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IN VITRO ANTIMICROBIAL ACTIVITY OF AQUEOUS, ETHANOL AND METHANOL EXTRACT OF SOME MEDICINAL PLANTS AGAINST HUMAN PATHOGENS

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ABSTRACT

The present study was aimed to evaluate the antimicrobial activity of aqueous, ethanol and methanol extract of some medicinal plants such as *Ocimum gratissimum* Linn and *Andrographis Paniculata* Nees against multi-drug resistant pathogenic gram positive (*Staphylococcus aureus*) and gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris*) bacteria. The sensitivity of gram positive and gram negative pathogenic multi-drug resistant bacteria to aqueous, ethanol and methanol extracts of *O. gratissimum* and *A. Paniculata* plants used as popular medicine in India was studied in vitro by the disk diffusion method and minimal inhibitory concentration (MIC). All the bacterial strains were found to be sensitive to aqueous, ethanol and methanol extracts. But, it is evident that the ethanol and methanol extracts were comparatively more effective than aqueous extracts. It can be concluded that the extracts of these two medicinal plants possess antimicrobial activity against human pathogens.

KEYWORDS

Folk medicine, Multi-drug resistant bacteria, Antimicrobial activity and Disk agar diffusion.

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INTRODUCTION

Resistance of microbes to available antimicrobial agents is a major global public health problem. Plants have a great potential for producing new drugs of great benefit to mankind. There are many approaches to search for biologically active principles in plants[1]. Medicinal plants represent a rich source of antimicrobial agents. Wide range of different parts of medicinal plants was used for

extract as raw drugs and they possess varied medicinal properties. Some of these raw drugs are collected in smaller quantities by local use while many other raw drugs are collected in larger quantities and traded in market as raw material for many herbal industries[2]. The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the screening of several medicinal plants for their potential antimicrobial activity[3]. In addition, in developing countries, synthetic drugs are not only expensive and inadequate for the treatment of diseases but also have adulteration and side effects. This kind of situation stresses the need to search a new drug for treating such infection[4]. Usually the discovery of new drug to cure pathogenic disorders is done by using pathogens obtained from commercial laboratories. The screening of drugs by using such microbial strains comparatively for many years may not be effective due to the evolution of resistant strains at high rate. So the antibacterial screening for any drug by using freshly collected clinical samples will be more potent. The revival of interest in plant derived drugs is mainly due to the current wide spread belief that ″green medicine″ was safe and more dependable than the costly synthetic drugs which may have adverse effect[1]. Hence, researchers are increasingly turning their attention to natural products looking for new leads to develop better drugs against microbial infection[5].

Ocimum gratissimum Linn (*O. gratissimum*), a tropical, much branched, annual herb is commonly used in folk medicine. *O. gratissimum* is an important medicinal herb which is commonly known as "Ram Tulshi". It belongs to the family of 'Labiaceae'. *O. gratissimum* is associated with chemo-preventive, anti-carcinogenic, free radical scavenging, radio protective and numerous others pharmacological use[6]. *O. gratissimum* is used to treat different diseases, e.g., upper respiratory tract infections, diarrhea, headache, ophthalmic, skin diseases, pneumonia, and also as a treatment for cough, fever and conjunctivitis[7, 8]. Earlier reports have shown the smooth muscle contracting lipid soluble principles, and antimutagenic activity in organic solvent extracts of *O. gratissimum* leaves[8, 9]. This medicinal plant has also potential role as antibacterial, antifungal, antimicrobial, anthelmintic, and *in vitro* antidermatophytic agent[10-17]. The aqueous leaf extract and seed oil showed anti-proliferative and chemo-preventive activity on HeLa cells[18]. Nangia-Makker *et al*. reported that, aqueous extract of *O. gratissimum* leaves inhibits tumor growth and angiogenesis by affecting tumor cell proliferation, migration, morphogenesis, stromal apoptosis and induction of inducible cyclooxygenase -2 (COX-2)[19].

Andrographis paniculata Nees (*A. paniculata*) is herbaceous plant in the family *Acanthaceae*, native to India and Sri Lanka. It is widely cultivated in Southern and Southeastern Asia, where it is used to treat infections and some diseases, often being used before antibiotics were created. Mostly the leaves and roots were used for medicinal purposes[20]. Water extract of *A. paniculata* Nees have potential antibacterial activities against *Staphylococcus aureus* and methicillin resistant *Staphyloococcus aureus*[21]. Sukesh *et al*., reported that the hexane extract of *A. paniculata* was effective against penicillin resistant *Staphylococcus aureus* MTCC 87[22].

The present study has been designed to determine the role of aqueous, ethanol and methanol extract of *Ocimum gratissimum* Linn and *Andrographis Paniculata* Nees for potential antimicrobial activity against multi-drug resistant pathogenic *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Proteus vulgaris* (*P. vulgaris*).

MATERIALS AND METHODS

Culture Media and Chemicals

Mueller-Hinton broth, nutrient agar, luria broth, tryptic soy broth, agar powder, RPMI-1640, Fetal bovine serum, crystal violet, cell culture grade DMSO were purchased from Himedia, India. Tris-Hcl, Tris buffer, sodium chloride, potassium dihydrogen phosphate (KH_2PO_4), di potassium hydrogen phosphate (K_2HPO_4), NaOH, ethanol,

methanol, cell culture grade DMSO, isopropanol were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. 3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Co., USA. All other the chemicals, reagents, were purchased from Himedia, India; SRL Pvt. Ltd. Mumbai, India and were of the highest grade available.

Bacterial Strains and Culture of Microorganisms

Six multi-drug resistant pathogenic *S. aureus*, *E. coli*, *P. aeruginosa* and *P. vulgaris* strains were selected for this study. Multi-drug resistant *S. aureus* strains were isolated from human post operative pus samples and *E. coli* strains were isolated from urine samples of urinary tract infection patient[23, 24]. Multi-drug resistant *P. aeruginosa* and *P. vulgaris* strains were obtained from Microbiology laboratory of Midnapore Medical College and Hospital. Bacterial culture was done at 37ºC throughout the experiment.

Plant Material Collection

O. gratissimum Linn. and *A. paniculata* Nees were collected from the campus of Abasgarh, Paschim Medinipur, West Bengal, India in June-July, 2010 and air-dried. A voucher specimen has been deposited to the herbarium of the Dept. of Botany, Vidyasagar University under the accession number PHYSIOL/Og-76 and PHYSIOL/Ap-77.

Preparation of Aqueous, Ethanol and Methanol Extract of Medicinal Plants

Aqueous, ethanol and methanol extract of *O. gratissimum* and *A. paniculata* was prepared according to our previous laboratory report[25-27]. In brief, the fresh aerial part of *O. gratissimum* and *A. paniculata* was dried, blended and extracted with distilled water (for aqueous extract), ethanol (for ethanol extract) and methanol (for methanol extract) [10:1]. The mixture was filtered with Whatman filter paper (No.1) and concentrated at 38 °C by a rotary evaporator, then allowed to stand at room temperature over night. This concentrated solution was then centrifuged at 2,000×*g* for 10 min and supernatant was freeze dried to obtain the crude extract.

Antibacterial Assay by Agar-Well Diffusion Method

The assay was conducted by agar well diffusion method[28]. The bacterial strains grown on nutrient agar at 37 ºC for 18 h were suspended in a saline solution (0.85% NaCl) and adjusted to a turbidity of 0.5 McFarland standards (10^8 CFU/ mL). The suspension was used to inoculate into petri plates (90 mm in diameter) with a sterile non-toxic cotton swab on a wooden applicator. Wells (6 mm in diameter) were punched in the agar and filled with 50 µL of 2000 µg/mL extracts. The dissolution of the ethanol and methanol extracts was aided by 1% (v/v) dimethylsulphoxide (DMSO) and that of the aqueous extract was aided with water, which did not affect the growth of microorganisms, in accordance with our control experiments. The surfaces of media were inoculated with bacteria from a broth culture. High-potency bio-discs were placed on the agar. After 18 h of incubation at 37 ºC, the plates were examined and the diameters of the inhibition zones were measured to the nearest millimeter.

Determination of Minimum inhibitory concentration (MIC) by Dilution Method

These tests were performed in sterile 96-well microplates and macroplates. The microdilution was performed in 96-well microtiter plates with U-shaped wells, while the macrodilution technique was as described by the National Committee for Clinical Laboratory Standards[29,30]. In brief, the cultures were diluted in Mueller-Hinton broth at density adjusted to 0.5 McFarland turbidity. The final inoculum was 5×10^5 CFU/mL of bacterial colony. Controls with 0.5 mL of culture medium only or others with plant extracts were used in the tests. The wells were filled with 100 µL of sterile H_2O, and 100 µL of the plant extracts were added to the wells by serial two-fold dilution from the suspension of plant extract stock solution. Each well was inoculated with 100 µL of 0.5 McFarland standard bacterial suspensions so that each well got 5×10^5 CFU/mL. The plates were covered, placed in plastic bags and incubated at 37ºC for 24 h. In this study, the MIC was the lowest concentration of plant extracts that exhibited the

growth of the organisms in the wells by visual reading.

Ethical Approval

The experimental protocol of this study was followed as per National Committee for Clinical Laboratory Standards (NCCLS) and Centre for Disease Control and prevention (CDC) guidelines and duly approved by the Institutional Ethical Committee.

Statistical Analysis

The experiments were performed three times and the data are presented as mean±S.E.M., n=6. Since the readings of control (distilled water and DMSO) experiments in the *in vitro* antibacterial studies of those plants were zero, the data were analyzed by simple arithmetic means of the different extracts, and the standard errors were compared with the control.

RESULTS AND DISCUSSION

The antimicrobial activities of aqueous, ethanol and methanol extracts of *Ocimum gratissimum* Linn and *Andrographis Paniculata* Nees against multi-drug resistant pathogenic *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris* were shown in Table No.1. In Table No.1, all the bacterial strains were found to be sensitive to aqueous, ethanol and methanol extracts. But, it is evident that the ethanol and methanol extracts were comparatively more effective than aqueous extracts. Aqueous, ethanol and methanol extracts of *Andrographis Paniculata* were comparatively more effective than *Ocimum gratissimum*. MIC value of the tested plant extracts against the tested microorganisms was shown in Table No.2. The antimicrobial compounds extracted from *Ocimum gratissimum* Linn and *Andrographis Paniculata* Nees might inhibit bacteria by a different mechanism to that of currently used antibiotics and have therapeutic values as antibacterial agents.

All types of extracts of these two plants showed varied antimicrobial efficacies against all the reference bacteria. The methanol extracts showed the best result followed by ethanol and aqueous extracts. Aqueous extracts showed less activity than ethanol and methanol extracts possibly because of the presence of similar active substances in aqueous extracts, in low concentrations or active substances were soluble in organic solvents and, therefore, not present in water extracts as also suggested by Sharma *et al*. 2011[31]. In general, the plant antibiotic substances appear to be more inhibitory to gram-positive organisms than gram-negative type. Unlike gram-positive bacteria, the lipopolysaccharide layer along with proteins and phosholipids are the major components in the outer surface of gram-negative bacteria[32-35]. The outer lipopolysaccharide layer hinders access of most compounds to the peptidoglycan layer of the cell wall. This explains the resistance of gram negative strains to the lytic action of most extracts.

The MIC of crude extracts of individual plants varies against different test strains. The relationship between zone of inhibition and MIC value may or may not be related. The crude extracts have mixture of phytoconstituents, which may influence the diffusion power of the active constituents. Several workers have made similar observations by using essential oils or complex mixture from higher plants[36-38]. For example some water-soluble compounds may have a higher diffusion power and lower antimicrobial activity[39].

Therefore, direct relationship of zone inhibition size with MIC value is expected with pure compounds not with crude extracts. On the other hand, these test strains may have different levels of intrinsic tolerance to antimicrobials and thus the MIC values differ from isolate to isolate. However, in such cases, the potency of crude extract on the basis of mean MIC values may be helpful in defining the relative potency of the extracts. The activity against both the types of bacteria may be indicative of the presence of broad spectrum antibiotic compounds or simply general metabolic toxins. Although this study investigated the in vitro antimicrobial activity, the results substantiate the ethnobotanical use of *O. gratissimum* and *A. Paniculata* for the treatment of bacteria related diseases.

Table No.1: Antibacterial activities of specific concentrations (30 mg/disc) of aqueous, ethanol and methanol extracts (arranged chronologically) of *Ocimum gratissimum* Linn and *Andrographis Paniculata* Nees plants compared with control (distilled water and DMSO) (mean±SE)

S.No	Solvents	Concentrations (mg/ml)	*Ocimum gratissimum*				*Andrographis Paniculata*			
			1	2	3	4	1	2	3	4
1	Aqueous	0[a]	+	+	+	+	+	+	+	+
		5	+	+	+	+	+	+	+	+
		10	+	+	+	+	+	+	+	+
		15	+	-	-	+	-	+	-	+
		20	-	-	-	-	-	-	-	-
		25	-	-	-	-	-	-	-	-
2	Ethanol	0[a]	+	+	+	+	+	+	+	+
		5	+	+	+	+	+	+	+	+
		10	+	-	-	+	+	+	-	-
		15	-	-	-	-	+	-	-	-
		20	-	-	-	-	-	-	-	-
		25	-	-	-	-	-	-	-	-
3	Methanol	0[a]	+	+	+	+	+	+	+	+
		5	+	+	+	+	+	+	+	+
		10	-	+	-	-	+	+	-	-
		15	-	-	-	-	+	-	-	-
		20	-	-	-	-	-	-	-	-
		25	-	-	-	-	-	-	-	-

Table No.2: Minimum Inhibitory Concentration of different extracts by dilution method

S.No	Plants	Extraction solvent	Antimicrobial activity			
			S. aureus	***E. coli***	***P. aeruginosa***	***P. vulgaris***
1	*Ocimum gratissimum*	Aqueous	12.36±0.78	10.13±0.58	11.85±0.58	8.35±0.64
		Ethanol	17.41±0.66	16.67±0.71	15.37±0.67	14.39±0.57
		Methanol	21.17±0.66	18.44±0.59	19.54±0.53	17.14±0.61
2	*Andrographis Paniculata*	Aqueous	10.95±0.58	7.06±0.71	8.03±0.78	10.5±0.88
		Ethanol	15.37±0.67	7.06±0.61	3.12±0.58	15.21±0.72
		Methanol	13.21±0.56	11.26±0.7	7.89±0.55	14.85±0.66
3	Distilled water		0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
4	DMSO		0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

CONCLUSION

All the bacterial strains were found to be sensitive against aqueous, ethanol and methanol extracts of these two medicinal plants but the ethanol and methanol extracts were comparatively more effective than aqueous extracts. It can be concluded that the extracts of these two medicinal plants possess antimicrobial activity against human pathogens. In conclusion, it is suggested that these medicinal plants may be used to discover natural bioactive products that might lead to the development of new drugs. On the basis of present work, a new drug therapy may be developed to fight against microbial infection mediated disorder.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

BIBLIOGRAPHY

1. Parekh J, Chandren S. *In vitro* antimicrobial activities of extracts of *Launaea procumbens* Roxb (Labiateae), *Afr J Biomed Res*, 9, 2006, 89-93.
2. Uniyal S K, Singh K N, Jomwal P *et al*. Traditional use of medicinal plants among the tribal communities of Chota Bengal, Western Himalaya, *J Ethnobiol Ethnomed*, 2, 2006, 1-14.
3. Elizabeth K M. Antimicrobial activity of *Terminalia bellerica, Indian J Clin Biochem*, 20, 2005, 150-153.
4. Sieradzki K, Wu S W, Thomasz A. Inactivation of the amphicillin resistance gene *mecA* in vancomycin-resistant *Staphylococcus aureus*, *Microbial Drug Resist*, 5(4), 1999, 253-257.
5. Saravanan R, Dhachinamoorthi D, Senthilkumar K *et al*. Antimicrobial activity of various extracts from various parts of *Calophyllum inophyllum L*, *J Appl Pharm Sci* 1(3), 2007, 102-106.
6. Gupta S K, Prakash J, Srivastava S. Validation of traditional claim of tulsi, *Ocimum sanctum Linn.* as a medicinal plant, *Ind J Exp Biol*, 40, 2002, 765-773.
7. Ilori M, Sheteolu A O, Omonibgehin E A *et al*. Antidiarrhoeal activities of *Ocimum gratissimum* (Lamiaceae), *J Diarrhoeal Dis Res*, 14, 1996, 283-285.
8. Onajobi F D. Smoth muscle contracting lipidicsoluble principles in chromatographic fractions of *Ocimum gratissimum, J Ethnopharmacol*, 18, 1986, 3-11.
9. Obaseiki-Ebor E E, Odukoya K, Telikepally H *et al*. Antimutagenic activity of extracts of leaves of four common edible vegetable plants in Nigeria (West Africa), *Mutat Res*, 302, 1993, 109-117.
10. Nakamura C V, Ueda-Nakamura T, Bando E *et al*. (1999) Antibacterial activity of *Ocimum gratissimum* L. essential oil, *Mem Inst Oswaldo Cruz*, 94, 1999, 675-678.
11. Orafidiya L O, Oyedele A O, Shittu A O *et al.* The formulation of an effective topical antibacterial product containing *Ocimum gratissimum* leaf essential oil, *Int. J. of Pharmaceutics*, 224, 2001, 177-183.
12. Nwosu M O, Okafor J I. Preliminary studies of the antifungal activities of some medical plants against Basidiobolus and some other pathogenic fungi, *Mycoses*, 38, 1995, 191-195.
13. Nakamura C V, Ishida K, Faccin L C *et al*. *In vitro* activity of essential oil from *Ocimum gratissimum* L. against four Candida species, *Research in Microbiol*, 155, 2004, 579-586.
14. Lemos J A, Passos X S, Ferna O F L *et al*. Antifungal activity from *Ocimum gratissimum L.* towards *Cryptococcus neoformans, Mem Inst Oswaldo Cruz, Rio de Janeiro*, 100, 2005, 55-58.
15. Sartoratto A, Machado A L M, Delarmelina C *et al*. Composition and antimicrobial activity of essential oils from aromatic plants used in Brazil, *Braz. J. of Microbiol*, 35, 2004, 275-280.
16. Pessoa L M, Morais S M, Bevilaqua C M L *et al.* Anthelmintic activity of essential oil of *Ocimum gratissimum* Linn. and eugenol against *Haemonchus contortus*, *Veterinary Parasitol*, 109, 2002, 59-63.

17. Silva M R, Oliveira J G Jr, Fernandes O F *et al*. Antifungal activity of *Ocimum gratissimum* towards dermatophytes, *Mycoses,* 48, 2005, 172-175.
18. Prakash J, Gupta S K, Singh N *et al*. Antiproliferative and chemo preventive activity of *Ocimum sanctum* Linn, *Int J Med Biol Environ,* 27, 1999, 165.
19. Nangia-Makker P, Tait L, Shekhar M P V *et al*. Inhibition of breast tumor growth and angiogenesis by a medicinal herb: *Ocimum gratissimum*, *Int J Cancer,* 121, 2007, 884-894.
20. Thiyagarajan P, Deepak H B, Agarwal A. *In vitro* modulation of LPS/calcimycin induced inflammatory and allergic mediators by pure compounds of *Andrographis paniculata* (King of bitters) extract, *Int. Immunopharmacol,* 11(1), 2001, 79-84.
21. Zaidan M R S, Noor R A, Badrul A R *et al. In vitro* screening of five local medicinal plants for antibacterial activity using disc diffusion method, *Trop. Biomed,* 22(2), 2005, 165-170.
22. Sukesh K, Shafi T T, Densingh J. Phytochemical investigation and antibacterial activity of *Gymnema sylvestre* and *Andrographis paniculata* from western ghats, *Int. J. of Phytomed.*, 3, 2011, 254-260.
23. Chakraborty S P, Kar Mahapatra S, Bal M *et al*. Isolation and identification of Vancomycin Resistant *Staphylococcus aureus* from post operative pus sample, *Al Ameen J. of Med. Sci*., 4(2), 2011, 152-168.
24. Dash S K, Chakraborty S P, Mandal D *et al.* Isolation and characterization of multi drug resistant uropathogenic *Escherichia coli* from urine sample of urinary tract infected patients, *Int. J. of Life Sci. and Pharma Res,* 2(1), 2012, L25-L39.
25. Singha P K, Roy S, Dey S. Protective activity of andrographolide and arabinogalactan proteins from *Andrographis paniculata* Nees. against ethanol-induced toxicity in mice, *J. of Ethnopharmacol.*, 111, 2007, 13-21.
26. Kar Mahapatra S, Chakraborty S P, Das S *et al*. Methanol extract of *Ocimum gratissimum* protects murine peritoneal macrophages from nicotine toxicity by decreasing free radical generation, lipid and protein damage and enhances antioxidant protection, *Oxidative Medicine and Cellular Longevity*, 2(4), 2009, 1-9.
27. Kar Mahapatra S, Chakraborty S P, Roy S. Aqueous extract of *Ocimum gratissimum* Linn and ascorbic acid ameliorate nicotine-induced cellular damage in murine peritoneal macrophage, *Asian Pac J of Trop Med,* 10, 2010, 775-782.
28. Perez C, Paul M, Bazerque P. An antibiotic assay by the agar-well diffusion method, *Acta Biol Med Exp,* 15, 1990, 113-115.
29. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disc susceptibility tests, *PA: NCCLS Publications,* 1993, M2-A5.
30. Paiva S R, Figueiredo M R, Aragao T V *et al*. Antimicrobial activity in vitro of plumbagin isolated from Plumbago species, *Mem Inst Oswaldo Cruz,* 98, 2003, 959-961.
31. Sharma C, Aneja K R, Kasera R. Screening of *Berberis aristata* D C. For antimicrobial potential against the pathogens causing ear infection, *Int J Pharmacol*, 7(4), 2011, 536-541.
32. Raja R D A, Jeeva S, Prakash J W *et al.* Antibacterial activity of selected ethnomedicinal plants from South India, *Asian Pac J Trop Med,* 4(5), 2011, 375-378.
33. Madhumitha G, Saral A M. Preliminary phytochemical analysis, antibacterial, antifungal and anticandidal activities of successive extracts of Cross and rain fund ibuliformis, *Asian Pac J Trop Med,* 4(3), 2011, 192-195.
34. Johnson M, Wesely E G, Kavitha M S *et al*. Antibacterial activity of leaves and inter-nodal callus extracts of *Mentha arvensis* L, *Asian Pac J Trop Med,* 4(3), 2011, 196-200.
35. Peixoto J R O, Silva G C, Costa R A *et al. In vitro* antibacterial effect of aqueous and ethanolic Moringa leaf extracts, *Asian Pac J Trop Med,* 4(3), 2011, 201-204.

36. Arjunan I, Gopinath D, Murthy N S. Role of informal care providers in home based long term care in diabetes mellitus at Kaiwara Primary Health Center area, Karnataka, India, *Asian Pac J Trop Dis*, 1(2), 2011, 127-130.
37. Tewari V, Tewari A, Bhardwaj N. Histological and histochemical changes in placenta of diabetic pregnant females and its comparision with normal placenta, *Asian Pac J Trop Dis*, 1(1), 2011, 1-4.
38. Idogun E S, Kasia B E. Assessment of microalbuminuria and glycated hemoglobin in type 2 diabetes mellitus complications, *Asian Pac J Trop Dis*, 1(3), 2011, 203-206.
39. Nang H L L, May C Y, Ngan M A *et al*. Extraction and identification of water soluble compounds in palm pressed fiber by SC-CO_2 and GC-MS, *Am J Environ Sci*, 3(2), 2007, 54-59.