African Journal of Pharmacy and Pharmacology

Volume 8 Number 1, 8 January, 2014
ISSN 1996-0816

Academic Journals

ABOUT AJPP

The **African Journal of Pharmacy and Pharmacology (AJPP)** is published weekly (one volume per year) by Academic Journals.

African Journal of Pharmacy and Pharmacology (AJPP) is an open access journal that provides rapid publication (weekly) of articles in all areas of Pharmaceutical Science such as Pharmaceutical Microbiology, Pharmaceutical Raw Material Science, Formulations, Molecular modeling, Health sector Reforms, Drug Delivery, Pharmacokinetics and Pharmacodynamics, Pharmacognosy, Social and Administrative Pharmacy, Pharmaceutics and Pharmaceutical Microbiology, Herbal Medicines research, Pharmaceutical Raw Materials development/utilization, Novel drug delivery systems, Polymer/Cosmetic Science, Food/Drug Interaction, Herbal drugs evaluation, Physical Pharmaceutics, Medication management, Cosmetic Science, pharmaceuticals, pharmacology, pharmaceutical research etc. The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles published in AJPP are peer-reviewed.

Submission of Manuscript

Submit manuscripts as e-mail attachment to the Editorial Office at: ajpp@academicjournals.org. A manuscript number will be mailed to the corresponding author shortly after submission.

The African Journal of Pharmacy and Pharmacology will only accept manuscripts submitted as e-mail attachments.

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author.

Editors

Sharmilah Pamela Seetulsingh- Goorah
Associate Professor,
Department of Health Sciences
Faculty of Science,
University of Mauritius,
Reduit,
Mauritius

Himanshu Gupta
University of Colorado- Anschutz Medical Campus,
Department of Pharmaceutical Sciences, School of Pharmacy Aurora, CO 80045,
USA

Dr. Shreesh Kumar Ojha
Molecular Cardiovascular Research Program
College of Medicine
Arizona Health Sciences Center
University of Arizona
Tucson 85719, Arizona,
USA

Dr.Victor Valenti Engracia
Department of Speech-Language and Hearing Therapy Faculty of Philosophy and Sciences, UNESP
Marilia-SP, Brazil.l

Prof. Sutiak Vaclav
Rovníková 7, 040 20 Košice,
The Slovak Republic,
The Central Europe,
European Union
Slovak Republic
Slovakia

Dr.B.RAVISHANKAR
Director and Professor of Experimental Medicine
SDM Centre for Ayurveda and Allied Sciences,
SDM College of Ayurveda Campus,
Kuthpady, Udupi- 574118
Karnataka (INDIA)

Dr. Manal Moustafa Zaki
Department of Veterinary Hygiene and Management
Faculty of Veterinary Medicine, Cairo University
Giza, 11221 Egypt

Prof. George G. Nomikos
Scientific Medical Director
Clinical Science
Neuroscience
TAKEDA GLOBAL RESEARCH & DEVELOPMENT CENTER, INC. 675 North Field Drive Lake Forest, IL 60045
USA

Prof. Mahmoud Mohamed El-Mas
Department of Pharmacology,

Dr. Caroline Wagner
Universidade Federal do Pampa
Avenida Pedro Anunciação, s/n
Vila Batista, Caçapava do Sul, RS - Brazil

Editorial Board

Prof. Fen Jicai
School of life science, Xinjiang University,
China.

Dr. Ana Laura Nicoletti Carvalho
Av. Dr. Arnaldo, 455, São Paulo, SP.
Brazil.

Dr. Ming-hui Zhao
Professor of Medicine
Director of Renal Division, Department of Medicine
Peking University First Hospital
Beijing 100034
PR. China.

Prof. Ji Junjun
Guangdong Cardiovascular Institute, Guangdong General Hospital, Guangdong Academy of Medical Sciences,
China.

Prof. Yan Zhang
Faculty of Engineering and Applied Science,
Memorial University of Newfoundland,
Canada.

Dr. Naoufel Madani
Medical Intensive Care Unit
University hospital Ibn Sina, Univesity Mohamed V Souissi, Rabat,
Morocco.

Dr. Dong Hui
Department of Gynaecology and Obstetrics, the 1st hospital, NanFang University,
China.

Prof. Ma Hui
School of Medicine, Lanzhou University,
China.

Prof. Gu HuiJun
School of Medicine, Taizhou university,
China.

Dr. Chan Kim Wei
Research Officer
Laboratory of Molecular Biomedicine,
Institute of Bioscience, Universiti Putra,
Malaysia.

Dr. Fen Cun
Professor, Department of Pharmacology, Xinjiang University,
China.

Dr. Sirajunnisa Razack
Department of Chemical Engineering, Annamalai University,
Annamalai Nagar, Tamilnadu,
India.

Prof. Ehab S. EL Desoky
Professor of pharmacology, Faculty of Medicine
Assiut University, Assiut,
Egypt.

Dr. Yakisich, J. Sebastian
Assistant Professor, Department of Clinical Neuroscience
R54
Karolinska University Hospital, Huddinge
141 86 Stockholm ,
Sweden.

Prof. Dr. Andrei N. Tchernitchin
Head, Laboratory of Experimental Endocrinology and Environmental Pathology LEEPA
University of Chile Medical School,
Chile.

Dr. Sirajunnisa Razack
Department of Chemical Engineering,
Annamalai University, Annamalai Nagar, Tamilnadu,
India.

Dr. Yasar Tatar
Marmara Unıversıty,
Turkey.

Dr Nafisa Hassan Ali
Assistant Professor, Dow institude of medical technology
Dow University of Health Sciences,Chand bbi Road, Karachi,
Pakistan.

Dr. Krishnan Namboori P. K.
Computational Chemistry Group, Computational Engineering and Networking,
Amrita Vishwa Vidyapeetham, Amritanagar, Coimbatore-641 112
India.

Prof. Osman Ghani
University of Sargodha,
Pakistan.

Dr. Liu Xiaoji
School of Medicine, Shihezi University,
China.

African Journal of Pharmacy and Pharmacology

Table of Contents: Volume 8, Number 1 8 January, 2014

ARTICLES

Research Articles

academicJournals

8(1), pp. 1-8, 8 January, 2014
DOI: 10.5897/AJPP2013.3939
ISSN 1996-0816 © 2014 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Orthogonal test design for optimizing the extraction of total flavonoids from *Flos pueraria*

Fei Cai, Chen Xiao, Qingjie Chen, Changhan Ouyang, Ruixue Zhang, Jiliang Wu* and Chao Liu*

Hubei Province Key Laboratory on Cardiovascular, Cerebrovascular, and Metabolic Disorders, Hubei University of Science and Technology, Xianning, 437100, China.

Accepted 16 December, 2013

The conditions for extraction of the total flavonoids from *Flos pueraria* was optimized and the purification and separation process were conducted to identify the main constituents of the total flavonoids as well. The solvent for extraction and its concentration, the solid-to-liquid ratio, extraction duration, temperature and ultrasonic frequency were investigated through a single-factor experiment. An orthogonal design (L9 (3^4)) was constructed to achieve the best extraction conditions. The crude extract was then purified sequentially by petroleum ether, ethanol and chloroform, n-butyl alcohol, and eluted gradually with mixed mobile phase of methanol-chloroform solution in the silica gel column system. The ingredients were further separated by color reaction, ultraviolet spectrophotometry, high performance liquid chromatography, infrared and mass spectral analysis. The optimum extraction condition for the total flavonoids from *F. pueraria* was as follows: extraction by 50% (v/v) methanol solution, the solid-to-liquid ratio at 1:30, extraction duration 2.0 h, the temperature of 70°C, ultrasound 3 times and 30 min each time. Five isoflavones were separated and identified as irisolidone, genistein, daidzein, kakkalide and puerarin, respectively. Under these optimal conditions, the yield of total flavonoids in the extracts was up to 17.5%, while the output rate previously reported is about 8% in general. Our study provided means for further development and utilization of the bioactive components from *F. pueraria*.

Key words: *Flos pueraria*, flavonoids, orthogonal design, purification, ultrasound-assisted extraction.

INTRODUCTION

Flos puerariae, a well-known Chinese medicine compound is the dry bud of *Pueraria lobata* which is a plant in the genus *Pueraria* in the pea family Fabaceae, subfamily Faboideae and it is widely cultivated in East Asian countries. It has been traditionally used for headache, dizziness, polydipsia and vomiting associated with alcoholism since ancient time. Previous studies have shown that *F. pueraria* possesses extensive pharmacological effects such as antioxidant (Xiong et al., 2010), antidiabetic (Choi et al., 2004), antivirus (Tsuchihashi et al., 2009), antitumor, liver protective (Lee et al., 2003), detoxification of alcohol (Lee et al., 2001) and estrogen-like action. There are a lot of bioactive components in *F. pueraria* and of these, flavonoids are the most important class of compounds with 2-benzene chromone nucleus. They are widely distributed throughout the plant kingdom and possess a variety of biological activities. Flavonoids intake is reported to be negatively correlated with the incidence of some chronic diseases including cardiovascular diseases (Koon et al., 2011), type II diabetes (Jung et al., 2006), neurodegenerative diseases (Ramassamy, 2006), cancers (Lin et al., 2009) etc. Conventional flavonoid extraction methods include Soxhlet extraction, maceration and heat reflux extraction.

*Corresponding author. E-mail: xn_liuchao@163.com, xywjl@163.com. Tel: 0086-715-826-3051. Fax: 0086-715- 815-1230.

These methods generally result in limited output due to oxidation and hydrolysis. It is known that the extraction conditions such as temperature, the solvent, the solid-to-liquid ratio, and the duration all contribute to the output efficiency. Additionally, ultrasound-assisted extraction (UAE) has been widely used for extraction of the bio-active substances from food and medicinal plant research industries, and compared with conventional extraction methods, UAE has many advantages such as less solvent consumption, reduced time, higher extraction rates and greater environmental protection. It is previously reported that the output rate of total flavonoids from *F. pueraria* is about 8% in general (Zhu et al., 1995).

In this study, we tried to optimize the procedure for extraction of the flavonoids from *F. pueraria* with the assistance of ultrasonic treatment which has not been reported before. An orthogonal array is employed to analyze the interaction among these operating factors.

MATERIALS AND METHODS

Reagent and drug

The flowers of *P. lobata* (*F. pueraria*) was acquired from Green Mountain *Pueraria mirifica* powder factory in Hengfeng city of Jiangxi province. 200 mesh column chromatography silica gel was obtained from Sinopharm Chemical Reagent Co. Ltd. The standard Rutin was obtained from Shanghai shunbo biotech co., Ltd. Other chemicals were of analytical grade and purchased from local markets.

Equipment

SB-5200 ultrasonic cleaning device (Ningbo xinzhi bio-tech Co.,Ltd.); SBS-100 digital meter automatic collectors (Shanghai huxi Analysis Instrument Factory co., Ltd); RE-5299 rotary evaporation instrument (Yingyuyuhua Apparatus Factory of Gongyi city); SHB-β water circulating multi-purpose vacuum pump (Zhengzhou Greatwall Scientific Industrial and Trade Co., Ltd); 50-Conc. UV-Vis spectrophotometric meter (VARIAN Corporation in the United States); LCQ Deca XP-type mass spectrometer (Thermo Finnigan companies in the United States); VECTOR22 fourier transform near infrared spectrometer (Bruker Corporation in Germany); RD-3 melting point apparatus (Tianjin Chuangxin electronic equipment manufacturing Co., Ltd).

Ultrasound-assisted extraction (UAE)

10 g *F. pueraria* was air-dried to constant weight at room temperature. It was mixed with 300 ml of 50% (v/v) of varied solvent (methanol, ethanol or n-butanol); varying solvent concentrations from 0 to 100% (v/v); varying solid-to-liquid ratios from 1:10 to 1:50. The extraction temperature was varied from 20, 30, 40, 50, 60, 70 to 80°C. The extraction duration was varied from 0, 0.5, 1.0, 1.5, 2.0 to 2.5 h. The ultrasound was set at 40 KHz, 30 min each time and varied from 1 to 3 times. At the end of each process, the extract solution was cooled to room temperature and complemented to 300 ml if there is any loss by evaporation. After that, 1 ml of the final extract solution was drawn and centrifuged at 4,000 rpm for 15 min. 0.1 ml of the supernatant was diluted 50-fold with 90% ethanol analyzed using an ultraviolet spectrophotometer. The absorbance was set at 265 nm and the content of total flavonoids was calculated from the standard curve. The blank control was set with solvents.

Optimization design

In order to optimize of the UAE conditions for *F. pueraria*, an orthogonal array ($L9(3^4)$) was constructed to evaluate the effects of the following factors: solvent concentration (A), solid-to-liquid ratio (B), extraction temperature (C), and extraction duration (D). Factors and experimental data are displayed in Table 1.

Purification, separation and structure identification

Besides flavonoids, the extract of *F. pueraria* also contains other substances such as proteins, carbohydrates, nucleic acids, pigments, volatile oils and so on. In order to purify the flavonoids, petroleum ether, absolute alcohol, chloroform and n-butanol was further used to remove lipids, polysaccharides, proteins and others. The purified extracts were then separated by silica gel column chromatography. The separation condition was as follows: The column was 7.0 × 50 cm. The column temperature was set at 25°C. The sample volume is 10 ml and gradients sequentially eluted by chloroform-methanol mixture (v/v = 50:1, 20:1, 10:1, 5:1, 4:1, 3:1, 2:1, 1:1, respectively). The average velocity was 3 ml/min. A tube was collected every 2 min and each gradient was collected for 25 tubes. UV spectrophotometry was used for track scanning and high performance liquid chromatography, infrared spectrum and mass spectrum were used for identification of the isolated monomer compounds. The conditions for high-*performance liquid chromatography* (HPLC) to identify the isolated monomer compounds in the final extract were as follow: Shimadzu Labsolution2010-UV; column: Eclipse XDB-C18 (4.6 mm × 150 mm, 5 μm); mobile phase consisted of A (acetonitrile) and B (1‰ phosphoric acid in water); 1 to 20 min (14% A: 86% B); 20 to 30 min (25% A: 75% B); 30 to 40 min (28% A: 72% B); 40 to 50 min (30% A: 70% B); 50 to 60 min (40% A: 60% B); flow rate: 0.8 ml/min; UV wavelength: 270 nm; column temperature: 30°C.

Statistical analysis

Values are presented as mean ± standard error of mean (SEM). One-way analysis of variance (ANOVA) was used to determine the significant differences among the groups, and values less than 0.05 were considered significant. Date analysis was performed using the statistical program for social science (SPSS) 13.0.

RESULTS AND DISCUSSION

Calibration and linearity of the rutin

90% ethanolic solution containing rutin standard (0.021 mg/ml) was prepared. This solution was then diluted in 90% ethanol to obtain concentrations corresponding to 0, 0.0042, 0.0084, 0.0168, 0.0336 and 0.0672 mg/ml. All solutions were stored at 4°C. λ_{max} 265 nm was obtained in the ultraviolet scanning from 200 to 500 nm, and standard curve was drawn as $Y = 30.812X + 0.0017$, $R^2 = 0.9999$. There is a good linearity in the range of 4.2 to 67.2 μg/ml (Figure 1A and B).

Effect of suitable solvent and its concentration on flavonoid yield

Solvents with varied concentration possess different

Table 1. Orthogonal design factors and levels.

Factors	Concentration (%) A	Solid-to-liquid ratio (g/ml) B	Temperature (°C) C	Duration (h) D
Level 1	40	1/20	50	1.0
Level 2	50	1/30	60	1.5
Level 3	60	1/40	70	2.0

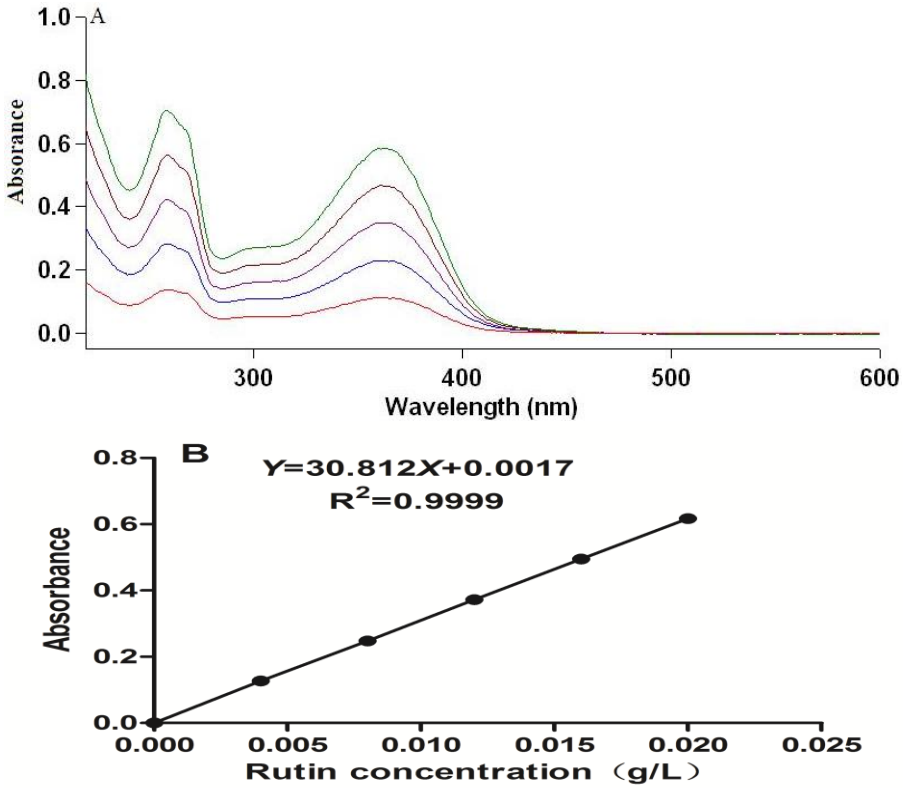

Figure 1. Calibration and linearity of the rutin. 90% ethanolic solution containing rutin corresponding to the concentrations of 0, 0.0042, 0.0084, 0.0168, 0.0336 and 0.0672 mg/ml was prepared.λmax265 nm was used for the ultraviolet scanning. A: The scan curve of rutin. B: The standard curve of absorption capacity of standard rutin. Y=30.812X+0.0017, R^2=0.9999 with a good linearity in the range of 4.2-67.2 μg/ml

polarities which can lead to different component types and quantities of extracts yield. Thus, the selection of a suitable solvent and its concentration is an important step for an optimal extraction yield. In this study, different solvents of methanol, ethanol, n-buty alcohol were tested as extraction solvents. As shown in Figure 2A, the flavonoid yield in methanol solution was the highest among the three solvents and the extraction efficiency increased in parallel with the increment of the methanol concentration within the scope of 0 ~ 40%, got to steady level within the scope of 40 ~ 60%, and then declined if the methanol concentration further rose. Thus, 50% (v/v) methanol solution was selected as the solvent for the exaction in the subsequent experiments (Figure 2B).

Effect of solid-to-liquid ratio on flavonoid yield

The solid-to-liquid ratio is another important factor which greatly influenced the flavonoid yield. Figure 3 shows that when the *F. pueraria* quality and methanol concentration were kept fixed, the extraction yield reached plateau as the solid-to-liquid ratio increased to 1: 30. This was probably because the increment in the solid-to-liquid ratio enhanced the solvent to penetrate and dissolve the target components, and a greater volume of solvent caused sufficient swelling of the material. But more solvent consumption may have negative effects on energy conservation and cost reduction. Our results showed that 1: 30 is the most appropriate ratio of solid-to-liquid for

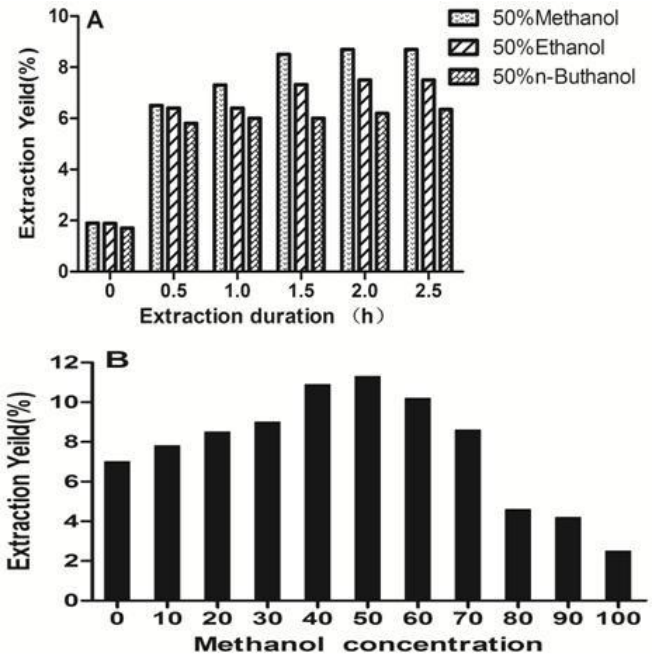

Figure 2. Effect of suitable solvent and its concentration on flavonoid yield. A: Effects of different solvents of methanol, ethanol, n-buty alcohol on the extraction yield; B: Effects of different methanol solution concentrations on the extraction yield. (amount of flowers of Pueraria lobata: 10g; solid-to-liquid ratio: 1:30; extraction duration: 2.0 h; extraction temperature: 70°C; ultrasound time: 3 times and 30 min each time)

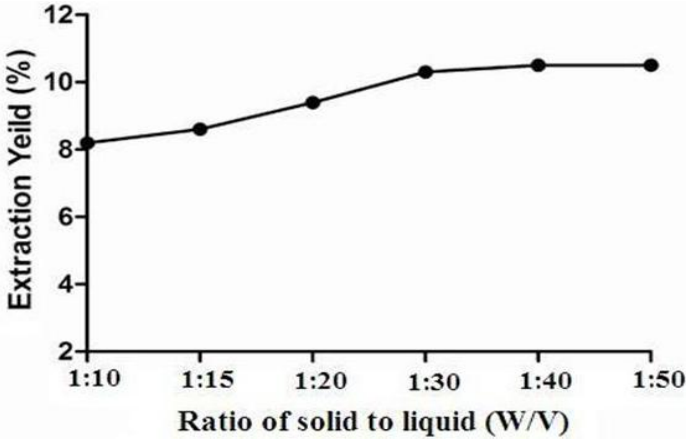

Figure 3. Effect of solid-to-liquid ratio on flavonoid yield. (amount of flowers of Pueraria lobata: 10g; solvent: 50% (v/v) methanol; extraction duration: 2.0 h; extraction temperature: 70°C; ultrasound time: 3 times and 30 min each time)

flavonoid yield.

Effect of extraction duration on flavonoid yield

As shown in Figure 4, the flavonoid yield from *F. pueraria* increased a lot within 1.5 h but very little when the extraction time exceeds 1.5 h under the same conditions. One reason may be the flower petals are very thin so that the flavonoids are rapidly released and reached equilibrium. On the other hand, more flavonoids is oxidized and degraded when the extraction duration

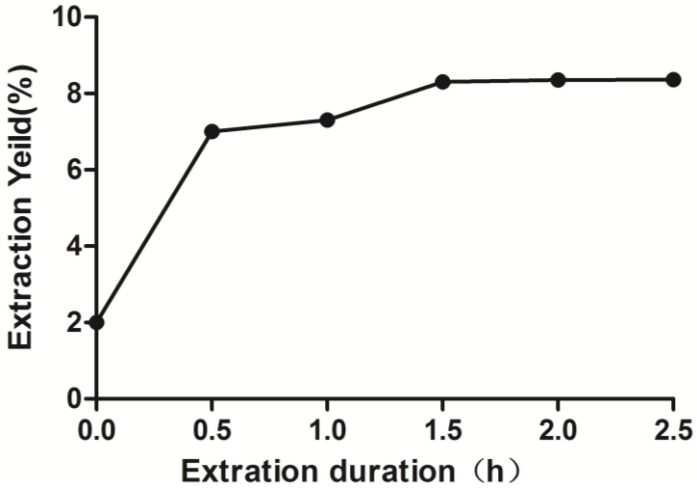

Figure 4. Effect of extraction duration on flavonoid yield. (amount of flowers of Pueraria lobata: 10g; solvent: 50% (v/v) methanol; solid-to-liquid ratio: 1:30; extraction temperature: 70°C; ultrasound time: 3 times and 30 min each time)

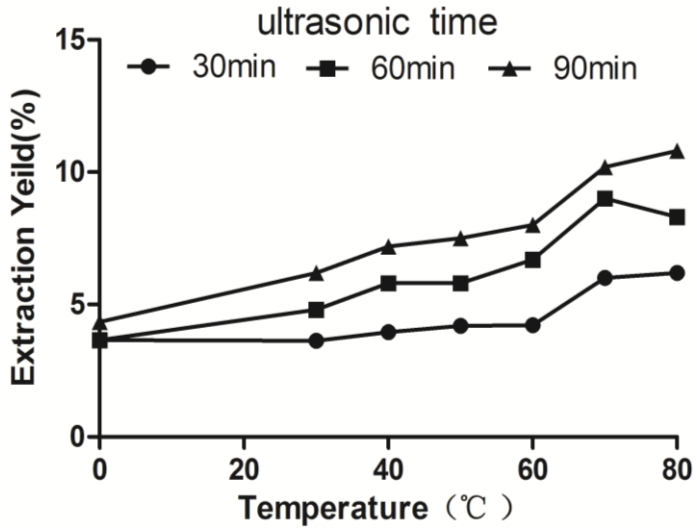

Figure 5. Effect of temperature with different ultrasonic time on flavonoid yield. (amount of flowers of Pueraria lobata: 10g; solvent: 50% (v/v) methanol; solid-to-liquid ratio: 1:30; extraction duration: 2.0 h)

extended. Thus, 1.5 h was chosen as the ultrasound-assisted extraction time in subsequent experiments.

Effect of temperature with different ultrasonic time on flavonoid yield

The effect of temperature and different ultrasonic time on flavonoid yield was also evaluated in the present study under the optimal conditions described. Ultrasound can cause temperature increase in the extraction. As shown in Figure 5, the total flavonoid yields consistently elevated during extraction when ultrasonic time was 30, 60 and 90 min, respectively. It suggested that ultrasound and increased temperature leads to accelerated diffusion of active ingredients and increased extraction efficiency, and the solvent viscosity and surface tension would decrease as the temperature increased, which is contributed to the sample wetting and matrix penetration. The flavonoid solubility also increased as a result of the temperature improvement. But once the extraction temperature exceeded 70°C, the extraction efficiency changed slightly.

Table 2. Results of $L_9(3^4)$ orthogonal test.

n	Concentration (%) A	Solid/liquid (g/ml) B	Duration (h) C	Temperature (°C) D	Yield (%)
1	40	1/20	1.0	50	10.12
2	40	1/30	1.5	60	14.83
3	40	1/40	2.0	70	16.94
4	50	1/20	1.5	70	15.26
5	50	1/30	2.0	50	14.21
6	50	1/40	1.0	60	13.27
7	60	1/20	2.0	60	14.87
8	60	1/30	1.0	70	15.02
9	60	1/40	1.5	50	11.33
k_1	13.963	13.417	12.803	11.887	-
k_2	14.247	14.687	13.807	14.323	-
k_3	13.740	13.847	15.340	15.740	-
R	0.507	1.270	2.537	3.853	-

Table 3. Variance analysis of orthogonal test.

Origins	SS	v	F	Critical value	P
A	0.387	2	1.000	19.000	-
B	2.503	2	6.468	19.000	-
C	9.792	2	25.302	19.000	P<0.05
D	22.792	2	58.894	19.000	P<0.05
SD	0.39				

Table 4. The proof experiment for optimum process of ultrasound-assisted extraction.

Times	Extraction yield (%)
1	17.41
2	17.54
3	17.48
$\overline{X}$	17.48
RSD (%)	2.17

It may be due to enhanced degradation or conversion along with the higher temperature in the extraction. Hence, 90 min for ultrasound time and the temperature 70°C were the most appropriate to achieve total flavonoid yield via UAE.

Orthogonal design

According to single factor experiment results, and in accordance with the $L_9(3^4)$ orthogonal experiment design for the four factors (solvent concentration, solid-to-liquid ratio, extraction time, extraction temperature) and three levels, the optimal extraction condition was obtained (Tables 1 and Table 2). As shown in Table 3, there is significant difference between factors C and D on the extraction efficiency ($P < 0.05$). No significant difference was found between factor A and factor B ($P > 0.05$). Thus, the most optimal conditions for total flavonoids extraction were as follows: $A_2B_2C_3D_3$, that is: methanol solution concentration of 50%, solid-to-liquid ratio of 1:30, extraction duration of 2 h, at temperature of 70°C, ultrasound 3 times and 30 min each time, and the validation experiments of 3 batches confirmed that the total flavonoids yield rate was (17.48 ± 2.17)% under these optimum conditions (Table 4).

Purification of total flavone

The total flavonoids yield with the optimum extraction conditions of UAE were further isolated by silica gel column chromatography. Combined with the melting point and the color reaction, UV scan, HPLC, IR, electron impact: mass spectroscopy (EI-MS), five isolated monomer compounds were identified as: irisolidone, genistein, daidzein, kakkalide, and puerarin (Bai et al., 2010; Kim et al., 2003; Yasuda et al., 1995) (Figure 6).

Conclusion

Through this optimization study, an efficient UAE method was developed to extract total flavonoids from *F. pueraria*. Under the most optimal conditions of 50% (v/v) methanol as the solvent, solid-to-liquid ratio of 1: 30, extraction duration of 2.0 h, ultrasound 3 times and 30 min each time, at the temperature of 70° C, the total flavonoids yield rate was almost up to 17.5% in the extracts.

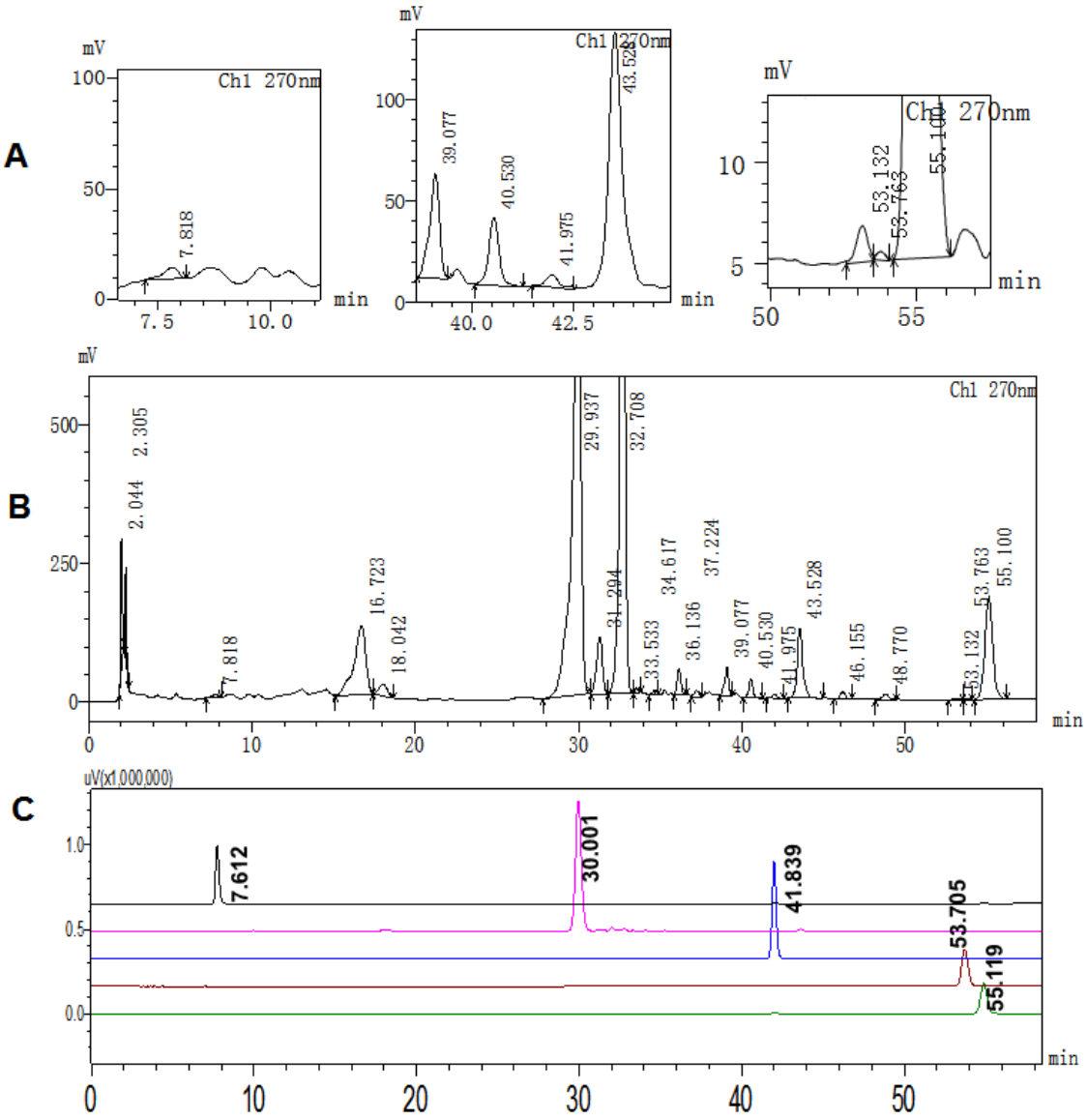

Figure 6. HPLC chromatogram for five isolated monomer compounds identified in the final extract as irisolidone, genistein, daidzein, kakkalide, and puerarin. A: Sample enlarge; B: Sample; C: Reference substance; $t_{puerarin}$=7.612min; $t_{kakkalide}$=30.001min; $t_{genistein}$=41.839min; $t_{irisolidone}$=53.705min; $t_{daidzein}$=55.119min

ACKNOWLEDGEMENTS

This work was financially supported by Colleges and Universities of Hubei Province Outstanding Young Scientific and Technological Innovation Team Plan (No.T201213), Hubei Province Natural Science Foundation Project of Outstanding Young Talents (No.2012FFA005).

REFERENCE

Bai X, Xie Y, Liu J, Qu J, Kano Y, Yuan D (2010). Isolation and identification of urinary metabolites of kakkalide in rats. Drug Metab. Dispos: the biological fate of chemicals. 38(2):281-286.

Choi J, Shin MH, Park KY, Lee KT, Jung HJ, Lee MS, Park HJ (2004). Effect of kaikasaponin III obtained from *Pueraria thunbergiana* flowers on serum and hepatic lipid peroxides and tissue factor activity in the streptozotocin-induced diabetic rat. J. Med. Food 7(1):31-37.

Lin YJ, Hou YC, Lin CH, Hsu YA, Sheu JJ, Lai CH, Chen BH, Lee Chao PD, Wan L, Tsai FJ (2009). Puerariae radix isoflavones and their metabolites inhibit growth and induce apoptosis in breast cancer cells. Biochem. Biophys. Res. Commun. 378(4):683-688.

Jung UJ, Lee MK, Park YB, Kang MA, Choi MS (2006). Effect of citrus flavonoids on lipid metabolism and glucose-regulating enzyme mRNA levels in type-2 diabetic mice. Int. J. Biochem. Cell Biol. 38(7):1134-1145.

Kim C, Shin S, Ha H, Kim JM (2003). Study of substance changes in flowers of Pueraria thunbergiana Benth. during storage. Arch. Pharm. Res. 26(3):210-213.

Lee HU, Choo MK, Bae EA, Kim DH (2003). Beta-glucuronidase inhibitor tectorigenin isolated from the flower of *Pueraria*

thunbergiana protects carbon tetrachloride-induced liver injury. Liver Int. 23:221–226.
Koon CM, Woo KS, Leung PC, Fung KP (2011). Salviae Miltiorrhizae Radix and Puerariae Lobatae Radix herbal formula mediates anti-atherosclerosis by modulating key atherogenic events both in vascular smooth muscle cells and endothelial cells. J. Ethnopharmacol. 138(1):175-183.
Lee MK, Cho SY, Jang JY, Cho MS, Jeon SM, Jang MK, Kim MJ, Park YB (2001). Effects of Puerariae Flos and Puerariae Radix extracts on antioxidant enzymes in ethanol-treated rats. Am. J. Chin. Med. 29(2):343-354.
Ramassamy C (2006). Emerging role of polyphenolic compounds in the treatment of neurodegenerative diseases: a review of their intracellular targets. Eur. J. Pharm. 545(1):51-64.
Tsuchihashi R, Kodera M, Sakamoto S, Nakajima Y, Yamazaki T, Niiho Y, Nohara T, Kinjo J (2009). Microbial transformation and bioactivation of isoflavones from Pueraria flowers by human intestinal bacterial strains. J. Natural Med. 63(3):254-260.
Xiong Y, Yang Y, Yang J, Chai H, Li Y, Yang J, Jia Z, Wang Z (2010). Tectoridin, an isoflavone glycoside from the flower of Pueraria lobata, prevents acute ethanol-induced liver steatosis in mice. Toxicology 276(1):64-72.
Yasuda T, Kano Y, Saito K, Ohsawa K (1995). Urinary and biliary metabolites of puerarin in rats. Biol. Pharm. Bull. 18(2):300-303.
Zhu ZG，Zhang ZY，Cai GM (2009). Extraction process of total flavonoids in Pueraria lobata and anti-oxidant activity. Central South Pharm. 7(3):203-205. Chinese.

academicJournals

Vol.8(1), pp. 9-15, 8 January, 2013
DOI 10.5897/AJPP10.250
ISSN 1996-0816 © 2014 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full length research paper

Antiproliferative and cytotoxic properties of honey in human prostate cancer cell line (PC-3): Possible mechanism of cell growth inhibition and apoptosis induction

Saeed Samarghandian[1]*, Fariborz Samini[2] and MohammadrezaTaghavi[1]

[1]Department of Basic Medical Sciences, Neyshabur University of Medical Sciences, Neyshabur, Iran.
[2]Department of Neurosurgery, Mashad University of Medical Sciences, Mashhad, Iran.

Accepted 30 December, 2013

Honey has long been used in medicine for different purposes. Only recently, however, its antioxidant property and preventive effects against different diseases, such as cancer, have been highlighted. In this study, we investigated the potential of honey to induce cytotoxic and apoptosis effects in cultured carcinomic human prostate cells (PC-3), a commonly used cell culture system for *in vitro* studies on prostate cancer. The cells were cultured in Roswell Park Memorial Institute (RPMI) medium and treated with different concentrations of honey for three consecutive days. Cell viability was quantitated by MTT assay. Apoptotic cells were determined using Annexin-V-FITC by flow cytometry. Honey could decrease cell viability in malignant cells in a concentration and time-dependent manner. The IC_{50} values against PC-3 were determined at 14.3, 9.2 and 4.3% after 24, 48 and 72 h, respectively. Honey induced apoptosis of PC-3 cells, as determined by flow cytometry histogram of treated cells which inducing apoptotic cell death is involved in honey toxicity. It might be concluded that honey could cause cell death in PC-3 cells, in which apoptosis plays an important role. Honey could also be considered as a promising chemotherapeutic agent in prostate cancer treatment in future.

Key words: Anexin-V, apoptosis, cytotoxicity, honey, 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), carcinomic human prostate cells (PC-3), chrysin.

INTRODUCTION

Prostate cancer is one of the leading causes of human male deaths throughout the world. It is a group of cancerous cells (a malignant tumor) that grow mostly from the outer part of the prostate (Jemal et al., 2005). Prostate cancer is clinically diagnosed in men over 50 years of age; expectancy, with aging the incidence of prostate cancer is probably to increase worldwide (Jemal et al., 2005; Marta et al., 2013). There is a promising opportunity for its intervention using cancer chempreventive compounds that can prevent or slow the progression of this downloaded disease (Tsao et al., 2003; Schmitz-Dräger et al., 2012). For a variety of reasons, naturally occurring botanicals and dietary substances are gaining increasing attention as cancer chemopreventive agents (Kumar et al., 2013). Important for prostate cancer chemproprevention is the fact that in

*Corresponding author. E-mail: saeed1142@yahoo.com. Tel: +989151200945.

recent years, the use of dietary and natural substances and botanical products are showing sustained increase by patients. In prostate cancer, a fine balance between cell proliferation and apoptotic death is lost which contributes to increase in cellular mass and tumor progression (Bilbro et al., 2013). In this regard, for prostate cancer chemoprevention at the present time, there is considerable emphasis in identifying novel natural substance that selectively induces apoptosis and growth arrest of prostate cancer cells without producing cytotoxic effects on normal cells.

Developments of new drugs with better efficacy are gaining momentum. The search for food as medicine is constantly evolving and people exploit various antioxidant rich foods for this purpose. Antioxidant rich foods have several preventive effects against different disease such as cancer. Honey has been used as a traditional food source since ancient times. Honey has a long tradition of use in folk medicine for various purposes and has been referred to extensively in the medical literature of Egypt and Greece (Lusby et al., 2002; Gomez-Caravaca et al., 2006). In general, honey is also rich with antioxidants and honey is thought to exhibit a broad spectrum of biological activities and therapeutic properties including antibacterial, antifungal, cytostatic (Estevinho et al., 2008; Molan, 2006), wound healing properties (French et al., 2005) and anti-inflammatory activity (Aljadi and Kammaruddin, 2004). There are also many reports in the medical literature of honey being effective as a dressing for wounds (Ghedolf and Engeseth, 2002; Vijaya and Nishteswar, 2012), burns (Aderounmu et al., 2013) and ulcer (Jull et al., 2008). Honey has been used for the treatment of Fournier's gangrene, abdominal wound disruption, gastric ulcers (Ali et al., 1997), gastroenteritis and burns, and for the storage of skin grafts (Subrahmanyan, 1993). Moreover, honey is harmless and in fact enables faster healing of the wounds by forming new tissues. An important property of honey is its antioxidant capacity; this is mainly due to the presence of flavonoids and phenolic acids, although the exact action mechanism is unknown. Among the mechanisms proposed are free radical sequestration, hydrogen donation, or a combination of these acting as substrates for radicals such as superoxide and hydroxyl. The antioxidant activity of honey is linked to the observed anticancer and antiatherosclerosis effects of honey (Beretta et al., 2007; Gribel and Pashinskii, 1990a). Honey is then substance made when the nectar and sweet deposits from plants are gathered, modified and stored in the honeycomb by honeybees. The major components of honey are fructose and glucose and also consist of carbohydrates, proteins, amino acids, vitamins, water, minerals and enzymes.

Apoptosis, an important process in cell development and maintenance of tissue homeostasis, plays an essential role as a protective mechanism against carcinogenesis by eliminating damaged cells or abnormal excess cells (Schuchmann and Galle, 2004; Yin et al., 2012). Apoptosis is characterized by particular morphological changes, including plasma membrane bleb, cell shrinkage, depolarization of mitochondria, chromatin condensation, and DNA fragmentation (Gyulkhandanyan et al., 2012). The relationship between apoptosis and cancer has been a recent focus. Apoptosis provides a number of useful clues when generating effective therapies and many chemotherapeutic agents exert their anticancer effects by inducing apoptosis in cancer cells (Peng et al., 2014). Therefore, induction of apoptosis has become a principal mechanism by which anticancer therapy is effective (Chen et al., 2014; Sharp, 2009).

It has been shown that honey could induce apoptosis in T24, RT4, 253J and MBT-2 bladder cancer cell lines (Chinthalapally et al., 1993). They showed significant inhibition of the proliferation of T24 and MBT-2 cell lines by 1 to 25% honey and of RT4, 253J cell lines by 6 to 25% honey. Honey contained many biologically active compounds including caffeic acid, caffeic acid phenethyl ester 16 and flavonoid glycones, these compounds have been proved to have an inhibitory effect on tumor cell proliferation and transformation by the down regulation of many cellular enzymatic pathways including protein tyrosine kinase, cyclooxygenase and ornithine decarboxylase pathways (Gribel and Pashiniski, 1990b). Research indicates that honey possessed moderate antitumor and pronounced antimetastatic effects in five different strains of rat and mouse tumors (Ferreres et al., 1994; Martos et al., 1997). Since honey is one of the common foods for humans and also because of the properties and potential medical uses that was mentioned earlier, it prompted the investigatation of a potential candidate for prostate cancer treatment and also how honey affects cell growth and induction of apoptosis.

MATERIALS AND METHODS

Chemicals and reagents

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was purchased from Amerso (USA). Roswell Park Memorial Institute (RPMI) 1640 was purchased from Gibco BRL (Grand Island, NY, USA). Annexin V/FITC (fluorescein isothiocyanate) was obtained from Invitrogene Corporation (USA). Fetal bovine serum (FBS) was purchased from PAA Laboratories GmbH, Austria. All other chemicals were of the highest quality commercially available.

Cell culture

The human prostate cancer cells, PC-3, were obtained from Pasteur Institute of Iran, cultured in RPMI medium supplemented with 10% FBS, 100 U/ml of penicillin and 100 µg/ml streptomycin. PC-3 cells were cultured in CO_2 incubator MCO-17AI (Sanyo Electric Co., Ltd, Japan) at 37°C, in humidified atmosphere

enriched by 5% CO_2 and sub-cultured every 3 to 4 days.

Cell viability assay

Cell viability was measured using the MTT assay, which is based on the conversion of MTT to formazan crystals by mitochondrial dehydrogenases (Mosmann, 1983). Briefly, PC-3 cells were plated at a density of (1 ×10^3 cells/ml) in 96-well plates and allowed to attach for 24 h to keep the log phase growth at the time of drug treatment. After treatment with different concentrations honey (0, 5, 10, 15 and 20%) for 72 h, 10 µl MTT was added into each well. After 4 h incubation at 37°C, the solution was removed, and the produced formazan was solubilized in 100 µl dimethyl sulfoxide (DMSO). Absorbance was measured at 550 nm using an automated microplate reader (Bio-Rad 550). Cell viability was expressed as a percentage of the control culture value. The cytotoxic effects of honey extract on cell line (PC-3) was expressed as IC_{50} value (the drug concentration reducing the absorbance of treated cells by 50% with respect to untreated cells). All experiments were carried out in triplicate.

Assessment of apoptosis by Annexin V-FITC

Apoptotic cell death of honey was measured using flouresin isothiocynate (FITC)-conjugated Annexin V/PI assay kit by flow cytometry (Deng et al., 2009), briefly 5 ×10^5 cells were washed with ice-cold PBS, re-suspended in 100 µl binding buffer, and stained with 5 µl of FITC-conjugated Annexin V (10 mg/ml) and 10 µl of PI (50 mg/ml). The cells were incubated for 15 min at room temperature in the dark, then 400 µl of binding buffer was added, and analyzed by a FACScan flow cytometry (Becton-Dickinson, USA). For analysis, PC-3 cell lines were gated separately according to their granularity and size on forward scatter (FSC) versus side scatter (SSC) plot. Early apoptosis and late apoptosis were evaluated on fluorescence 2 (FL2 for propidium iodide) versus fluorescence 1 (FL1 for Annexin) plots. The percentage of cells stained with annexin V only was evaluated as early apoptosis; the percentage of cells stained with both annexin V and propidium iodide was evaluated as late apoptosis or necrotic stage.

Statistical analysis

All results were expressed as mean ± standard error of mean (SEM). The significance of difference was evaluated with ANOVA and Bonfrroni's test. A probability level of P<0.05 was considered statistically significant.

RESULTS

Effects of honey on cell viability

PC-3 cancerous cells were incubated with various concentrations of honey for 24, 48 and 72 h. The impact of honey on cell viability was quantitated by MTT assay. Exposure of PC-3 cells with honey showed after 24 h significantly high growth inhibitory effects on prostate cancer cell line in a concentration and time dependent manner (p<0.001). Although, there was no significant result at low concentration of honey (2.5%) (data not shown); however, there were significant decrease in viability at concentration of 5, 10, 15 and 20% (P<0.05, p<0.001 and P<0.001, respectively) versus control after 24 h (Figure 1). On the other hand, treatment of PC-3 cell line for 4 and 72 h at different dose of honey (2.5, 5, 10 and 20%) resulted in marked reduction of number of viable cells (p<0.001) (Figure 1). The dose inducing 50% cell growth inhibition (IC_{50}) against malignant cell (PC-3) was determined at 14.3 ± 0.8, 9.2 ± 0.5 and 4.3 ± 0.3% at 24, 48 and 72 h, respectively (Table1).

Table 1. Doses inducing 50% cell growth inhibition (IC_{50}) of honey extract against prostate cancer cell line (PC-3).

IC_{50}	24 h	48 h	72 h
PC-3 (%)	14±1.0	10±0.9	4±0.05

Cells were treated with different concentration of honey for 24 and 48 h. Viability was quantitated by MTT assay.

Quantification studies for apoptosis by honey

To study the roles of honey in apoptosis, honey was used to setup apoptosis system on PC-3 cell line. PC-3 cells were treated with concentrations of 5 and 20% of honey for 24 h. After treatment, the cells were harvested and apoptosis was examined by flow cytometry (Figure 2). Quantitative analysis using Annexin V/PI assay further showed that the proportion of early stage apoptotic cells (Annexin V+/PI-) increased significantly from 19.12 to 35.61%, while proportion of late stage apoptotic cell (Annexin V+/PI+) increased significantly from 27.89 to 41.93% when the cells were treated with the concentrations of 5 and 20% honey, respectively (Figure 3). Apoptosis induced from 5 and 20% of honey was statistically higher than control and the percentage of the early and late apoptotic cells significantly increased by increasing honey concentration (p<0.001). Although, at concentration of 2.5%, there was no significant difference between percentage of early and late apoptotic cells (data not shown), however, the percentage of the early and late apoptotic cells significantly increased by increasing honey concentration (p<0.001), and also the number of the late apoptotic cells versus early apoptotic cells at concentration of 5 and 20% honey treated cells were statically significant (p<0.01, p<0.001) (Figure 3).

DISCUSSION

The most effective anticancer drugs currently used for treating different types of cancer have serious side effects. Current research has mainly focused on finding

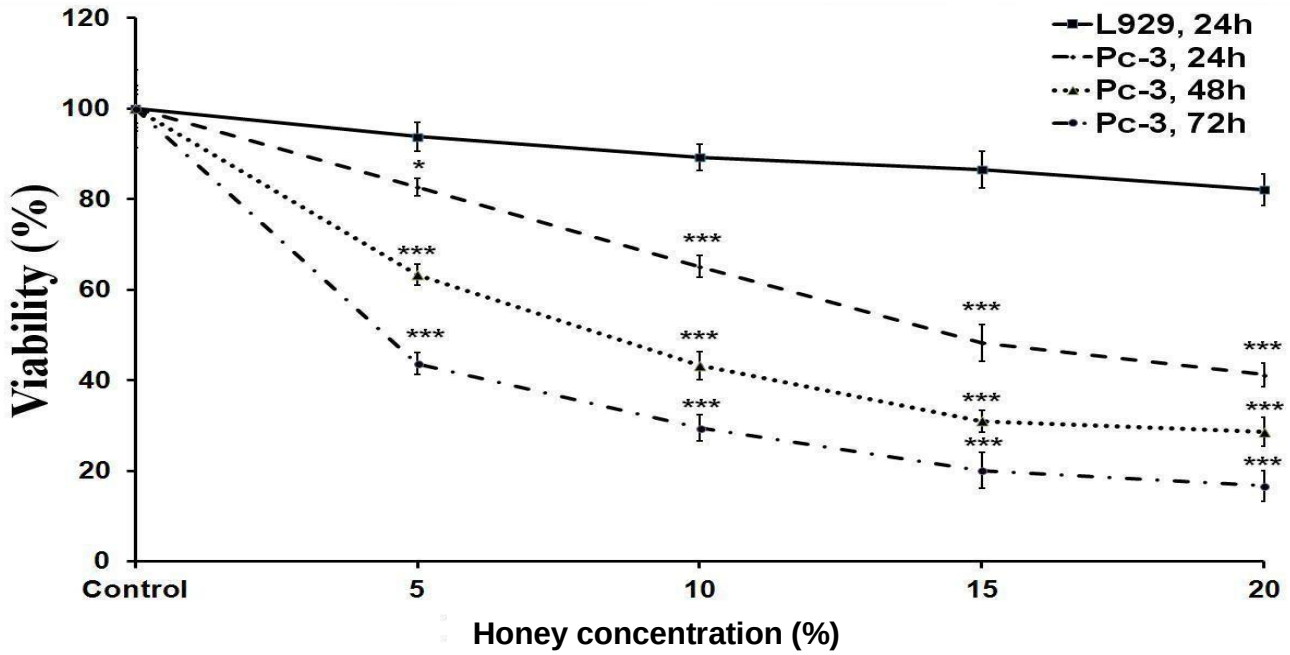

Figure 1. Effect of honey on cell viability of PC-3 cells. Cells were treated with different concentration of honey for 24, 48 and 72 h. Viability was quantitated by MTT assay. Results are mean ± SEM. The asterisks are indicator of statistically difference obtained separately at different time points compared to their controls shown in figure as *P<0.05; ***P<0.001.

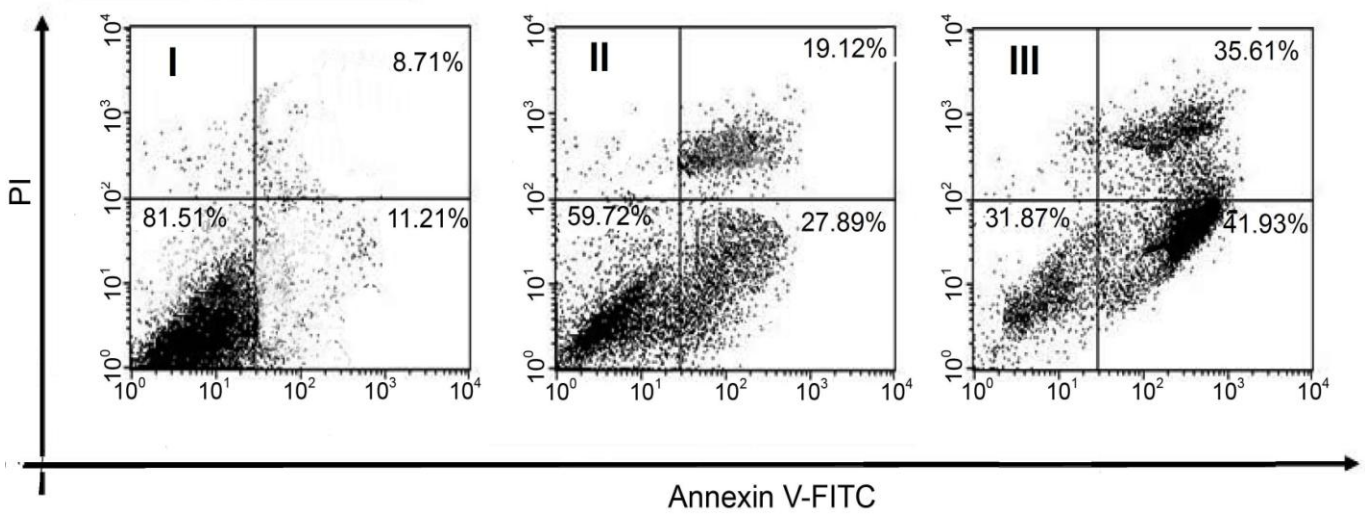

Figure 2. Assessment of apoptosis by Annexin V/PI on human prostate cancer cells (PC-3). The cells were treated with 5 and 20% (symbol II, III, resspectively) or media only (control symbol I), and apoptosis was examined by flow cytometry after Annexin V-PI double staining. Necrotic cells lose membrane integrity, permitting PI entry. Viable cells exhibit Annexin V (-)/PI (-); early apoptotic cells exhibit Annexin (+)/PI (-); late apoptotic cells or necrotic cells exhibit Annexin V (+)/PI (+).

ways of reducing these severe side effects (Alizadehnohi et al., 2012). The therapeutic activity of most anticancer drugs in clinical use is limited by their general toxicity to proliferating cells, including some normal cells. Although, chemists continue to develop novel cytotoxic agents with unique mechanisms of action, many of these compounds still lack tumor selectivity and have not been therapeutically useful (Rao et al., 2005; Chari, 2008).

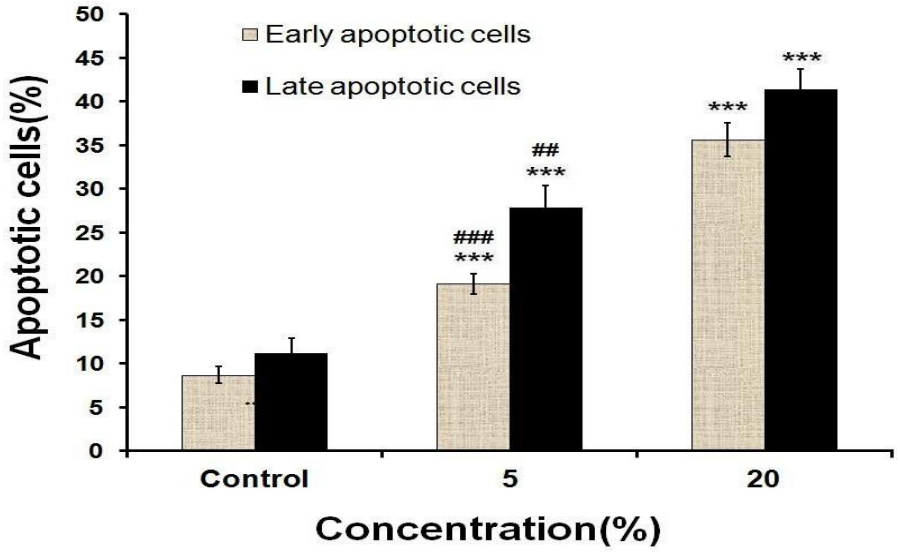

Figure 3. Assessment of apoptosis by Annexin-V/PI on renal cell carcinoma (ACHN). Percentage of cell death based on the assessment of apoptosis by Annexin-V/PI. ***P<0.001 and $^{##}$P<0.01, $^{###}$P<0.001 compared with the control and the other dose, respectively.

New targets for cancer therapy focus on interfering with specific targeted molecules needed for carcinogenesis and tumor growth in order to overcome the problems of traditional therapies (Goldman, 2003). Natural products are perceived as pure, and without side effects medication products (Montbriand, 2004). The purpose of the present study was to determine whether honey, through induction of apoptosis, possess an inhibitory effect on prostate cancer cells. It was demonstrated that honey can induce apoptosis in PC-3 cells. Analysis of cytotoxicity by MTT assay confirmed the cytotoxic effects of honey in a time- and dose-dependent manner.

A wide variety of natural food and products have been recognized to induce apoptosis in various tumor cells. It is thus considered important to screen apoptotic inducers and good candidate for development of anti cancer drug and also offering an opportunity to study the molecular mechanism of tumor genesis (Amit et al., 2009). Many patients with cancer or other chronic conditions use alternative therapies, often herbal or natural products (Montbriand, 1995; Montbriand, 2000). Honey, which is one of the most complex mixtures of carbohydrates produced in nature, has a long history as a medicinal substance. It is a known natural product with several biological activities. Some bioactive compounds have been found in honey such as chrysin which have been used to prevent cancer (Omene et al., 2012; Samarghandian et al., 2011).

For the protection of human health, considerable attention is currently focused on the consumption of functional foods. In particular, the role of dietary antioxidants capable of scavenging the oxidants and free radicals responsible for initiating various diseases has been intensively discussed (Samarghandian et al., 2013a, b). Systematic investigations of the antioxidant properties of various foods, beverages, spices and herbs have been performed (Butkovic et al., 2004; Samini et al., 2013) and the number of papers addressing the health-protective and antioxidant characteristics of honey is increasing. Honey has for a long time been used as a natural source of sugars, as well as an important ingredient in traditional medicine, having antimicrobial and antiinflammatory properties (Ku et al., 2007). Honey contains a variety of compounds including cafeic acid, benzoic acid and esters, substitute's phenolic acids and esters, flavonoid glycones and beeswax (Greenaway et al., 1987; Heby, 1981). Some of the observed biological activities of honey may be traced to its chemical constituents (Spector and Moore, 1988; Honn et al., 1989). Cafeic acid (3, 4-dihydroxycinnamic acid) ester derivates, which are present in honey at levels of 20 to 25%, are thought to exhibit a broad spectrum of activities that possibly include tumor inhibition. Several cellular components that have been associated with cell proliferation, such as polyamines and polyamine synthetic enzyme activities including ornithine decarboxylase, are present at high levels in proliferating normal and neoplastic cells. In addition many kinases, such as tyrosine protein kinase (TPK), mediate proliferative as well as metabolic signals in the cells. Eicosanoids, the metabolits of arachidonic acid through the lipoxygenase and cyclooxygenase pathways, exerts a

variety of biological activities. The health-protective and therapeutic impacts of honeys were formerly attributed to the presence of various antioxidant components, such as flavonoids, phenolic acids, organic acids, enzymes and vitamins (Wang et al., 2004; Samarghandian et al., 2011).

The mechanism of the antitumor effect shown in this study is unclear, but it may be related to the inhibitory effect of caffeic acid esters and flavonoid glycones on TPK, lipoxygenase, and cyclooxygenase pathways metabolites. Honey also contains various simple polyphenols which were found to exert significant antiproliferative potential against various cancer cells *in vitro* (Samarghandian et al., 2011). The apoptosis inducing potential of the honey could be attributed to its phenolic constituents (Samarghandian et al., 2010). Various signaling pathways, including stimulation of tumour necrosis factor-alpha (TNF-alpha) release, inhibition of cell proliferation, induction of apoptosis and cell cycle arrest, as well as inhibition of lipoprotein oxidation, mediate the beneficial effects exerted by honey and its major components such as chrysin and other flavonoids (Woo et al., 2004).

Anti-tumor and anti-cancer effects of honey have been shown in few studies. In this study, the cytotoxic and proapoptotic effects of honey in PC-3 cell lines were investigated. To the author's knowledge, this is the first report on honey-induced apoptosis in human prostate cancer cells. Our data confirmed that honey has cytotoxic activity against carcinomic human prostate cancer cell line, which is consistent with previous studies indicating that honey and its ingredients possess antitumor and anticarcinogenic activities (Samarghandian et al., 2011). Different studies have shown the anti proliferative activity of honey on human breast cancer cell line (MCF7) (Yaacob et al., 2013). The ability to induce tumor cell apoptosis is an important property of a candidate anticancer drug, which discriminates between anticancer drugs and toxic compounds (Samarghandian et al., 2010). The MTT assay is thought to be produced by the mitochondrial enzyme succinate dehydrogenase and can be dissolved and quantified by measuring the absorbance of the resultant solution. The absorbance of the solution is related to the number of live cells. A multiwell spectrophotometer assay can be semiautomated to process a large number of samples and provide a rapid object measurement of cell number (Samarghandian et al., 2011). Our study showed that honey exerted a significant proliferation inhibitory activity against PC-3 cells in a dose-dependent manner (Figure 1). Much effort has been directed towards the effect of saffron on apoptosis and understanding their mechanisms of action. The apoptosis evoked by honey was confirmed by the Annexin V-FITC (Figure 2). In the present study, honey-induced apoptosis was involved in cell death. Apoptosis is characterized by distinct morphological features including chromatic condensation, cell and nuclear shrinkage, membrane blebbing and oligonucleosomal DNA fragmentation. As shown in Figures 2 and 3, honey at 5 and 20% induced significant cell toxicity in the PC-3 cells in dose-dependent manner. Apoptosis only partially contributed in this toxicity, it might be suggested that non-apoptotic cell death to be also involved in honey-induced toxicity in these cells.

Overall, this study showed that honey may contain bioactive compounds that inhibit the proliferation of human prostate cell lines (PC-3) with the involvements of apoptosis or programmed cell death. Further studies are needed to fully recognize the mechanism involved in cell death, honey could be considered as promising chemotherapeutic agent in lung cancer treatment.

ACKNOWLEDGEMENTS

The authors would like to thank Research Affairs of Neyshabur University of Medical Sciences for financially supporting this work.

REFERENCES

Aderounmua A, Omonisi A, Akingbasote J, Makanjuola M, Bejide R, Orafidiya L, Adelusola K (2013). Wound-healing and potential anti-keloidal properties of the latex of calotropis procera (aiton) asclepiadaceae in rabbits. Afr. J. Tradit. Complement Altern. Med. 10: 574-579.

Ali AT, al-Swayeh OA, al-Humayed MS (1997). Natural honey prevents ischemia-reperfusion-induced gastric mucosal lesions and increased vascular permeability in rats, Eur. J. Gastroenterol. Hepatol. 9:1101–1105.

Alizadehnohi M, Nabiuni M, Nazari Z, Safaeinejad Z, Irian S (2012). The synergistic cytotoxic effect of cisplatin and honey bee venom on human ovarian cancer cell line A2780cp. J. Venom. Res. 3:22-27.

Aljadi AM, Kammaruddin MY (2004). Evaluation of the phenolic contents and antioxidant capacities of two Malaysian floral honeys. Food Chem. 85:513-518.

Amit S, Tomoo I (2009). Mutant p53 Gain of Oncogenic Function: In Vivo Evidence, Mechanism of Action and Its Clinical Implications, Fukuoka Acta Med. 100:217—228.

Beretta G, Orioli M, Maffei Facino R (2007). Antioxidant and radical scavenging activity of honey in endothelial cell cultures (EA.hy926). Planta Med. 73:1182-1189.

Bilbro J, Mart M, Kyprianou N (2013). Therapeutic Value of Quinazoline-based Compounds in Prostate Cancer. Anticancer Res. 33:4695-4700.

Butkovic V, Klasinc L, Bors W (2004). Kinetic study of flavonoid reactions with stable radicals. J. Agric. Food Chem. 52:2816–2820.

Chari RV (2008). Targeted cancer therapy: conferring specificity to cytotoxic drugs. Acc. Chem. Res. Rev. 41:98-107.

Chen SF, Nien S, Wu CH, Liu CL, Chang YC, Lin YS (2013). Reappraisal of the anticancer efficacy of quercetin in oral cancer cells. J. Chin. Med. Assoc. 76:146-1452.

Chinthalapally V, Dhimant D, Barbara S, Nalini K, Shantu A, Bandaru R (1993). Inhibitory effect of caffeic acid esters on azoxymethane-induced biochemical changes Antineoplastic activity of bee honey 219 and aberrant crypt foci formation in rat colon. Cancer Res. 53:4182–4188.

Deng L, Hu S, Baydoun AR, Chen J, Chen X, Cong X (2009). Aspirin induces apoptosis in mesenchymal stem cells requiring Wnt/beta-

catenin pathway. Cell Prolif. 42:721-730.
Estevinho L, Pereira AP, Moreira L, Dias LG, Pereira E (2008). Antioxidant and antimicrobial effects of phenolic compounds extract of Northeast Portugal honey. Food Chem. Toxicol. 46:3774-3779.
Ferreres F, Blazquez M A, Gil MI, Tomasbarberan FA (1994). Separation of honey flavonoids byFrench VM, Cooper RA and Molan PC (2005). The antibacterial activity of honey against coagulase-negative staphylococci. J. Antimicrob. Chemother. 56:228-231.
Ghedolf N, Engeseth NJ (2002). Antioxidant capacity of honeys from various floral sources based on the determination of oxigen radical absorbance capacity and inhibition of in vivo lipoprotein oxidation in human serum samples. J. Agric. Food Chem. 50:3050-3055.
Goldman B (2003). Combinations of targeted therapies take aim at multiple pathways. J. Natl. Cancer Inst. 22:1656-1657.
Gomez-Caravaca AM, Gomez-Romero M, Arraez-Roman D, Segua-Carretero A, Fernandez-Gutierrez A (2006). Advances in the analysis of phenolic compounds in products derived from bees. J. Pharmacol. Biol. Anal. 41:1220-1234.
Greenaway W, Scaysbrook T, Whately FR (1987). The analysis of bud exudates and propolis by gas chromatographymass spectrometry, Biol. Sci. 232:249–254.
Gribel NV, Pashiniski VG (1990a). Antitumor properties of honey. Vop. Onkol. 36:704–707.
Gribel NV, Pashinskii VG (1990b). The antitumor properties of honey. Vopr. Onkol. 36:704-709.
Gyulkhandanyan AV, Mutlu A, Freedman J, Leytin V (2012). Markers of platelet apoptosis: methodology and applications. J. Thromb. Thrombolysis 33:397-411.
Heby O (1981). Role of polyamines in the control of cell proliferation and differentiation. Differentiation 19:1–5.
Honn KV, Grossi IM, Taylor JD (1989). Lipoxygenase regulation of membrane expression of tumor cell glycoproteins and subsequent metastasis. Adv. Prostaglandin Thromboxane Leukotriene Res. 19:439–43.
Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ ,Thun MJ (2005). Cancer statistics. CA Cancer J. Clin. 55:10-30.
Jull A, Walker N, Parag V, Molan P, Rodgers A (2008). Randomized clinical trial of honey-impregnated dressings for venous leg ulcers, Br. J. Surg. 95:175–182.
Ku¨c_u¨k M, Kolaylı S, Karaog˘lu S, Ulusoy E, Baltacı C, Candan F (2007). Biological activities and chemical composition of three honeys of different types from Anatolia, Food Chem. 100:526–534.
Kumar N, Kale RK, Tiku AB (2013). Chemopreventive Effect of Lagenaria siceraria in Two Stages DMBA Plus Croton Oil Induced Skin Papillomagenesis. Nutr. Cancer 65:991-1001.
Lusby PE, Coombes AL, Wilkenson JM (2002): Honey: a potent agent for wound healing? J. Wound Ostomy Continence Nurs. 29:295-300.
Marta GN, Hanna SA, Fernandes da Silva JL, Carvalho Hde A (2013). Screening for prostate cancer: an updated review. Expert. Rev. Anticancer Ther. 13:101-108.
Martos I, Cossentini M, Ferreres F, Tomas-Barberan FA (1997). Flavonoid composition of micellar electrokinetic capillary chromatography., J. Chromatogr. 669:268-274.
Molan PC (2006). The evidence supporting the use of honey as a wound dressing. Int. J. Low. Extrem. Wounds 5:40-54.
Montbriand MJ (1995). Alternative therapies as control behaviours used by cancer patients. J. Adv. Nurs. 22:646-654.
Montbriand MJ (2000). Alternative therapies. Health professionals' attitudes. Can. Nur. 96:22-26.
Montbriand MJ (2004). Herbs or natural products that protect against cancer growth part three of a four-part series. Oncol. Nurs. Forum Rev. 31:127-146.
Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods. 983:55–63.
Omene CO, Wu J, Frenkel K (2012). Caffeic Acid Phenethyl Ester (CAPE) derivd from propolis, a honeybee product, inhibits growth of breast cancer stem cells. Invest New Drugs. 30:1279-1288.
Peng SF, Lee CY, Hour MJ, Tsai SC, Kuo DH, Chen FA, Shieh PC, Yang JS (2014). Curcumin-loaded nanoparticles enhance apoptotic cell death of U2OS human osteosarcoma cells through the Akt-Bad signaling pathway. Int. J. Oncol. 44:238-246.
Rao JS, Gondi C, Chetty C, Chittivelu S, Joseph PA, Lakka SS (2005). Inhibition of invasion, angiogenesis, tumor growth, and metastasis by adenovirus-mediated transfer of antisense uPAR and MMP-9 in nonsmall cell lung cancer cells. Mol. Cancer Ther. 9:1399-1408.
Samarghandian S, Afshari JT, Davoodi S (2011). Chrysin reduces proliferation and induces apoptosis in the human prostate cancer cell line pc-3. Clinics (Sao Paulo). 66:1073-1079.
Samarghandian S, Afshari JT, Davoodi S (2011). Honey induces apoptosis in renal cell carcinoma. Pharmacogn. Mag. 7:46-52.
Samarghandian S, JT Afshari JT, Davoodi S (2010). Modulation of programmed cell death by honey bee in human prostate adenocarcinoma. J. Med. Plant Res. 4:2551-2558.
Samarghandian S, Borji A, Delkhosh MB, Samini F (2013). Safranal treatment improves hyperglycemia, hyperlipidemia and oxidative stress in streptozotocin-induced diabetic rats. J. Pharm. Pharm. Sci. 16:352-362.
Samarghandian S, Boskabady MH, Davoodi S (2010). Use of in vitro assays to assess the potential antiproliferative and cytotoxic effects of saffron (Crocus sativus L.) in human lung cancer cell line. Pharmacogn Mag. 6:309-314.
Samarghandian S, Shabestari MM (2013). DNA fragmentation and apoptosis induced by safranal in human prostate cancer cell line. Indian J. Urol. 29:177-183.
Samini F, Samarghandian S, Borji A, Mohammadi G, Bakaian M (2013). Curcumin pretreatment attenuates brain lesion size and improves neurological function following traumatic brain injury in the rat. Pharmacol. Biochem. Behav. 110:238-244.
Schmitz-Dräger BJ, Lümmen G, Bismarck E, Fischer C (2012). Prevention strategies for prostate cancer. Minerva. Urol. Nefrol. 64:225-231.
Schuchmann M, Galle PR (2004). Sensitizing to apoptosis – sharpening the medical sword. J. Hepato. 40:335–336.
Sharp A (2009). Beneficial effects of honey dressings in wound management, Nurs. Stand. 24:66-68.
Spector AA, Moore SA (1988). Hydroxyeicosatetraenoic acids (HETEs). Prog. Lipid Res. 27: 271–276.
Subrahmanyan M (1993). Storage of skin grafts in honey, Lancet. 341:63–66.
Tsao AS, Kim ES, Hong WK (2004). Chemoprevention of cancer, CA Cancer J. Clin. 54:150-180.
Vijaya KK, Nishteswar K (2012). Wound healing activity of honey: A pilot study. Ayu. 33:374-377.
Wang XH, Gheldof N, Engeseth NJ (2004). Effect of processing and storage on antioxidant capacity of honey. J. Food Sci. 69:96–101.
Woo KJ, Jeong YJ, Park JW, Kwon TK (2004). Chrysin-induced apoptosis is mediated through caspase activation and Akt inactivation in U937 leukemia cells, Biochem. Biophys. Res. Commun. 325:1215–1222.
Yaacob NS, Nengsih A, Norazmi MN (2013). Tualang honey promotes apoptotic cell death induced by tamoxifen in breast cancer cell lines. Evid. Based. Compl. Altern. Med. 1-9.
Yin PH, Liu X, Qiu YY, Cai JF, Qin JM, Zhu HR, Li Q (2012). Anti-tumor activity and apoptosis-regulation mechanisms of bufalin in various cancers: new hope for cancer patients. Asian Pac. J. Cancer Prev. 13:5339-5343.

academicJournals

8(1), pp. 16-20, 8 January, 2014
DOI: 10.5897/AJPP2013.3707
ISSN 1996-0816 © 2014 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Evaluation of the modulatory and antibacterial activity of the ethanolic extract and fractions of *Duguetia furfuracea* A. St.-Hil.

Cicera Norma Fernandes[1], Heloisa Helena Ferreira Sousa[1], Maria Cristina Melo Borges[1], Celestina Elba Sobral Souza[2], Glaucia Morgana Melo Guedes [2], Fernando Gomes Figueredo[2]*, Saulo Relison Tintino[2], José Galberto Martins Costa[3], Henrique Douglas Melo Coutinho[2], Irwin Rose Alencar Menezes[1], Cícero Francisco Bezerra Felipe[1] and Marta Regina Kerntopf[1]

[1]Laboratory of Molecular Pharmacology and Chemistry, Regional University of Cariri, Crato (CE), Brazil.
[2]Laboratory of Molecular Biology and Microbiology, Regional University of Cariri, Crato (CE), Brazil.
[3]Research Laboratory of Natural Products, Regional University of Cariri, Crato (CE), Brazil.

Accepted 30 December, 2013

Medicinal plants have been the subject of research in several countries such as Brazil. The *Duguetia furfuracea* A. St.-Hil., popularly known as araticum-bravo, ata-brava and ata de lobo, has been used in folk medicine as anti-rheumatic drugs, for the treatment of renal dysfunction, spinal pain and stomach, and against pediculosis. This work aimed to analyze the antibacterial effect of the crude extract and fractions obtained from the leaves of *D. furfuracea*. The characterization of secondary metabolites was carried out through phytochemical prospection, being checked for the presence of tannins, flavonoids and alkaloids. The minimum inhibitory concentration (MIC) was determined by using broth microdilution method and its modulating activity of antibiotic activity in sub-inhibitory (MIC/8) concentration. When standard bacterial strain is used for the MIC and multidrug-resistant strains of modulation, all samples had a MIC ≥ 1024 µg/ml. The samples when combined with aminoglycosides demonstrated synergistic activity against the *Escherichia coli* 27 and *Staphylococcus aureus* 358. The results of this study indicate the species *D. furfuracea* as a promising source in combating bacterial multidrug resistance, increasing the potential of antibiotics.

Key words: *Duguetia furfuracea* A. St.-Hil., modulation, antibacterial activity, aminoglycosides.

INTRODUCTION

The use of medicinal plants for the treatment of diseases is one of the oldest practices of mankind, and the information for their use comes through popular culture (Funke and Melzig, 2006; Biavatti et al., 2007; Oliveira et al., 2007; Agra et al., 2007). The use of these plants, especially in South America contributes significantly to the basic health care. For the treatment of common infections, many plants are used in Brazil in the form of crude extract, infusions or plasters, without any scientific evidence of its effectiveness (Nakamura et al., 1999).

With respect to pathogenic bacteria, bacterial resistance to antibiotics is a growing problem and concern (Georgopapadakou, 2005; Nostro et al., 2004). The constant use of antibiotics has caused a lot of problems among which we can highlight the imbalance of human ecology and microbial resistance. This context shows that

*Corresponding author. E-mail: fgfigueredo@gmail.com. Tel: +55(88)31021212. Fax: +55(88) 31021291.

there is a need to search for new antibiotics that are effective, opening paths for the development of research, because the development of any new antimicrobial comes with resistance of microorganisms; the emergence of resistant pathogens is a threat to these advances (Moellering, 2000).

Research on natural products with antimicrobial activity has increased significantly in recent years. Medicinal plants have been the subject of research in several countries such as Brazil. This country holds a rich biodiversity and possessor of a diverse flora. In this way, the diversity of molecules found in plants makes them promising sources of new antimicrobials (Di Stasi et al., 2002; Estevam et al., 2009). The Annonaceae family has approximately 135 genera and 2,500 species of tropical and subtropical trees and shrubs, fruit trees (Fechine et al., 2002; Chatrou et al., 2004), and in Brazil 33 genera and 250 species were identified (Souza and Lorenzi, 2005). Within this family, the genre *Duguetia* stands by the various structural classes found: alkaloids, amides, diterpenes, steroids, flavonoids and roundness (Pontes et al., 2004; Carrolo et al., 2006).

The species *Duguetia furfuracea* St.-Hil., occurs in several regions in Brazil, the state of Mato Grosso do Sul; this species often becomes an invading plant when the cerrado is turned into pastures. Popularly known as araticum-bravo, ata-brava and ata de lobo, has been used in folk medicine as anti-rheumatic drugs, for the treatment of renal dysfunction, spinal pain and stomach, and against pediculosis (Rodrigues and Carvalho, 2001; Carolo et al., 2006; Pott and Pott, 1994; Gottsberge, 1987; Gavilanes and Brandão, 1998).

This work aimed to analyze the antibacterial effect of the crude extract and fractions obtained from the leaves of *D. furfuracea*.

MATERIALS AND METHODS

Bacterial

The bacterial strains used in this study were four standard strains of bacteria (Gram-positive and Gram-negative): *E. coli* (ATCC10536) and clinical isolated (EC27), *Staphylococcus aureus* (ATCC25923) and clinical isolated (SA358), *Pseudomonas aeruginosa* (ATCC15442) and *Klebsiella pneumoniae* (ATCC4362) with resistance profile shown in Table 1. The microorganisms used in the tests were obtained from Instituto Nacional de Controle de Qualidade em Saúde (INCQS) da Fundação Oswaldo Cruz, Ministério da Saúde. All strains were maintained on Heart Infusion Agar (HIA; Difco laboratories Ltda), being subsequently cultivated by 24 h in Brain-Heart Infusion (BHI – Difco, Laboratories Ltda).

Plant

The leaves of *D. furfuracea* were collected in June 2010, at Sítio Barreiro Grande, in the municipality of Crato, Ceará, Brazil. A plant was prepared and sent for identification in the Herbarium Dárdano Andrade Lima of Regional University of Cariri (URCA) and registered under number # 5508.

Preparation of crude extract and fractions

The ethanolic extract was prepared from the fresh leaves of *D. furfuracea* (637 g) by cold extraction method. The leaves of the species were previously washed in running water, crushed and macerated and then subjected to solvent extraction in ethanol P.A (Dinâmica, Brasil) for 72 h. After this period the ethanolic solvent has been distilled using a Rotary evaporator unit at 60°C under reduced pressure, yield getting of 1.57%. After this process, it was made the fractionation of ethanolic extract (10 g) under vacuum filtration, using three solvents (according to polarity scale): hexane, ethyl acetate and methanol.

Drugs

Neomycin, Kanamycin, Gentamicin and Amikacin were obtained from Sigma Chemical Laboratory Corp., St. Louis, MO, USA. All drugs were dissolved in sterile water.

Phytochemical prospecting

The phytochemical prospecting of ethanolic extract of *D. furfuracea* leaves was done through the methodology of Matos (1997); where to identify the classes of secondary metabolites it was observed that the color changed the formation of precipitates after addition of specific reagent. The results obtained after the tests are described as shown in Table 2.

Test of modulatory and antibacterial activity of aminoglycosides

The solutions of the crude ethanolic extract and fractions (hexane, ethyl acetate and methanol) were prepared using 10 mg samples dissolved in 1 ml of dimethyl sulfoxide (DMSO), obtaining an initial concentration of 10 mg/ml, if required, diluted in distilled water reaching concentration of 1024 µg/ml.

The minimum inhibitory concentration (MIC) was determined through microdilution method in 10% BHI with suspension of 10^5 UFC/ml, from the inoculum of 100 µl of each standard lineage of bacteria and then added 100 µl of each natural product, being diluted in serial manner with final concentrations of samples ranging from 512 to 8 µg/ml (Javadpour et al., 1996). For the evaluation of the samples as modulator of antibiotic activity, the MIC of antibiotics were determined in the presence and absence of natural products in sub-inhibitory concentrations (MIC/8) (Coutinho et al., 2008). Aminoglycosides have been assessed in concentrations that varied from 2,500 to 1.22 µg/ml. The plates were incubated at 35°C for 24 h.

RESULTS AND DISCUSSION

The results of the MIC to all samples were ≥1024 µg/ml, this value does not demonstrate clinical relevance. However, in modulating activity of aminoglycosides was checked, synergistic activity with some of the antibiotics tested.

Table 1. Bacterial origin and profile of antibiotic resistance.

Bacteria	Origin	Resistance profile
E. coli 27	Surgical wound	Ast, Ax, Amp, Ami, Amox, Ca, Cfc, Cf, Caz, Cip, Clo, Im, Can, Szt, Tet, Tob
E. coli ATCC 10536	-	-
K. pneumoniae ATCC 4362	-	-
P. aeruginosa ATCC 15442	-	-
S. aureus 358	Surgical wound	Oxa, Gen, Tob, Ami, Can, Neo, Para, But, Sis, Net
S. aureus ATCC 25923	-	-

Ast: Aztreonan; Ax: Amoxicillin; Amp: Ampicilin; Ami: Amikacin; Amox: Amoxilina; Ca: Cefadroxil; Cfc: Cefaclor; Cf : Ceftazidime; Cip: Ciprofloxacin; Clo: Chloramphenicol; Im: Imipenem; Can: kanamycin; Szt: Sulfametrin; Tet: Tetracycline; Tob: Tombramicina; Oxa: Oxacillin; Gen: Gentamicin; Neo: Neomycin; Para: Paromomycin; But: Butirosin; Sis: Sisomycin; Net: Netilmicin.

Table 2. Classes of secondary metabolites found in the crude ethanolic leaves of *Duguetia furfuracea*.

Classes of metabolites	(+) Presence(-) Absence
Phenols	-
Pyrogallic tannins	-
Condensed tannins	+
Anthocyanins	-
Anthocyanidins	-
Flavones	-
Flavonols	-
Xanthones	-
Chalcones	+
Aurones	+
Flavanonol	-
Leucoanthocyanidin	-
Catechin	+
Flavanone	+
Alkaloids	+

Tables 3 and 4 show synergistic or antagonistic modulating activity to the antibiotics kanamycin, amikacin, neomycin or gentamicin in association to the crude ethanolic extract and fractions. Against multi-drug resistant bacteria *E. coli* (27), the ethyl acetate and methanol fractions demonstrated synergism when associated to the antibiotic kanamycin, showing a reduction of MIC to 625 µg/ml. In the association of amikacin with hexane and ethyl acetate fractions showed a reduction of the MIC to 78, 12 and 312.5 µg/ml, respectively. To neomycin, both the crude extract and the hexane and methanol fractions promoted reduction of MIC in 156.25 µg/ml. In relation to multiresistant bacteria *S. aureus* (358), the crude ethanolic extract when associated with the antibiotic kanamycin, obtained a MIC reduction of 78.12 µg/ml. However, the mikacin when combined with the crude ethanolic extract and with hexane and methanol fractions promoted a reduction of MIC in 156.25 µg/ml. The crude ethanolic extract association with gentamicin, presented the synergism reducing the MIC from the antibiotic to 2.44 µg/ml.

Natural compounds, either from plant and animal origin can cause alteration of the effect of antibiotics, either increasing or antagonizing the antibiotic activity (Coutinho et al., 2008; Rodrigues et al., 2009; Tintino et al., 2013). We note that both extract and fractions acted synergistically when associated with aminoglycosides, showing a catalyzing effect, favoring an antibacterial activity to them. This was also observed as an antagonistic effect of natural products in association with aminoglycoside gentamicin against *E. coli* (27). This same effect has also been observed in studies by Veras et al. (2011), which reported a significant increase in MIC among natural products and aminoglycosides. According to Granowitz and Brown (2008), the antagonistic effects of combined use of antibiotics can be assigned to mutual chelation. According to Behling et al. (2004), the antagonistic effects of combined use of antibiotics can be

Table 3. Modulatory activity of *Duguetia furfuracea* against bacteria *E. coli* (27) with: crude ethanolic extract, hexane fraction, ethyl acetate fraction and methanolic fraction.

E. coli 27	+EEDF	+FH	+FAE	+FM	Control
Kanamycin	2.500	2.500	625	625	2.500
Amikacin	1.250	78,12	312,5	2.500	>2.500
Neomycin	156.25	156.25	625	156.25	625
Gentamicin	1.250	625	312.5	1.250	19.53

EEDF: Ethanolic extract of *Duguetia furfuracea*; FH: hexane fraction; FAE: ethyl acetate fraction; FM: methanolic fraction.

Table 4. Modulatory activity of *Duguetia furfuracea* against bacteria *S. aureus* (358) with: crude ethanolic extract, hexane fraction, ethyl acetate fraction and methanolic fraction.

S. aureus 358	+EEDF	+FH	+FAE	+FM	Control
Kanamycin	78.12	312.5	312.5	312.5	312.5
Amikacin	156.25	156.25	625	156.25	625
Neomycin	312.5	312.5	312.5	312.5	312.5
Gentamicin	2.44	19.53	19.53	19.53	19.53

EEDF: Ethanolic extract of *Duguetia furfuracea*; FH: hexane fraction; FAE: ethyl acetate fraction; FM: methanolic fraction.

assigned to mutual chelation. This possibly explains the reduction in the activity of aminoglycoside antibiotics in the presence of ethanolic extract of leaves of *D. furfuracea*, which demonstrated the presence of flavonoids by phytochemical survey carried out.

The results observed in species confirm with those found by Bento (2010), where the leaf extract of *Annona muricata* demonstrated a catalyzing effect against the same strains and species belonging to the same family. It is suggested that the synergistic effect brought by the extract and fractions can be due to the presence of compounds that exhibit antibacterial activity such as tannins and flavonoids. In tannins, antibacterial properties can be associated with the hydrolysis of an ester linkage between gallic acid, thereby serving as a natural defense mechanism against infections (Ho et al., 2001). However, the flavonoids have activity that probably is due to its ability to form complexes with soluble extra-cel proteins that bind to the bacterial cell wall (Tsuchiya et al., 1996).

The data obtained indicate the species *D. furfuracea* as a promising source in combating bacterial multidrug resistance, presenting itself as potentiating antibiotic.

REFERENCES

Behling EB, Sendão MC, Francescato HDC, Antunes LMG, Bianchi MLP (2004). Flavonoid quercetin: general aspects and biological actions. Aliment. Nutr. 15:285-292.

Bento EB (2010). Pharmacological Bioprospecting of Medicinal Plants Grown in Northeast Brazil: Evaluation of Antimicrobial and cytoprotective effect of Annona muricata Linnaeus (Soursop). Dissertation (Master in Molecular Bioprospecting), Regional University of Cariri.

Biavatti MW, Marensi V, Leite SN, Reis A (2007). Ethnopharmacognostic survey on botanical compendia for potential cosmeceutic species from Atlantic Forest. Rev. Bras. Farmacogn. 17:640-653.

Carollo CA, Hellmann-Carollo AR, Siqueira JM, Albuquerque S (2006). Alkaloids and a flavonoids from aerial parts (leaves and twigs) of *Duguetia furfuracea* - Annonaceae. J. Chem. Soc. 51:837-841.

Chatrou LW, Rainer H, Maas PJM (2004). Annonaceae (Soursop Family). In: Smith N, Mori SA, Henderson A, Stevenson DW & Heald SV (Eds.):Flowering Plants of the Neotropics: 18-20. The New York Botanical Garden, New York.

Coutinho HDM, Costa JGM, Lima EO, Falcão-Silva VS, Siqueira-Júnior JP (2008). Enhancement of the Antibiotic Activity against a Multiresistant Escherichia coli by *Mentha arvensis* L. and Chlorpromazine. Chemotherapy 54:328-330.

Di Stasi LC, Oliveira G P, Carvalhaes MA, Queiroz-Junior M, Tin OS, Kakinami SH (2002). Medicinal plants populary used in Brazilian Tropical Atlantic Forest. Fitoterapia 73:69-91.

Estevam CS, Cavalcanti AM, Cambui EVF, Araújo Neto V, Leopoldo PTG Fernandes RPM, Araujo BS, Porfírio Z, Sant'ana AEG (2009). Perfil fitoquímico e ensaio microbiológico dos extratos da entrecasca de Maytenus rígida Mart. (Celastraceae). Rev. Bras. Farmacogn. 19:299-303.

Fechine IM, Navarro VR; Da Cunha EVL, Silva MS, Maia JGS, Barbosa Filho JM (2002). Alkaloids and volatile constituents from *Duguetia flagellaris*. Biochem. Syst. Ecol. 30: 267-269.

Funke I, Melzig MF (2006). Traditionally used plants in diabetes therapy- phytotherapeutics as inhibitors of α-amylase activity. Rev. Bras. Farmacogn. 16:1-5.

Gavilanes ML, Brandão M (1998). Plantas consideradas medicinais ocorrentes na Reserva Biológica Municipal do Poço Bonito, Município de Lavras, Minas Gerais. Daphne 8:57-68.

Georgopapadaku NH (2005). Infectious disease 2001: drug resistence, new drugs. Drug resistence updates: reviews and commentaries in antimicrobial and anticancer chemotherapy. 5:181-191.
Gottsberge IS (1987). O cerrado como potencial de plantas medicinais e tóxicas. In: Simpósio de Plantas Medicinais do Brasil. Oréades, pp. 15-30.
Granowitz EV, Brown RB (2008). Antibiotic adverse aeactions and drug interactions. Crit. Care Clin. 24:421–442.
Ho KY, Tsai CC, Huang JS, Chen CP, Lin TC, Li CC (2001). Antimicrobial activity of tannin components from *Vaccium vitisidaea* L. J. Pharm. Pharmacol. 53:187-191.
Javadpour MM, Lo WC, Bishop SM, Alberty JB, Cowell SM, Becker CL, Mclaughlin ML (1996). De novo antimicrobial peptides with low mammalian cell toxicity. J. Med. Chem. 39:3107-3113.
Matos FJA (1997). Introdução in Phytochemistry experimental. 2. ed. Fortaleza: Editora UFC.
Moellering Jr RC (2000). Novos desafios no campo das doenças infecciosas. In: Patógenos emergentes nas doenças infecciosas: Relatório Especial Hospital Práctice. Euromédice. Ed. Médicas.
Nakamura CV, Ueda-Nakamura T, Bando E, Melo AFN, Cortez DAG, Dias Filho BP (1999). Antibacterial activity of *Ocimum gratissimum* L. essential oil. Mem. Inst. Oswaldo Cruz. 94:675-678.
Nostro A, Blanco AR, Cannatelli MA, Enea V, Flamini G, Morelli I (2004). Susceptibilityof methicillin-resistant staphylococci to oregano essential oil, carvacrol andthymol. FEMS Microbiol. Lett. 230:191–195.
Oliveira FQ, Gobira B, Guimarães C, Batista J, Barreto M, Souza M (2007). Espécies vegetais indicadas na odontologia. Rev. Bras. Farmacogn. 17:466-476.
Pontes AF, Barbosa MRV, Maas PJM (2004). Flora paraibana: Annonaceas Juss. Acta Bot. Bras. 18:281-293.
Pott A, Pott VJ (1994). Plantas do pantanal. EMBRAPA-Centro Nacional de Pesquisa do Pantanal. Corumbá: EMBRAPA-SPI, p. 320.
Rodrigues FFG, Costa JGM, Coutinho HDM (2009). Synergy effects of the antibiotics gentamicin and the essential oil of *Croton zehtneri*. Phytomedicine 16:1052-1055.
Rodrigues VEG, Carvalho DA (2001). Role of Endogenous Nitric Oxide and Prostaglandin in Duodenal Bicarbonate Response Induced by Mucosal Acidification in Rats. Dig. Dis. Sci. 46:1208-1216.
Souza VC, Lorenzi H (2005). Botânica Sistemática: guia ilustrado para identificação das famílias de Angiospermas da flora brasileira, baseado em APG II. São Paulo: Nova Odessa, Instituto Plantarum, p. 640.
Tintino SR, Cunha FAB, Santos KKA, Guedes GMM, Souza CES, Matias EFF, Braga MFBM, Andrade JC, Costa JGM, Freita MA, Coutinho HDM (2013). Atividade extracts in modulating ethanol and hexane root of Costus cf. arabicus of antimicrobial drugs. Rev. Bras. Biosci. 11:157-162.
Tsuchiya H, Sato M, Miyazak T, Fujiwara S, Tanigaki S, Ohyama M, Tanaka T, Jinuma M (1996). Comparative study on the antibacterial activityof phytochemical flavones against methicilin-resistant *Sthaphylococcus aureus*. J. Ethnopharmacol. 50:27-34.
Veras HNH, Santos IJM, Santos ACB, Fernandes CN, Matias EFF, Leite GO, Souza HHF, Costa JGM, Coutinho HDM (2011). Comparative evaluation of antibiotic and antibiotic modifying activity of quercetin and isoquercetin *in vitro*. Curr. Top. Nutraceutical Res. 9:25-30.

academicJournals

8(1), pp. 21-23, 8 January, 2014
DOI: 10.5897/AJPP12.1020
ISSN 1996-0816 © 2014 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Short Communication

Wound healing activity of leaf methanolic extract of *Ficus hispida* Linn.

Ramandeep Singh[1]*, Preeti Thakur[1], Alok Semwal[1] and Satinder Kakar[2]

[1]Department of Pharmacy, Himachal Institute of Pharmacy, Paonta Sahib (H.P), India.
[2]Department of Pharmacy, Doon valley Institute of Pharmacy and Medicine, Karnal (Haryana), India.

Accepted 30 December, 2013

In this study, the wound healing activity of methanolic extract of the leaves of plant *Ficus hispida* Linn. was evaluated on excision wound model. The leaves of *F. hispida* Linn. were successively extracted by using solvent methanol and screened for phytochemical constituents. Wistar rats (either sex) were given orally doses of 75 and 150 mg/kg body weight at 24 h intervals and their wound contraction area were calculated and wound healing were evaluated at day 0, 4, 8, 12, 16 and 20th. The results of phytochemical screening revealed that methanolic extract showed positive result for carbohydrates, saponins, sterols and tannin. The methanolic extract revealed significance increase in wound healing activity. The present investigation revealed that methanolic extract of *F. hispida* Linn. leaves increase the number of collagen tissue and this mechanism is responsible for wound healing activity. This could provide a rationale for the use of this plant as wound healer in folk medicines.

Key words: Wound healing, herbs, *Ficus hispida*, phyto-medicines.

INTRODUCTION

Wounds generally termed as physical injuries that result in an opening or breaking of the skin. There are different types of wounds which range from mild to potentially fatal. Wound healing is impaired in diabetic patients with infection or hyperglycemia. Diabetes mellitus is one of the major contributors to chronic wound healing problems. Diabetic patients with ulcer are at high risk for major complications which include infection and amputation. In traditional medicine, plants are generally used for treatment of various acute and chronic diseases and abnormalities in the body. Due to the present fast life of the humans, a drastic increase in chronic disease conditions mainly diabetes has been observed. Most of these patients tend to face a tremendous problem when they get an infected wound. Hence, in the current review, a list of the plants used in traditional medicine for the treatment of wounds and diabetes were screened. The work includes a list of traditionally claimed plants used for diabetes and wounds which are scientifically proved as well as scientifically not proved (Sandhya and Hygeia, 2011).

Ficus hispida Linn. (Moraceae)

Plants of *Ficus* species are used extensively in various parts of the world against a wide range of ailment .The synergistic action of its metabolic production is most probably responsible for beneficial effects of the plant (Bakshi et al., 2001). *Ficus* is a large genus of trees or shrubs, often climbers with milky juice, widely distributed throughout the tropical or hemispheres, but particularly abundant in south-East Asia and Polynesia. About 50 species of *Ficus* occur in India. The genus is remarkable

*Corresponding author. E-mail: ramandeep_pharma@yahoo.com. Tel: +91-9736922900 or +91-9017138383.

Table 1. Phytochemical screening of extract of leaves of *Ficus hispida* Linn.

S/N	Phytochemical tests	Inference
1	Glycosides	+
2	Alkaloids	-
3	Carbohydrates	+
4	Sterols	+
5	Saponins	+
6	Tannins	+
7	Flavonoids	+
8	Triterpenoids	+

for the large variation in the habitat of its species. It contains some of the giants of the vegetable kingdom such as Banyan tree, Pipal tree. Traditionally, various parts of the plants of the *Ficus* spp. are used for medicinal purpose. Chemical constituents e.g. oleanolic acid, β-sitosterol, triterpenoids, flavonoids, hispidin, β-amyrin are present (Peraza et al., 2002). According to Ayurveda, it is astringent to bowels; useful in treatment of biliousness, ulcers, erysipelas, vomiting, vaginal complains, fever, inflammations, leukoderma, psoriasis, hemorrhoids, ulcers and leprosy (Ayyanar and Ignacimuthu, 2009). Pharmacologically, it is used for treatment of diseases like hypoglycemia, cancer, inflammation, diarrhea, etc. (Kirtikar and Basu, 1975).

MATERIALS AND METHODS

Leaves collection and identification

F. hispida Linn. (Family: Moraceae) were collected fresh from Devi nager, Paonta sahib, Himachal Pardesh, India in the month of May 2011. The plant was authenticated by botanist Prof. Jaswant Saini, Department of Botany, Govt. Degree College, Paonta Sahib (H.P), India. The specimens vouchers (HIP/02/11/Herbarium/1110) were deposited. The leaves were air dried in the room away from sun light. After dried leaves were crushed to make powder.

Preparation of leaf extract

Extraction of leaves was carried out by the process of maceration. Fresh leaves of the plants were cleaned from extraneous materials, shade dried, powdered mechanically, weighed and stored in air tight container. About 250 g of powdered material was soaked in 1000 ml methanol for 72 h in beaker and mixture was stirred every 18 h using a sterile glass rod. Filtrate was obtained with the help of Whatman filter paper No. 1 and the solvent was removed by rotary evaporator under reduced pressure at leaving a dark brown residue.

Preliminary phytochemical screening of different extracts

The extract of *F. hispida* Linn. leaves were subjected to qualitative chemical tests for identification of various constituents such as alkaloids, carbohydrates, glycosides, proteins, tannins, sterols, saponins, amino acids, etc (Table 1).

Animal

Albino Wistar rats of the either sex (180 to 200 g) were used for the past study. They were maintained under standard environmental conditions and were fed with standard pellet diet as per CPCSEA guidelines.

Excision wound model

Animals was anesthetized prior to and during creation of the wounds, with 1 ml of chloroform with cotton in desiccators (10 mg/kg). The rats were inflicted with excision wounds as described by Morton et al. (1972) and Kamath et al. (2003). An impression was made on the dorsal thoracic region 1 cm away from vertebral column and 5 cm away from ear on the anaesthetized rat. The dorsal fur of the animals was shaved with an electric clipper and the anticipated area of the wound to be created was outlined on the back of the animals with methylene blue using a circular stainless steel stencil. A full thickness of the excision wound of circular area of 500 mm^2 and 2 mm depth was created along the markings using toothed forceps, scalpel and pointed scissors. Haemostasis was achieved by blotting the wound with cotton swab soaked in normal saline. The entire wound was left open (Lowry et al., 1951). All surgical procedures were performed under aseptic conditions (Table 2).

RESULTS AND DISCUSSION

Various substances of plant origin have been used in folk medicine of different culture as wound healer, some of which have been identified pharmacologically to exert their effects on the epithelial tissues. Furthermore, ancient literature alluded to the use of numerous plants/preparations including *F. hispida* Linn. as wound healer without any scientific evidence. To understand the scientific reason behind this claim, we investigated the effect of methanolic extract of *F. hispida* Linn. leaves in this study. In this investigation, treatment of the rats (either sex) with the methanolic extract of *F. hispida* Linn., leaves enhance the wound healing in rats. The

Table 2. Percentage of wound contraction by *Ficus hispida* Linn. extracts and standard drug on excision wound model in rats.

Group dose	0th Day	4th Day	8th Day	12th Day	16th Day	20th Day
Control (%)	502.7±3.29	384.3±3.32 (23.54)	302.7±2.40 (39.78)	236.3±2.27 (52.99)	133.7±2.65 (73.40)	24.67±1.52 (95.09%)
Standard (%)	502.7±2.108	323.7±3.32 (35.60)	172.3±3.63 (65.72)	102.3±2.75 (79.64)	19.33±1.43 (96.15)	-[100)
Ficus-75 (%)	502.3±3.70	367.3±3.52 (26.87)	219.5±2.55 (56.30)	135.0±2.29 (73.12)	75.33±2.56 (85.00)	--[100)
Ficus-150 (%)	503.0±2.51	345.0±3.29 (31.41)	208.3±4.04 (58.58)	120.3±3.20 (76.08)	21.33±1.76 (95.75)	--[100)

Wound area (mm^2) mean ± SEM and percentage of wound contraction.

healing proceeds with an increase in the number of collagen tissues. New tissues are formed in the wound area and the wound starts healing. The significant increase in wound healing by extract of *F. hispida* Linn. leaves at dose of 75 and 150 mg/kg body weight on day 0, 4, 8, 12, 16 and 20th successively is an indication of enhanced healing effect of the plant on wound.

ACKNOWLEDGEMENTS

The authors are thankful to the Director and Chairman, Himachal Institute of Pharmacy, Paonta Sahib (HP) for providing facilities for this research work.

REFERENCES

Ayyanar M, Ignacimuthu S (2009). Herbal medicines for wound healing among tribal people in Southern India Ethnobotanical and Scientific evidences. Int. J. Appl. Res. Nat Prod. 2(3):29-42

Bakshi DNG, Sharma PS, Pal DC (2001). A Lexicon of Indian Medicinal Plants. Nayaprakashan, New Delhi 2:190

Kamath JV, Rana AC, Chowdhury AR (2003). Pro- healing effect of. *Cinnamomum zeylanicum* bark. Phytother. Res. 17:970-972.

Kirtikar KR, Basu BD (1975). Indian Medicinal Plants. Periodical Experts, New Delhi., 2:2338.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

Morton JJ, Malone MH (1972). Evaluation of vulnerary activity by open wound procedure in rats, Arch. Int. Pharmacodyn. Ther. 196:117-120.

Peraza-Sánchez SR, Chai H, Shin Y, Santisuk T, Reutrakul V, Farnsworth NR (2002). Constituents of the leaves and twigs of *Ficus hispida*. Planta Med. 68:186–188.

Sandhya S, Hygeia JD (2011).Plants as Potent Anti diabetic and wound healing agents- A Review. Int. Res. J. Pharm. 3(1):11-19.

academicJournals

8(1), pp. 24-30, 8 January, 2014
DOI: 10.5897/AJPP12.1310
ISSN 1996-0816 © 2014 Academic Journals
http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Application of *Ipomoea batatas* starch as suspending agent in acetaminophen suspension

Onyishi Ikechukwu V.*, Chime Salome A. and Kanu Ifeoma

Department of Pharmaceutical Technology and Industrial Pharmacy, University of Nigeria, Nsukka 410001, Nigeria.

Accepted 16 December, 2013

Natural plant mucilage has gained importance over synthetic ones because of low toxicity, low cost and good availability. The objective of the study was to search for a cheap and effective natural raw material that can serve as an alternative suspending agent in the formulation of acetaminophen suspension. The phytochemical and the physicochemical properties of the mucilage of *Ipomoea batatas* L. were studied. The suspending properties of mucilage extract of *I. batatas* L. were evaluated comparatively with that of acacia and sodium alginate using model formulations at concentrations of 5, 10, 15 and 20% w/v. The suspensions were evaluated for rheological properties, sedimentation profile and stability studies. The results showed the presence of flavonoids, saponin, protein, carbohydrate and reducing sugars. The rheological properties of suspension showed that increase in concentration increased the viscosity of suspension, while increase in temperature did not significantly increased the viscosity of suspension ($p < 0.05$). The order of stability of suspension in terms of sedimentation profile ranked thus: E (20% w/v potato starch) > D (15% w/v potato starch) > F (10% w/v sodium alginate) > C (10% w/v potato starch) > B (5% w/v potato starch). These results indicate that mucilage from *I. batatas* L. in acetaminophen suspension has low sedimentation rate, medium viscosity and easily dispersible and can therefore serve as suspending agent in formulations of suspensions of sparingly soluble drugs.

Key words: Suspensions, potato starch powder, acetaminophen, rheological properties, physicochemical properties.

INTRODUCTION

Pharmaceutical suspensions are liquid dosage forms that require the addition of suspending agents in order to stabilize their system (Mahmud et al., 2010). These suspending agent increase sedimentation volume, ease redispersibility, enhance pourability and prevent compact cake formation. Suspending agents are grouped into three classes: synthetic, semi synthetic and the natural polysaccharides, in which class acacia, tragacanth and starch belong to the latter class (Mbang et al., 2004; Mahmud et al., 2010). The challenge of suspension formulation concerns sedimentation, caking and resuspension (Ogaji and Hoag, 2011). A suspension should not settle rapidly, it should be sufficiently fluid to flow easily under the condition of administration. As a suspension is energetically unstable, the particles that have settled tend to interact to form a cake or hard crystalline network. It is required that suspensions are formulated such that caking is minimized and so that the particles that have settled may be readily redispersed upon shaking (Ogaji and Hoag, 2011). In a pharmaceutical suspension, a suspending agent helps the drug stay suspended thereby reducing caking at the bottom of the preparation. Consistency of the solute throughout the suspending medium is facilitated with the drug or solute staying suspended in the continuous phase (Ogaji and Hoag, 2011). In recent years,

*Corresponding author. E-mail: docikeonyishi@gmail.com, ikechukwu.onyishi@unn.edu.ng. Tel: + 2348033763348. Fax: +234-42-771709.

pharmaceutical scientists have been paying increasing attention to the extraction, development and use of starches in the formulation of dosage forms (Garr and Bangudu, 1991; Alebiowu and Itiola, 2003). Natural polymers have advantages over the synthetic and semi-synthetic polymers which include better biocompatibility, relatively cheap, easily accessible, physiologically inert and do not exert pharmacological effect. Therefore, the danger of use of synthetic polymer matrix materials which often goes along with detrimental effects on incorporated drug during manufacturing of formulations or after application are completely avoided (Reithmeier et al., 2001). Potato starch is a tropical tuber that store edible material in subterranean roots or tubers (Oke, 1967). It mainly consists of starch, which is the only qualitatively important digestible polysaccharides being regarded a nutritionally superior to low molecular carbohydrate or sugars (Malcolm, 1990). The objectives of the study are to formulate acetaminophen suspension using starch from sweet potatoes as the suspending agent and to compare the suspending properties of potato starch with that of acacia and sodium alginate in acetaminophen suspension.

MATERIALS AND METHODS

Acetaminophen (May and Baker, England), acacia, sodium alginate (BDH Chemicals Ltd., England), saccharin sodium (Sigma-Aldrich, Germany), distilled water (Lion water, Nsukka, Nigeria). *Ipomoea batatas* starch was obtained from a batch processed in our laboratory. All other reagents and solvents were analytical grade and were used as supplied.

Extraction and purification of potato starch

I. batatas L. were purchased from the market of Nsukka, Nigeria in early December, 2011. The potatoes were cleaned by removing the soil and stones and washed with water. The bark were properly peeled and rewashed with clean water containing 1% sodium metabisulphite (an anti-oxidant). After washing, the potatoes were reduced to a fine pulp in a rasping machine of hammer mill type. The pulp was separated from the rasped potato by means of a muslin cloth and agitation was provided using hands. During screening, the water used contained 1% sodium metabisulphite in order to avoid discolouration of the starch by oxidative enzymes. The obtained starch suspension was allowed to settle under gravity and the supernatant was decanted. The starch suspension was washed severally using three times its volume of water for 3 days with intermittent shaking and changing of water. Dewatering was done using a bag with pores of about 100 mm and pressure was applied using hand. The drying of the starch was carried out under the sun (Ajali, 2004).

Phytochemical screening

Phytochemical tests were carried out on the potato starch for the presence of tannins, saponins, flavonoids, resins, fats and oils, glycosides, proteins, carbohydrates and reducing sugars. The tests were carried out using standard procedures of analysis (Harborne, 1993; Sofowora, 1993; Trease and Evans, 2002).

PHYSICOCHEMICAL ANALYSIS OF POTATO STARCH

Micromeritic properties of potato starch

Bulk and tapped densities

A 50 g quantity of starch powder was weighed out and placed in a 100 ml graduated cylinder. The volume occupied by the sample was noted as the bulk volume. The bulk density was obtained by dividing the mass of the sample by the bulk volume, as shown in Equation 1 (Aulton, 2007; Ngwuluka et al., 2010):

$$\text{Bulk density} = \frac{\text{Mass of Powder (M)}}{\text{Bulk volume of powder } (V_B)} \quad (1)$$

The cylinder was tapped on a wooden platform by dropping the cylinder from a height of one inch at 2 s interval until there was no significant change in volume reduction. The volume occupied by the sample was then recorded as the tapped volume. The tapped density was calculated using the formula:

$$\text{Tapped density} = \frac{\text{Mass of sample (M)}}{\text{Tapped volume } (V_T)} \quad (2)$$

Flow rate and angle of repose

A funnel was properly clamped onto retort stand. The funnel orifice diameter, base diameter and efflux tube length were appropriately measured. A 50 g quantity of the sample was weighed out and gradually placed into the funnel with the funnel orifice closed with a shutter. The time taken for the entire sample in the funnel to flow through the orifice was noted. The flow rate was gotten by dividing the mass of the sample by the time of flow in seconds. The static angle of repose was determined using the fixed base cone method (Aulton, 2007; Ngwuluka et al., 2010). About 50 g of the sample was transferred into an open-ended cylinder placed on a static base cone on a horizontal surface. The cylinder was gradually withdrawn vertically and the sample formed a cone-shaped heap. The height of the sample was determined using a cathetometer; the radius was gotten by dividing the fixed diameter by two. Angle of repose (ɵ) for each sample was obtained using the equation:

$$\Theta = \frac{\tan^{-1} \text{height}}{\text{radius}} \quad (3)$$

Also, the moisture content and ash content were determined.

Proximate analysis

Moisture and ash contents of the *I. batata* starch samples were determined by standard methods of analysis (AOAC, 1990).

Solubility

The solubility of potato starch powder was tested in water (cold and hot), methanol, ethanol and chloroform.

Rheological analysis

Potato starch powder mucilage (2% w/v) was prepared in distilled

water and the viscosities in cp were determined using a viscometer (Universal torsion viscometer, Gallenkamp, England) (Onyechi, 2008).

Effects of concentration on viscosity

Potatoes starch mucilage containing 0.5, 1, 2, 3, 4 and 5% w/v of starch were prepared in distilled water and the viscosities were recorded at room temperature (28°C).

Effects of temperature on viscosity

Also, about 2% w/v of potatoes starch powder were prepared and the viscosities were determined at 28, 40, 60, 80 and 100°C, respectively.

Effects of pH and electrolytes on the viscosity

A 0.1 M of HCl and NaCl, respectively were added to 2% w/v of starch mucilage and the viscosities were determined. A small amount of conc. HCl and NaOH were added to different portions of starch mucilage to modify the pH, and the pH was recorded. The viscosities of the different mucilages were recorded.

Formulation of acetaminophen suspension

Acetaminophen suspensions containing 2.4% w/v of acetaminophen were prepared using acacia (10% w/v), sodium alginate (10% w/v) or potatoes starch (0, 5, 10, 15 and 20% w/v) as the suspending agent as shown in Table 1. Mucilages of the suspending agent were prepared by hydration using part of the vehicle. The solid components of the formulation were finely triturated with the aid of mortar and pestle. The suspending agent was added to the powdered drug and triturated until homogeneous slurry was obtained. Sodium benzoate (0.02%) was used as the preservative and saccharine (1%) was used as the sweetener. This was transferred into a 100 ml beaker and the remaining vehicle was used to rinse the mortar to make up the required volume.

Evaluation of acetaminophen suspension

Sedimentation volume

The sedimentation volume of the suspensions was determined by measuring the volume of the sediments in the suspension placed in the measuring cylinders. The sedimentation volume was recorded at 30 min interval for 6 h, then at 12, 24 and 48 h. The sedimentation volume (F) was calculated using the formula:

$$F = \frac{V_u}{V_o} \quad (4)$$

Where V_u = height of sediment at a given time and V_o = original volume of sediment before settling occurred (Mahmud et al., 2010).

Rheological measurements

The viscosity of each batch of the suspension was determined using a viscometer (Universal torsion viscometer, Gallenkamp, England).

pH stability studies

The pH of the suspensions were determined using pH meter (pH ep® Hanna instrument, Padova, Italy) in time dependent manner (1 day, 30 and 60 days).

Statistical analysis

Statistical analysis was done using statistical package for social sciences (SPSS) version 14.0 (SPSS Inc. Chicago, IL.USA). All values were expressed as mean ± standard deviation (SD). Data were analysed by one-way analysis of variance (ANOVA). Differences between means were assessed by a two-tailed student's T-test. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Phytochemical properties

The result of phytochemical analysis of potato starch presented in Table 2 showed that the starch contains flavonoids, glycosides, saponin, protein, carbohydrate, reducing sugars, and fats and oils. The presence of these constituents showed some benefits of natural products since not only will they act as a suspending agent but will provide some nutritional and pharmacological activity. Phytochemicals are non-nutritive plant chemicals that have protective or disease preventive properties. Plants produce these chemicals substances to protect themselves, and they are also believed to protect humans against certain diseases (Edeoga et al., 2005).

Physicochemical properties

Solubility properties of potato starch

Potato starch was found to be insoluble in cold water, ethanol, methanol, acetone and chloroform, but it was soluble in hot water. Therefore, potato starch exhibited properties as a good suspending agent.

Micromeritic properties

The results of the micromeritic properties of potato starch presented in Table 3 indicate that the starch exhibited poor flowability values. However, flow rate and angle of repose obtained were within the acceptable limits for powder fluidity. Angle of repose was used as an indirect method of assessing flowability of powder because of their relationship with interparticle cohesion. The starch had angle of repose of 30° which showed that the starch had high interparticulate friction and hence exhibited poor flow. Hausner's ratio ≤ 1.25 indicates good flow, while > 1.25 indicates poor flow (Yüksel et al., 2007; Chime et al., 2012). Also, Carr's index in the range of 5 to 16 indicates good flow, 18 to 21 shows fair flow, while values above

Table 1. Composition of acetaminophen suspension.

Batch	Acetaminophen (%)	Acacia (%)	Sodium alginate (%)	Potatoes starch (%)	Sodium benzoate (%)	Saccharine (%)
A	2.4	10	0	0	0.02	1.0
B	2.4	0	0	5	0.02	1.0
C	2.4	0	0	10	0.02	1.0
D	2.4	0	0	15	0.02	1.0
E	2.4	0	0	20	0.02	1.0
F	2.4	0	10	0	0.02	1.0

A-F represents various formulations of acetaminophen suspension.

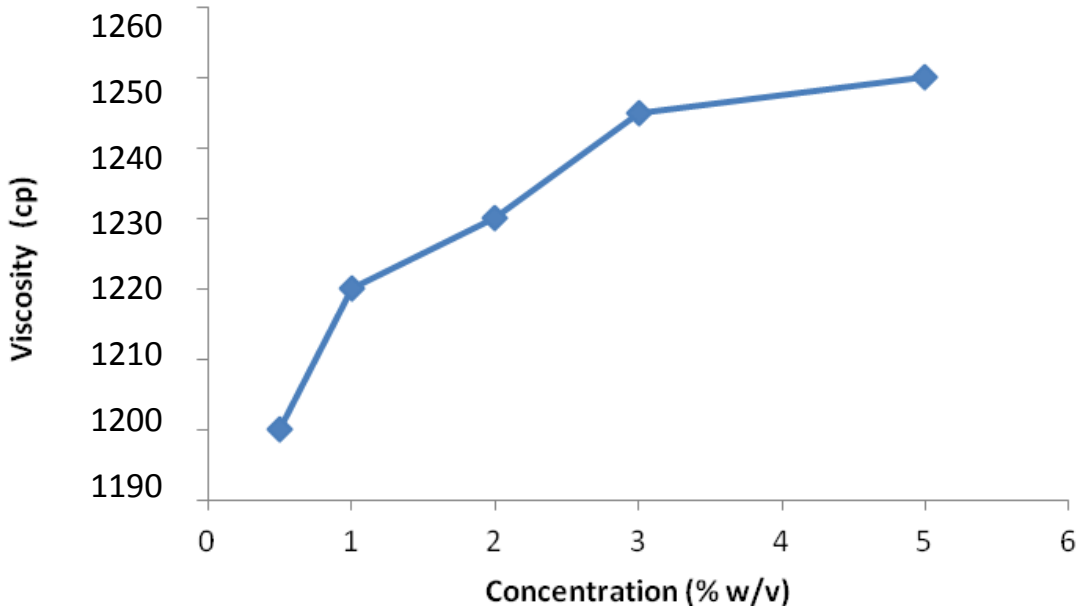

Figure 1. Effect of concentration on the viscosity of potato starch dispersion.

Table 2. Phytochemical content of *Ipomoea batatas* starch.

Chemical component	Results
Flavonoids	+
Saponin	+
Carbohydrates	+
Reducing sugars	+
Tannins	+
Resins	+
Glycosides	+
Proteins	+
Fats and oil	+

+ Present.

38 shows very poor flow (Yüksel et al., 2007; Chime et al., 2012). The results of Hausner's quotient also shown in Table 3 showed that potato starch had values significantly lower than 1.25 and therefore had poor flow ability due to high interparticulate friction in the bulk powder. The results of Carr's compressibility index also shown in Table 3 showed that potato starch had values of approximately 38° and hence exhibited poor flow.

Rheological properties of starch

The rheological properties of potato starch dispersions in distilled water showed that the viscosity of starch dispersions had a close to linear relationship at concentrations 0.5 to 5% w/v of potato starch. The viscosity increased continually up to a concentration of 5 % w/v as shown in Figure 1. Potato starch powder on initial increase in temperature absorbed more water showing a high initial peak viscosity. The results indicated that the gelatinization temperature of potato starch

Table 3. Physicochemical properties of potato starch powder.

Sample	BD (g/ml)	TD (g/ml)	Flow rate (g/s)	A.R (°)	HQ	CI (%)	MC (%)	AC (%)
Potato starch	0.60±0.10	0.96±0.17	0.52±0.07	29.80	1.60	37.50	4.50	0.50

BD = bulk density, TD = tapped density, A.R= angle of repose, HQ= Hausner's quotient and CI= Carr's compressibility index, MC= moisture content, AC= ash content.

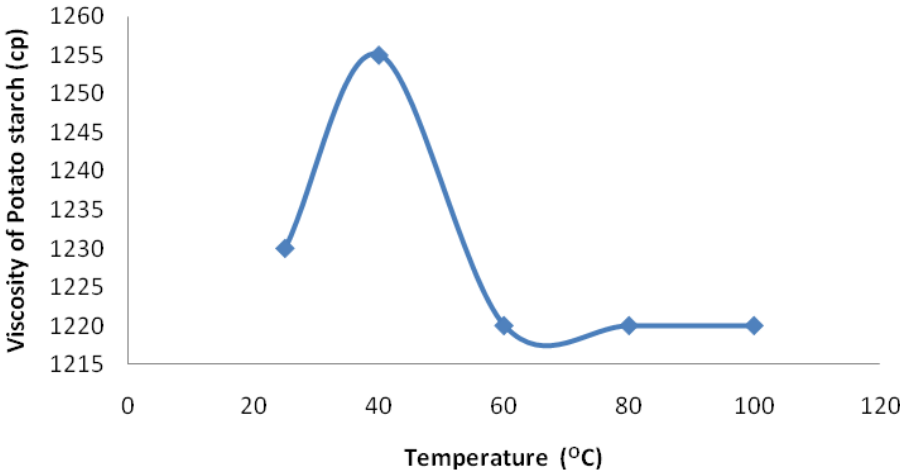

Figure 2. Effect of temperature on the viscosity of potato starch dispersion.

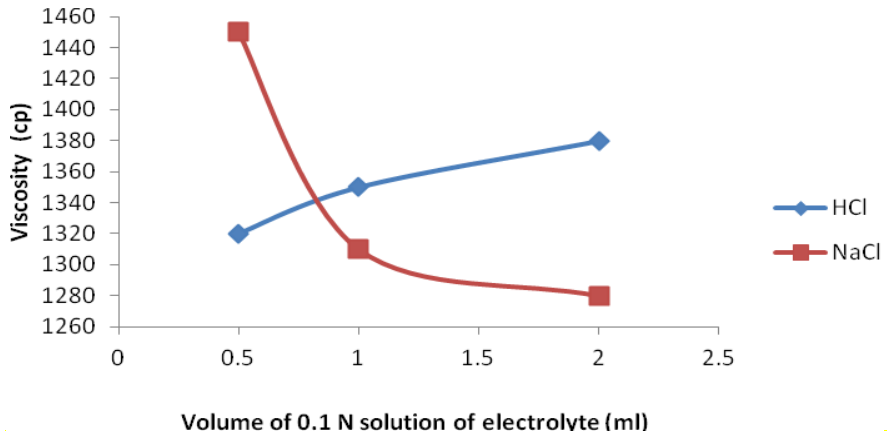

Figure 3. Effect of electrolytes on the viscosity of potato starch dispersions.

powder is low leading to the dispersion thickening quickly as the temperature increased. The rise to a peak temperature leading to high viscosity dropped rapidly on further increase in temperature, indicating little tendency to retrograde as shown in Figure 2. However, the presence and type of electrolytes significantly affected the viscosity of the starch dispersion ($p < 0.05$) as shown in Figure 3; sodium chloride caused a decrease in the viscosity of starch suspension as the concentration increased, while HCl caused an increase in the viscosity of suspension of starch as the concentration introduced increased.

Rheological properties of acetaminophen suspensions

Figure 4 shows the rheological properties of acetaminophen suspension formulated with potato starch and two reference suspending agents, acacia and sodium alginate. The results showed that acacia exhibited the highest viscosity of 1,265 cp significantly ($p < 0.05$) different from the tests suspending agent, potato starch. Also, sodium alginate showed higher viscosity value than potato starch. However, increase in concentration of potato starch above 10% w/v did not increase the viscosity

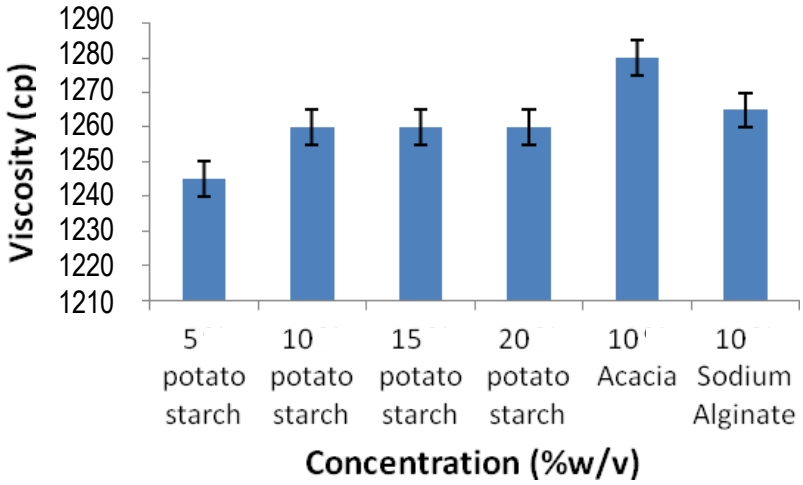

Figure 4. Effect of concentration on the viscosity of acetaminophen suspension formulated with varying concentrations of potato starch compared with sodium alginate and acacia.

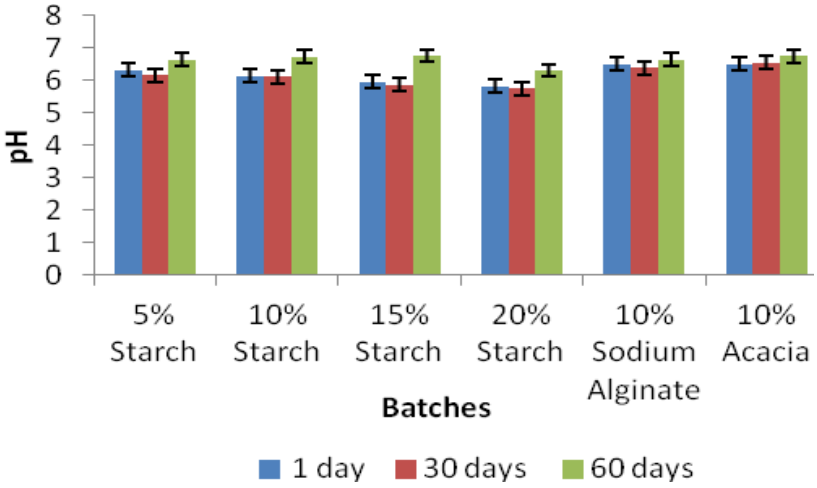

Figure 5. pH stability studies of acetaminophen suspension formulated with potato starch and reference suspending agents, acacia and sodium alginate.

of the suspension of acetaminophen as shown in Figure 4. Therefore, acacia and sodium alginate showed better rheological properties than potato starch in acetaminophen suspensions.

Time dependent pH stability

The results of pH stability studies of acetaminophen suspension are shown in Figure 5. From the results, the pH of all the suspensions slightly increased with time ($p < 0.05$). The results however, showed that the suspensions may require a buffer to keep the pH more stable. Change in pH could be a function of degradation of the active pharmaceutical ingredient (API) or excipients. A prior stable API may be affected by degradation of excipients with storage through generation of unfavorable pH (increase or decrease) or reactive species for the API (Chime et al., 2012; Attama et al., 2009).

Sedimentation volume of suspension

The results of sedimentation volume of acetaminophen suspension presented in Figure 6 showed that suspension formulated with 20% w/v potato starch was more stable than those formulated with 10% w/v sodium alginate in terms of sedimentation volume. The order of stability of suspension in terms of sedimentation volume could be ranked thus: E (20% w/v potato starch) > D

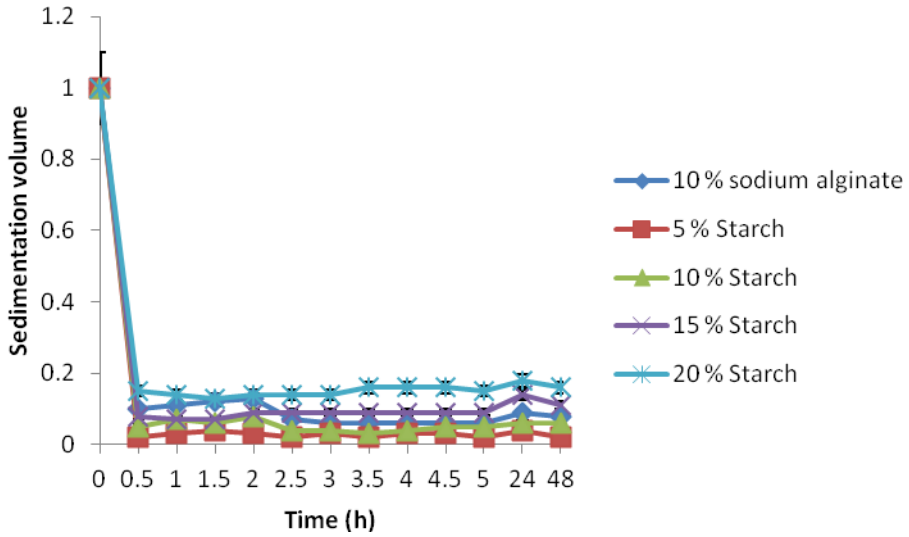

Figure 6. Sedimentation volume of acetaminophen suspension formulated with potato starch and reference suspending agent.

(15% w/v potato starch) > F (10% w/v sodium alginate) > C (10% w/v potato starch) > B (5% w/v potato starch).

Conclusion

Acetaminophen suspensions were successfully formulated using different ratios of potato starch as the suspending agent. The physicochemical properties of potato starch studied showed that it could be used in the formulation of suspensions. The properties of acetaminophen suspensions prepared were comparable to the properties of the reference suspending agents, sodium alginate and acacia used in the study but has advantage over the references which include: the viscosity of potato starch suspension is not affected by temperature fluctuations, it is tasteless, odourless, easy to prepare and relatively cheap.

REFERENCES

Ajali U (2004). Chemistry of Bio-Compounds. Rhyce Kerex Publishers, Ogui, Enugu, Nigeria: 8-13.

Alebiowu G, Itiola AO (2003). The effects of starches on mechanical properties of paracetamol tablet formulations: Pregelatinization of starch binders. Acta Pharm. 53:231-237.

AOAC, Association of Official Analytical Chemists (1990). Official Methods of Analysis of the Association of Official Analytical Chemists. Washington, D.C.

Attama AA, Okafor CE, Builders PF, Okorie O (2009). Formulation and *in vitro* evaluation of a PEGylated microscopic lipospheres delivery system for ceftriaxone sodium. J. Drug Deliv. 16:448–616.

Aulton ME (2007). Pharmaceutics; The Science of Dosage Form Design, 3rd Edn. Churchill Living Stone, Edinburgh; 197-210.

Chime SA, Attama AA, Agubata CO, Ogbonna JD, Onunkwo GC (2012). Micromeritic and antinociceptive properties of lyophilized indomethacin-loaded SLMs based on solidified reverse micellar solutions. J. Pharm. Res. 5(6):3410-3416.

Edeoga HO, Okwu DE, Mbaebie BO (2005). Phytochemical Constiuents of some Nigerian medicinal plants. Afr. J. Biotechnol. 4 (7):685-688.

Garr JSM, Bangudu AB (1991). Evaluation of sorghum starch as a tablet excipient, Drug Dev Ind Pharm. 17:1–6.

Harborne JB (1993). Phytochemistry. Academic Press, London, pp. 89-131.

Mahmud HS, Oyi AR, Allagh TS and Gwarzo MS (2010). Evaluation of the Suspending Property of *Khaya snegalensis* Gum in Co-Trimoxazole Suspensions. Res. J. Appl. Sci. Eng. Tech. 2(1):50-55.

Malcolm PS (1990). Polymer Chemistry: An Introduction. Oxford University Press, New York; pp. 537-569.

Mbang NF, Musiliu OA and Taiwo OO (2004). Evaluation of the suspending properties of *Albizia zygia* gum on sulphadimidine suspension. Trop. J. Pharm. Res. 3(1):279-284.

Ngwuluka NC, Idiakhoa BA, Nep EI, Ogaji I, Okafor SI (2010). Formulation and evaluation of paracetamol tablets manufactured using the dried fruit of *Phoenix dactylifera* Linn as an excipient. Res. Pharm. Biotech. 2(3):25-32.

Ogaji JI, Hoag SW (2011). Effect of *Grewia* gum as a suspending agent on ibuprofen pediatric formulation. AAPS Pharm. Sci. Tech. 12(2):507-513.

Oke OL (1967). The Nutritive Value of Some Nigerian Foodstuff from Animal Origin. West Afr. J. Pharm. 9:52.

Onyechi JO (2008). Introductory formulation Science 2. Global Publishers Nig. Ltd. Nsukka; 25-30.

Reithmeier HJ, Herrmann, Gopferich A (2001). Development and characterisation of lipid microparticles as a drug carrier for somatostatin. Int. J. Pharm. 218:133–143.

Sofowora H (1993). Screening Plants for Bioactive Agents In: Medicinal Plants and Traditional Medicine in Africa, Spectrum Books Ltd., Sunshine House, Ibadan. Nigeria 2nd Edn. pp. 134-156.

Trease GE, Evans WC (2002). Pharmacology. 15thg Edn. Saunders Publishers, London, pp. 42-44, 221-249, 303 -393.

Yüksel N, Türkmen B, Kurdoğlu AH, Başaran B, Erkin J, Baykara T (2007). Lubricant efficiency of magnesium stearate in direct compressible powder mixtures comprising cellactose® 80 and pyridoxine hydrochloride. FABAD J. Pharm. Sci. 32:173-183.

African Journal of Pharmacy and Pharmacology

Related Journals Published by Academic Journals

- *Journal of Medicinal Plant Research*
- *African Journal of Pharmacy and Pharmacology*
- *Journal of Dentistry and Oral Hygiene*
- *International Journal of Nursing and Midwifery*
- *Journal of Parasitology and Vector Biology*
- *Journal of Pharmacognosy and Phytotherapy*
- *Journal of Toxicology and Environmental Health Sciences*

academicJournals