al*., 1982, Song and Xu., 2014)**. Recent studies examined the effects of natural compound amygdalin on female reproductive system concentrated on secretory activity of porcine ovarian granulosa cells (GC) *in vitro* **(Halenár *et al*., 2013a**). **Halenár *et al.* (2015)** have the investigated release of steroid hormone progesterone by GC from cyclic and non-cyclic porcine ovaries. The progesterone release was not significantly *(P>0.05)* affected by the amygdalin treatment at all experimental doses (1, 10, 100, 1000 and 10000 mg/ml) compared to the control group without addition. However, a significant stimulation *(P<0.05)* of the 17-β-estradiol release after amygdalin addition at the highest dose (10000 mg/ml) was observed. Other experimental doses of amygdalin (1, 10, 100 and 1000 mg/ml) did not cause differences in the 17β-estradiol secretion. **Kolesár *et al*., (2015)** in their review have described the characteristic, metabolism and possible effects of amygdalin on reproductive processes. Previous studies described the effects of natural compound amygdalin on female and male reproductive systems focused on process of steroidogenesis **(Halenár *et al*., 2013a, 2015)**, spermatozoa motility and morphological abnormalities of bull spermatozoa **(Tanyildizy and Bozkurt, 2004)**. Amygdalin significantly inhibited sperm hyaluronidase activity. The inhibition of hyaluronidase activity can cause a drop in the fertilization ability of bull spermatozoa due to the prevention of acrosomal reaction. However, amygdalin did not produce any morphological abnormality in bull spermatozoa. The inhibition of sperm hyaluronidase activity and spermatozoa motility showed that these compounds have deleterious effects on bull sperm in vitro **(Tanyildizy and Bozkurt, 2004)**.

Amygdalin is one of main pharmacological components of crude ingredients of Keishi-bukuryo-gan, Japanese herbal medicine **(Yasui et al., 2003)**. It has been used for induction of ovulation in women suffering from infertility **(Igarashi, 1988)**. *Keishi-bukuryo-gan* and its crude ingredients affected steroidogenesis in pre-ovulatory follicles **(Usuki, 1987, 1990, 1991)** and the corpus luteum **(Usuki, 1986, 1988)** in the rat ovary *in vivo* and *in vitro*.

The characterization and role of mTOR

Mammalian target of rapamycin (mTOR) is a *serine/threonine kinase*, which belongs to phosphatidylinositol-3 kinase (PI3K) related kinases (PIKKs) family. It regulates cellular metabolism, growth, proliferation, and therefore is a target for the development of a number of mTOR inhibitors **(Pópulo *et al*., 2012)**. Akt functions as a component of the PI3K cell survival pathway. In cancer, Akt activity is frequently elevated due to multiple mechanisms, including loss of function of the PTEN tumor-suppressor gene and mutations of the PIK3CA gene. Akt functions as a component of the PI3K cell survival pathway **(Manning et al., 2005)**. Akt acts as a survival kinase in many cancers **(Cheng *et al*., 2005)**. The PI3K pathway is implicated in cell survival and cell growth, and can be activated by growth factors binding to cell-surface receptors. It is an intricate signaling cascade that is among the most frequently activated pathways in different types of cancer. PI3K is the subject of extensive research **(Knight *et al*., 2007)**.

Qian *et al.* (2015) submitted a study where Western blot results showed that amygdalin had no significant impact on Akt and Rictor expression. Rictor as a subunit of mTOR plays an important role in the Akt-mTOR signaling pathway; its phosphorylation level is positively regulated by Akt **(Chen *et al*., 2010)**. But the determination of Akt and rictor phosphorylation level showed that amygdalin significantly reduced the phosphorylation level of these two proteins in highly metastatic cells, suggesting that amygdalin was able to regulate the activity of Akt and rictor signaling pathways **(Qian *et al*., 2015)**. The PI3K pathway is a key signal transduction system that links oncogenes and multiple receptor classes to many essential cellular functions, and is perhaps the most commonly activated signalling pathway in human cancer. This pathway therefore presents both an opportunity and a challenge for cancer therapy. Even as inhibitors that target PI3K isoforms and other major nodes in the pathway, including Akt and mTOR, reach clinical trials, major issues remain. **Liu *et al*., (2009)** describe the progress made in understanding of the PI3K pathway and discuss the potential of and challenges for the development of natural therapeutic agents that target this pathway in cancer (**Liu *et al.*, 2009)**. Thus, amygdalin might not only be an innovative tool to neutralize metastatic dissemination but also to complement mTOR-inhibitor based regimens **(Gupta *et al*., 2013)**.

SUMMARY, CONCLUSIONS, AND FUTURE PERSPECTIVES

This review described possible effects of natural bioregulators on various types of animal cells. In recent years, increasing attention has been paid to natural substances and their impact on specific pathways in the cell. Amygdalin, as natural product shows lot of evidences. This natural compound is known for its anticancer, anti-inflammatory activity and other medicinal benefits, but on the other side represents one of the most controversial substance. Although amygdalin itself is non-toxic but its production HCN splitted by some endogenous plant enzymes is toxic substance for animals including humans. These agents that inhibit the downstream protein kinase mTOR as well as agents that inhibit multiple kinases, including components of the PI3K-Akt pathway are under clinical evaluation. There are still only a few studies which could suggest the possible involvement of amygdalin in mTOR pathway and thus influence animal reproductive system. Therefore other in vitro and in vivo experiments are necessary.

Acknowledgments: This work was financially supported by the Ministry of Education, Science, Research and Sport of the Slovak Republic projects no. 1/0039/16, KEGA 011SPU-4/2016, Slovak Research and Development Agency, APVV-0304-12, and European Community under project no 26220220180: Building Research Centre „AgroBioTech".

REFERENCES

Abdel-Rahman M.K. (2011). Can apricot kernels fatty acids delay the atrophied hepatocytes from progression to fibrosis in dimethylnitrosamine (DMN)-induced liver injury in rats? *Lipids in Health and Disease.*10(1), 114.

Adewusi, S. R., Oke, O.L. 1985. On The Metabolism Of Amygdalin. 2. The Distribution Of Beta-Glucosidase Activity And Orally Administered Amygdalin In Rats. *Canadian Journal of Physiology* and *Pharmacology*, ., 63, 1084-1087. http://dx.doi.org/10.1139/y85-178

Aggarvall, B. B.,Van Kuiken, M. E., Iyer, L. H. - Sung, B. (2009). Molecular Targets of Nutraceuticals Derived from Dietary Spices: Potential Role in Suppression of Inflammation and Tumorigenesis. *Experimental Biology and Medicine*,. 234,8, 825-849. http://dx.doi.org/10.3181/0902-mr-78

Baytop T. *Türkiyede bitkilerle tedavi*. Istanbul: Istanbul Eczacilik Fakültesi Yayinlari; 1999. http://dx.doi.org/10.1501/ilhfak_0000001385
Bolarinwa, I. F., Orfila, C., & Morgan, M. R. (2014). Amygdalin content of seeds, kernels and food products commercially-available in the UK. *Food chemistry*, *152*, 133-139. http://dx.doi.org/10.1016/j.foodchem.2013.11.002
Brattsten, L.B., Samuelian, J.H., Long, K.Y., Kincaid, S.A., Evans, C.K., 1983. Cyanide as a feeding stimulant for the southern armyworm, Spodoptera eridania. Ecol. Entomology 8, 125–132. http://dx.doi.org/10.1111/j.1365-2311.1983.tb00490.x
Brower, V. (1998). Nutraceuticals: Poised for a healthy slice of the healthcare market? *Nature Biotechnology*, 16(8), 728-731. http://dx.doi.org/10.1038/nbt0898-728
Calderaro J, Rebouissou S, de Koning L, Masmoudi A, He´rault A. (2014) PI3K/AKT pathway activation in bladder carcinogenesis. *International Journal of Cancer* 134 (8) 1767–2018. http://dx.doi.org/10.1002/ijc.28518
Cantley LC. (2002). *Science*, 296, 1655–1657. http://dx.doi.org/10.3390/ijms13021886
Chedrese J P. ,Reproductive endocrinology: a molecular approach., Springer., 2009, p. 382. http://dx.doi.org/10.1007/978-0-387-88186-7
Davis, R.H., Nahrstedt, A., 1985. Cyanogenesis in insects. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology. Pergamon Press, Oxford, pp. 635– 654. http://dx.doi.org/10.1016/b978-0-08-030812-8.50020-4
Dirlewanger, E., Cosson, P., Tavaud, M., Aranzana, MJ., Poizat, C., Zanetto, A., Arús, P., Laigret, F., (2002) Development of microsatellite markers in peach [*Prunus persica* (L.) Batsch] and their use in genetic diversity analysis in peach and sweet cherry (*Prunus avium* L.). *Theoretical Applied Genetics*.105,127-138. http://dx.doi.org/10.1007/s00122-002-0867-7
Ellison N. M., Byar D. P., Newell G. R. (1978). *Journal of Medicine*. 299, 549-552. http://dx.doi.org/10.1056/nejm197809072991013
Eric E. Conn. (1969). Cyanogenic glycosid . *Journal of the Science of Food and Agriculture.*, *17* (3), 519-526 http://dx.doi.org/10.1021/jf60163a014
Flood, A., Velie, EM., Chaterjee, N., Subar, AF., Thompson, FE., Lacey, JV. Jr. (2002). Fruit and vegetable intakes and the risk of colorectal cancer in the Breast Cancer Detection Demonstration Project follow-up cohort. *American Journal of Clinical Nutrition*, 75, 936-943. http://dx.doi.org/10.1093/ije/dyg052
Fukuta T., Ito H., Mukainaka T., Tokuda H., Nishino H., Yoshida T. (2003). *Biological and Pharmaceutical Bulletin*. 26, 271-273. http://dx.doi.org/10.1248/bpb.26.271
Franciscu, I. A., Pinotti, M. H. P. Cyanogenic glycosides in plants. *Braz. Arch. Biol. Technol*., 2000, 43, 487–492. http://dx.doi.org/10.1590/s1516-89132000000500007
Gomez, E., Burgos, L., Soriano C., Marin, J. (1998). Amygdalin content in the seeds of several apricot cultivars. *Journal of the Science of Food and Agriculture* 77, 184-186. http://dx.doi.org/10.1002/(sici)1097-0010(199806)77:2<184::aid-jsfa22>3.0.co;2-h
Gonzales, I. and Sabatini, S. (1989). Cyanide poisoning: pathophysiology and current approaches to therapy. *The International* Journal of *Artificial Organs,* 12(6): 347-355. http://dx.doi.org/10.1111/aor.2000.24.issue-12
Gupta, S., Hau, A. M., Beach, J. R., Harwalker, J., Mantuano, E., Gonias, S. L., ... & Hansel, D. E. (2013). Mammalian target of rapamycin complex 2 (mTORC2) is a critical determinant of bladder cancer invasion. *Plos one* .,8 (11), 3-9 http://dx.doi.org/10.1371/journal.pone.0081081
Halenár,M., Medveďová,M., Maruniaková, N., Packová, D., Kolesárová, A., (2013), Amygdalin and its effects on animal cells. Journal *of Microbiology and Biotechnology*., 2 (1), 1414-1423 http://dx.doi.org/10.15414/jmbfs.2015.4.special2.19-22
Halenár,M., Medveďová,M., Maruniaková, N., Packová, D., Kolesárová, A., (2014). Dose-response of porcine ovarian granulosa cells to amygdalin treatment combined with deoxynivalenol. *Journal of Microbiology and Biotechnology*., 4(2), 19-22. http://dx.doi.org/10.15414/jmbfs.2015.4.special2.19-22
Halenár,M., Medveďová,M., Maruniaková, N.,Kolesárová, A.,(2015), Assessment of a potential preventive ability of amygdalin in mycotoxin-induced ovarian toxicity. *Journal of Environmental Science and Health*., 50, 411–416. http://dx.doi.org/10.1080/03601234.2015.1011956
Haque, M. R., Bradbury, J. H. Total cyanide determination of plants and foods using the picrate and acid hydrolysis methods. *Food Chem.*, 2002, 77, 107–114. http://dx.doi.org/10.1016/s0308-8146(01)00313-2
Hwang, H., Lee, H., Kim, C., Shim, I., Hahm, D., (2008). Inhibitory effect of amygdalin on lipopolysaccharide-inducible TNF-alpha and IL-1beta mRNA expression and carrageenan-induced rat arthritis. *Journal of Microbiology and Biotechnology*., 18(10), 1641-1647. http://dx.doi.org/10.1248/bpb.31.1559
Chang H. K., Yang H. Y., Lee T. H., Shin M. C., Lee M. H., Shin M. S., Kim C. J., Kim O. J., Hong S. P., Cho S. (2005). *Biological and Pharmaceutical Bulletin*., 28, 449-454. http://dx.doi.org/10.1248/bpb.28.449
Chen, C. C., Jeon, S. M., Bhaskar, P. T., Nogueira, V., Sundararajan, D., Tonic, I., ... & Hay, N. (2010). FoxOs inhibit mTORC1 and activate Akt by inducing the expression of Sestrin3 and Rictor. *Developmental cell*, *18*(4), 592-604. http://dx.doi.org/10.101 6/j.devcel.2010.03.008
Cheng JQ, Lindsley CW, Cheng GZ, Yang H, Nicosia SV.. (2005). The Akt/PKB pathway: molecular target for cancer drug discovery. *Oncogene*. 24,7482-7492 http://dx.doi.org/10.1038/sj.onc.1209088
Igarashi, M. (1988). Kampo medicine in endocrinology. In Recent Advancesin the Pharmacology of Kampo (Japanese herbal) medicines, E. Hosoya, Y. Yamamura (eds). Tokyo, *Excepta Medica*, 1988, 157-160. http://dx.doi.org/10.1055/s-2006-960085
Javorka J., Béder I., Béderová A., Buc M., Čalkovská A., Donič V., Hájek J., Zeman M., (2012) Lekárska fyziológia. Osveta, Martin, p. 453.
Jones, P.R., Andersen, M.D., Nielsen, J.S., Høj, P.B., Møller, B.L., 2000. The biosynthesis, degradation, transport and possible function of cyanogenic glucosides. In: Romero, J.T., Ibrahim, R., Varin, L., De Luca, V. (Eds.), Evolution of Metabolic Pathways. Elsevier Science, New York, pp. 191–247. http://dx.doi.org/10.1016/s0079-9920(00)80008-8
Kolesár, E., Halenár, M., Kolesárová A., Massányi P., (2015). Natural plant toxicant – cyanogenic glycoside amygdalin: characteristic, metabolism and the effect on animal reproduction. *Journal of microbiology, biotechnology and food sciences*.4(2),49-50. http://dx.doi.org/10.15414/jmbfs.2015.4.special2.49-50
Kolesárová, A., Capcarová, M., Baková, Z., Gálik, B., Juráček, M., Šimko, M., Sirotkin, A.V. (2011). The effect of bee pollen on secretion activity, markers of proliferation and apoptosis of porcine ovarian granulosa cells in vitro. *Journal of Environmental Science and Health*, Part B, 46(3) 207-212. http://dx.doi.org/10.1080/03601234.2011.540202
Knight ZA, Shokat KM. (2007). Chemically targeting the PI3K family. *Biochem Soc Trans,* 35, 245-249.
Kris-Etherton, PM., Hecker, KD., Bonanome, A., Coval, SM., Binkoski, AE., Hilpert, KF., et al.(2002). Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer. *American Journal Medicine*, 113, 71S-88. http://dx.doi.org/10.1016/s0002-9343(01)00995-0
Lamming, D. W., Mihaylova, M. M., Katajisto, P., Baar, E. L., Yilmaz, O. H., Hutchins, A., ... & Sabatini, D. M. (2014). Depletion of Rictor, an essential protein component of mTORC2, decreases male lifespan. *Aging cell*, *13*(5), 911-917. http://dx.doi.org/10.1111/acel.12256
Lechtenberg, M., Nahrstedt, A., 1999. Cyanogenic glucosides. In: Ikan, R. (Ed.), Naturally Occurring Glycosides. John Wiley & Sons, New York, pp. 147–191. http://dx.doi.org/10.1002/(sici)1099-1026(199911/12)14:6<426::aid-ffj846>3.0.co; 2-9
Liu P., Cheng H., Roberts T.M, Zhao J.J., (2009).Targeting the phosphoinositide 3-kinase pathway in cancer. *Nature Reviews* 8, 627-644.http://dx.doi.org/10.1038/nrd2926
Manning BD, Cantley LC. (2007). AKT/PKB signaling: navigating downstream. *Cell*. 129, 1261-1274.
Macheix, JJ., Sapis, J., Fleuriet, A., (1991). Phenolic compounds and polyphenoloxidase in relation to browning in grapes and wines. *Critical Reviews in Food Science and Nutrition* 30, 441-486. http://dx.doi.org/10.1080/10408399109527552
Mazur, W., Uehara,M., Wahala, K., Adlercreutz, H., (2000). Phyto-oestrogen content of berries, and plasma concentrations and urinary excretion of enterolactone after asingle strawberry-meal in human subjects. *British Journal of Nutrition*. 83, 381-387. http://dx.doi.org/10.1079/bjn19980007
Medveďová M., Kolesárová A, Capcarová M., Sirotkin A., Kováčik J., (2010), The release of progesterone by ovarian granulosa cells following cobalt experimental administration *Potravinárstvo,* 4, p. 33-336. http://dx.doi.org/10.1080/10934521003708968
Meyers, S.A., Rosenberger, A. E. 1999. A plasma membrane-associated hyaluronidase is localized to the posterior acrosomal region of stallion sperm and is associated with spermatozoa function. *Biology of. Reproduction.,* 61(2), 444-451 http://dx.doi.org/10.1095/biolreprod61.2.444 .
Moertel, C. G., Ames, M. M., Kovach, J. S., Moyer, T. P., Rubin, J. R., & Tinker, J. H. (1981). A pharmacologic and toxicological study of amygdalin. *Jama*, *245*(6), 591-594. http://dx.doi.org/10.1001/jama.245.6.591
Monget, P., Martin, G. B. (1997). Involvement of insuline-like growth factors in the interaction between nutrition and reproduction in female mammals. *Human Reproduction Volume*, 12 (1), 33-52. http://dx.doi.org/10.1093/humrep/12.suppl_1.33
Pak J. U., Moon S. J., Moon K., Won J. H. (1999). *Journal* of *Korean Oriental Oncology.* 5, 137-150. http://dx.doi.org/10.3346/jkms.1999.14.5.546
Nout MJ, Tuncel G, Brimer L. Microbial degradation of amygdalin of bitter apricot seeds (Prunus armeniaca). Int *J Food Microbiol.* 1995; 24: 407-412. http://dx.doi.org/10.1016/0168-1605(94)00115-m
Pak JU., Moon SJ., Moon K., Won JH.(1999) Effects of Seman Armenicae and Radix Trichosanthis on the iNOS expression and superoxide formation in the RAW264.7 cells. *Journal* of *Korean Oriental Oncology*. 5,137-150. http://dx.doi.org/10.4093/jkda.2006.30.5.336
Park, H. J., Yoon, S. H., Han, L. S., Zheng, L. T., Jung, K. H., Uhm, Y. K, et al. (2005). Amygdalin inhibits genes related to cell cycle in SNU-C4 human colon cancer cells. *World Journal of Gastroenterology*., 11, 5156-5161.

Pópulo, H., Lopes, J. M., & Soares, P. (2012). The mTOR signalling pathway in human cancer. *International journal of molecular sciences*, *13*(2), 1886-1918. http://dx.doi.org/10.3390/ijms13021886
Poulton J. E. (1990). Cyanogenesis in plants. *Review Plant Physiology*, 94, 401-405. http://dx.doi.org/10.1104/pp.94.2.401
Rauws, A.G. ,Olling, M. ,Timmerman, A. (1982) The pharmacokinetics of amygdalin. *Archive of toxicology*, 49, 1982, . 311-312. http://dx.doi.org/10.1007/bf00347879
Prunier, A., Quesnel, H. (2000). Influence of the nutritional status on ovarian development in female pigs. *Animal Reproduction Science*, 60(61), 185-197. http://dx.doi.org/10.1016/s0378-4320(00)00093-2
Qian, L., Xie, B., Wang, Y., & Qian, J. (2015). Amygdalin-mediated inhibition of non-small cell lung cancer cell invasion in vitro. *International journal of clinical and experimental pathology*, *8*(5), 5363. http://dx.doi.org/10.1016/j.ajpath.2012.11.019
Ruiz, D., Egea, J., Gil, MI., Tomas-Barberan, FA., (2006). Phytonutrient content in new apricot (Prunus armeniaca L.) varieties. *Acta Horticulturae* 717, 363-368. http://dx.doi.org/10.17660/actahortic.2006.717.73
Selmar, D., (1999). Cyanide in Foods. *Phytochemicals in human health protection, nutrition, and plant defense* (Vol.33).USA: Springer. http://dx.doi.org/10.1007/978-1-4615-4689-4_14
Shim B. S., Park J. K., Choi S. H. (2000). *Journal* of *Korean Oriental Oncology*.6, 19-28. http://dx.doi.org/10.4070/kcj.2000.30.2.227
Shragg, T.A., Albertson, T.E., Fisher, C.J., Jr. Cyanide poisoning after bitter almond ingestion. (1982) *Western Journal of Medicine* .136, 65-69. http://dx.doi.org/10.4103/1755-6783.140262
Subash, C., Gupta, J., Hye, K., Sahdeo, P., Bharat, B. (2010). Regulation of survival, proliferation; invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals. *Cancer Metastasis Reviews*, 29(3), 405-434. http://dx.doi.org/10.1007/s10555-010-9235-2
Suchard, J. R., Wallace, K. L., Gerkin, R. D. (1998). Acute cyanide toxicity caused by apricot kernel ingestion. *Annals of Emergency Medicine.* 32, 742-744. http://dx.doi.org/10.1016/s0196-0644(98)70077-0
Song Z., Xu X. (2014) Advanced research on anti-tumor effects of amygdalin, 10(5), 3-7. http://dx.doi.org/10.4103/0973-1482.139743
Tanyildizi, S., Bozkurt, T. (2004). In Vitro Effects of Linamarin, Amygdalin and Gossypol Acetic Acid on Hyaluronidase Activity, Sperm Motility and Morphological Abnormality in Bull Sperm. *Turkish Journal of Veterinary and Animal Sciences*, vol. 28, 2004, p. 819-824.
Usuki, S. 1986. Effects of Chinese herbal medicines on progesterone secretion by corpus luteum. In Japanese Journal of Fertility and Sterility, vol. 31, 1986, p. 482-486. http://dx.doi.org/10.1142/s0192415x86000260
Usuki, S. 1987. Effects of Hachimijiogan, Tokishakuyakusan and Keishibukuryogan on estrogen and progesterone secretion in ovarian follicles. In Japanese Journal of Fertility and Sterility, vol. 32, 1987, p. 276-283. http://dx.doi.org/10.1142/s0192415x8700014x
Usuki, S. 1988. Effects of Hachimijiogan, Tokishakuyakusan and Keishibukuryogan on progesterone and 17α-hydroxyprogesterone secretion by rat corpora lutea in vivo. In Japanese Journal of Fertility and Sterility, vol. 33, 1988, p. 60-66. http://dx.doi.org/10.1142/s0192415x88000078
Usuki, S. 1990. Effects of Tokishakuyakusan and Keishibukuryogan on steroidogenesis by rat preovulatory follicles in vivo. In American Journal of Chinese Medicine, vol. 18, 1990, p. 149-156. http://dx.doi.org/10.1142/s0192415x90000198
Usuki, S. (1991). Effects of Hachimijiogan, Tokishakuyakusan and Keishibukuryogan, Ninjinto and Unkeito on estrogen and progesterone secretion in preovulatory follicles incubated in vitro. *American Journal of Chinese Medicine*, 19, 1991, 65-71. http://dx.doi.org/10.1142/s0192415x91000107
Vavilov, N. I. (1951). The origin, variation, immunity and breeding of cultivated plants. *Soil Science*, 72(6), 482. http://dx.doi.org/10.1097/00010694-195112000-00018
Vetter, J. 2000 Plant cyanogenic glycosides, Review Toxicon, 38, p. 11–36. http://dx.doi.org/10.1016/s0041-0101(99)00128-2
Yan, Z., Denneboom, C., Hattendorf, A., Dolstra, O., Debener T, Stam P, Visser PB (2005) Construction of an integrated map of rose with AFLP, SSR, PK, RGA, RFLP, SCAR and morphological markers. *Theoretical Applied Genetics* 110, 766-777. http://dx.doi.org/10.1007/s00122-004-1903-6
Yasui, T. , Matsuzaki, T. , Ushigoe, K. , Kuwahara, A. , Maegawa, M. , Furumoto, H. , AONO, T. , Irahara, M. (2003). Stimulatory effect of the herbal medicine Keishi-bukuryo-gan on a cytokine-induced neutrophil chemoattractant, in rat ovarian cell culture. *American Journal of Reproductive Immunology*, 50, 2003, 90- 97. http://dx.doi.org/10.1034/j.1600-0897.2003.00055.x
Yau, MH., Che, CT., Liang, SM., Kong,YC., Fong WP.(2002). An aqueous extract of Rubus chingii fruits protects primary rat hepatocytes against tert-butyl hydroperoxide induced oxidative stress. *Life Science* 72, 329-338. http://dx.doi.org/10.1016/s0024-3205(02)02239-7
Yildirim AN, San B, Koyuncu F, Yildirim F (2010). Variability of phenolics, α-tocopherol and amygdalin contents of selected almond (Prunus amygdalus Batsch.) genotypes. Journal of Food Agriculture and Environment, 8(1): 76-79. http://dx.doi.org/10.1002/jsfa.2740350616
Zeisel, S. H. (1999). Regulation of nutraceuticals. *Science*, 285, 1853-1855. http://dx.doi.org/10.1126/science.285.5435.1853
Zhang, G. M., Jin, B. Q. (1986). Pharmacokinetics of amygdalin in rabbits. *Zhongguo Yao Li Xue Bao*, 7, 460-462. http://dx.doi.org/10.1086/591376
Zhou, C. ,Qian, L. , Ma, H. ,Yu, X , Zhang, Y. ,QU, W., Zhang, X, Xia, W. (2012). Enhancement of amygdalin activated with β-D-glucosidase on HepG2 cells proliferation and apoptosis. *Carbohydrate Polymers*, 90,. 516-523. http://dx.doi.org/10.1016/j.carbpol.2012.05.073