

Research Article

In Vitro* Evaluation of Antimicrobial Activity of Crude Extract from Plants *Diospyros peregrina*, *Coccinia grandis* and *Swietenia macrophylla

**S Dewanjee¹, M Kundu¹, A Maiti¹, R Majumdar², A Majumdar²
SC Mandal*¹**

¹Pharmacognosy and Phytotherapy Research Laboratory, Division of Pharmacognosy, Department of Pharmaceutical Technology, Jadavpur University, Kolkata-700032, India.

²Department of Pharmaceutical science, Birla Institute of Technology, Mesra, Jharkhand, India.

Abstract

Purpose: The aim of the present study was to investigate antimicrobial activity of methanol extract of *Diospyros peregrina* fruits (MEDP), *Coccinia grandis* leaves (MECG) and *Swietenia macrophylla* barks (MESM).

Methods: MEDP, MECG and MESM were examined against some selective gram positive and gram negative bacterial (20) and fungal (4) strains. Preliminary antimicrobial activity was evaluated by agar disc diffusion method. Minimum inhibitory concentration was determined by tube dilution (MIC) whilst minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined by agar diffusion method.

Results: MEDP and MESM both have shown highest sensitivity against *Escherichia coli* strains. MEDP was found resistant to *Sarcina luteus* and *Bacillus* spp whereas MESM was resistant to all *Shigella* strains. MECG has shown major activity against *Staphylococcus aureus*, *Escherichia coli*, *Shigella dysenteriae*, *Shigella sonnei* and *Pseudomonas aeruginosa*; whilst resistant to *Shigella flexneri* and *Shigella boydii*. Against fungi strains extracts were found effective at higher concentrations. *Candida albicans* has shown highest sensitivity whilst *Penicillium* spp. was least effective to all three extracts.

Conclusion: The study confirms that MEDP, MECG, MESM all possess antimicrobial activity with different potency against variety of selected microorganisms. The differentiating activities of these three extracts encourage developing a novel broad spectrum antimicrobial herbal formulation in future.

Key words: *Diospyros peregrina*, *Coccinia grandis*, *Swietenia macrophylla*, Antimicrobial activity, Ciprofloxacin, Griseofulvin.

*Corresponding Author: Tel: 0091-33-24676316 (Residential), 0091-33-24146126 (Office). Fax: 0091-33-28371078.
E-mail: subhashmandal@yahoo.com

INTRODUCTION

In recent times, the rapid development of multi-resistant bacterial and fungal strains of clinically important pathogens fetches the interest of scientist to develop newer broad spectrum antimicrobial agents¹. The less availability and high cost of new generation antibiotics necessitates looking for the substances from alternative medicines with claimed antimicrobial activity. A number of herbs with significant antimicrobial activity have been reported in different traditional literatures^{2, 3, 4}. Now it is aimed to explore scientifically the antimicrobial potential of three traditional plants and substantiate the folklore claims.

Diospyros peregrina Gurke. (Ebenaceae) is a small middle sized tree of costal West Bengal. The fruits have ethnomedicinal significance for the treatment of diarrhoea, dysentery, cholera, ulcer of mouth and in wounds^{5, 6}. The fruits contain triterpenes, alkanes, flavonoids and tannins^{7, 8, 9, 10}. *Coccinia grandis* (L.) Voigt. (Family: Cucurbitaceae) is a climbing perennial herb distributed almost all over the world. The leaves of the plant possess antidiabetic, anti-inflammatory, antipyretic, analgesic, antispasmodic, antimicrobial, cathartic, expectorant activities^{11, 12}. The leaves contain triterpenoids, alkaloids and tannins¹³. The plant *Swietenia macrophylla* (Family: Meliaceae) is a large evergreen tree native to tropical America distributed almost all over the world. The barks of this plant possess anti-HIV, antimicrobial, antimalarial, and antitumor activities¹⁴. The barks contain triterpenoids, limonoids, flavonoids and tannins^{15, 16}. The objective of this research was to authenticate the antimicrobial sensitivity of the methanol extract of unripe matured fruits of *Diospyros peregrina*, *Coccinia grandis* leaves and *Swietenia macrophylla* bark and against some selected bacterial and fungal strains to lengthen the queue of antimicrobial herbs.

MATERIAL AND METHOD

Plant material

Matured unripe fruits of *Diospyros peregrina* (Family: Ebenaceae) were collected in the month of June from the villages of South 24 Parganas, West-Bengal, India; the leaves of *Coccinia*

grandis (L) Voigt. (Family: Cucurbitaceae) and barks of *Swietenia macrophylla* King. (Family: Meliaceae) were collected in the month of April, from the villages of Midnapore (E), West Bengal, India. The plants were authenticated by the Botanical Survey of India. Voucher specimens number entitled CHN/1-1(69), CNH/1-1 (44) and CNH/1-1(64) were deposited at our institute for future reference.

Preparation of methanol extract

The powdered plant materials (matured unripe fruits of *Diospyros peregrina*, leaves of *Coccinia grandis* and barks of *Swietenia macrophylla*) of 600 g each were extracted separately with methanol using Soxhlet apparatus. The resulting extracts were evaporated in vacuum and finally lyophilized into solid mass devoid of solvent (Yield = 8.75, 13.02 and 13.62 % respectively) and stored in desiccators for future use.

Preparation of sample

In the study of antimicrobial activity, extracts were dissolved in Dimethyl sulphoxide (DMSO). The corresponding concentration was expressed in term of μg of extract per ml of solvent ($\mu\text{g/ml}$).

Chemicals

All chemicals and solvents used in this experiment were of analytical grade obtained from BDH, Poole, UK.

Microorganisms

Twenty different bacterial strains namely *Staphylococcus aureus* 29737, *Staphylococcus aureus* ML 267, *Sarcina luteus* 9341, *Bacillus pumilus* 8241, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 10536, *Escherichia coli* VC Sonawave 3:37 C, *Escherichia coli* CD/99/1, *Escherichia coli* RP₄, *Escherichia coli* 18/9, *Escherichia coli* K88, *Shigella dysenteriae* 1, *Shigella sonnei* 1, *Shigella sonnei* BCH 217, *Shigella flexneri* type 6, *Shigella boydii* 937, *Pseudomonas aeruginosa* ATCC 25619, *Vibrio cholerae* 2, *Vibrio cholerae* 785, *Vibrio cholerae* 1037 and four different fungal strains namely *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 6275, *Penicillium notatum* ATCC 11625, *Penicillium funiculosum* NCTC 287 were collected from institute of microbial technology, Chandigarh, India. The bacterial strains were

Table 1: Preliminary antimicrobial activity of MEDP, MEGC and MESM

	<i>Zone of inhibition diameters in mm</i>			
	MEDP (200 µg/ml)	MEGC (200 µg/ml)	MESM (200 µg/ml)	Ciprofloxacin (200 µg/ml)
Gram positive bacteria				
<i>Staphylococcus aureus</i> 29737	10.10 ± 0.26	12.56 ± 0.18	8.63 ± 0.12	14.13 ± 0.07
<i>Staphylococcus aureus</i> ML 267	10.07 ± 0.20	13.20 ± 0.20	9.03 ± 0.17	13.53 ± 0.67
<i>Sarcina luteus</i> 9341	-	10.00 ± 0.20	8.03 ± 0.13	12.63 ± 0.12
<i>Bacillus pumilus</i> 8241	-	8.03 ± 0.12	8.07 ± 0.09	13.03 ± 0.12
<i>Bacillus subtilis</i> ATCC 6633	-	8.00 ± 0.17	7.57 ± 0.03	13.60 ± 0.10
Gram negative bacteria				
<i>Escherichia coli</i> ATCC 10536	10.53 ± 0.13	12.53 ± 0.15	9.60 ± 0.10	13.50 ± 0.10
<i>Escherichia coli</i> VC	10.57 ± 0.09	12.57 ± 0.23	9.76 ± 0.03	13.00 ± 0.10
Sonawave3:37 C				
<i>Escherichia coli</i> CD/99/1	12.20 ± 0.09	12.50 ± 0.20	10.13 ± 0.13	12.63 ± 0.70
<i>Escherichia coli</i> RP ₄	11.63 ± 0.03	12.00 ± 0.15	9.53 ± 0.90	12.13 ± 0.07
<i>Escherichia coli</i> 18/9	12.50 ± 0.15	11.67 ± 0.13	10.30 ± 0.10	13.00 ± 0.12
<i>Escherichia coli</i> K88	12.56 ± 0.09	11.60 ± 0.10	10.67 ± 0.07	14.06 ± 0.09
<i>Shigella dysenteriae</i> 1	8.57 ± 0.13	13.03 ± 0.17	-	15.63 ± 0.07
<i>Shigella sonnei</i> 1	8.03 ± 0.13	13.07 ± 0.17	-	15.07 ± 0.13
<i>Shigella sonnei</i> BCH 217	8.50 ± 0.10	12.60 ± 0.15	-	15.57 ± 0.09
<i>Shigella flexneri</i> type 6	8.13 ± 0.07	-	-	15.07 ± 0.12
<i>Shigella boydii</i> 937	7.57 ± 0.03	-	-	14.43 ± 0.13
<i>Pseudomonas aeruginosa</i> ATCC 25619	8.50 ± 0.10	14.10 ± 0.15	8.10 ± 0.12	16.07 ± 0.13
<i>Vibrio cholerae</i> 2	10.00 ± 0.12	10.03 ± 0.12	8.63 ± 0.12	14.03 ± 0.13
<i>Vibrio cholerae</i> 785	10.00 ± 0.21	10.43 ± 0.13	8.67 ± 0.13	14.60 ± 0.06
<i>Vibrio cholerae</i> 1037	10.06 ± 0.03	11.63 ± 0.12	8.06 ± 0.12	14.07 ± 0.13
	MEDP (2000 µg/ml)	MEGC (2000 µg/ml)	MESM (2000 µg/ml)	Griseofulvin (2000 µg/ml)
Fungal strains				
<i>Candida albicans</i> ATCC 10231	10.70 ± 0.06	16.50 ± 0.15	11.20 ± 0.10	18.2 ± 0.20
<i>Aspergillus niger</i> ATCC 6275	8.26 ± 0.12	11.97 ± 0.17	9.60 ± 0.10	14.03 ± 0.09
<i>Penicillium notatum</i> ATCC 11625	8.60 ± 0.10	9.03 ± 0.17	8.53 ± 0.07	11.10 ± 0.10
<i>Penicillium funiculosum</i> NCTC 287	7.33 ± 0.13	7.03 ± 0.09	7.63 ± 0.13	12.06 ± 0.06

Key: - : no measurable zone. Values are mean ± S.E.M. of 3 replications. MEDP – methanol extract of mature fruits of *Diospyros peregrina*, MEGC – methanol extract of the leaves of *Coccinia grandis*, MESM – methanol extract of the bark of *Swietenia macrophylla*.

grown in MacConkey agar plates at 37 °C and maintained on nutrient agar slants, while fungi were grown at 30 °C and maintained in Saboraud glucose agar slants.

Preliminary screening for antimicrobial activity The test was performed by disc diffusion assay as per NCCLS, 1993¹⁷. The nutrient agar plates containing an inoculum size of 10⁶ cfu / ml for bacteria and 2 × 10⁵ spores for fungi on Saboraud glucose agar plates, were used¹⁸. Previously prepared extract impregnated disc (6

mm in diameter) at the concentrations of 200 µg/ml for bacterial and 2000 µg/ml for fungal strains were placed aseptically on sensitivity plates with appropriate controls. Ciprofloxacin (200 µg/ml) and griseofulvin (2000 µg/ml) were used as standard antibacterial and antifungal antibiotics respectively. Plates were incubated at 37 °C for 24 hours for bacteria and 30 °C for 3 days for fungal spores¹⁹. Sensitivity was recorded by measuring the clean zone of growth inhibition on agar surface around the disc.

Table 2: Minimum inhibitory concentration of the three methanol extracts

Name of the organisms	MEDP ($\mu\text{g/ml}$)		MECG ($\mu\text{g/ml}$)		MESM ($\mu\text{g/ml}$)	
	MIC	MBC	MIC	MBC	MIC	MBC
Gram positive bacteria						
<i>Staphylococcus aureus</i> 29737	100	100	10	25	200	200
<i>Staphylococcus aureus</i> ML 267	100	150	10	25	200	200
<i>Sarcina luteus</i> 9341	> 200	> 200	100	150	200	200
<i>Bacillus pumilus</i> 8241	> 200	> 200	200	> 200	200	200
<i>Bacillus subtilis</i> ATCC 6633	> 200	> 200	200	> 200	200	200
Gram negative bacteria						
<i>Escherichia coli</i> ATCC 10536	25	25	10	25	50	50
<i>Escherichia coli</i> VC Sonawave 3:37 C	25	25	10	25	50	50
<i>Escherichia coli</i> CD/99/1	10	10	10	25	50	75
<i>Escherichia coli</i> RP ₄	10	10	10	25	50	75
<i>Escherichia coli</i> 18/9	10	10	25	50	50	75
<i>Escherichiacoli</i> K88	10	25	25	25	50	75
<i>Shigella dysenteriae</i> 1	200	200	10	10	> 200	> 200
<i>Shigella sonnei</i> 1	200	200	10	25	> 200	> 200
<i>Shigella sonnei</i> BCH 217	200	200	10	25	> 200	> 200
<i>Shigella flexneri</i> type 6	200	> 200	> 200	> 200	> 200	> 200
<i>Shigella boydii</i> 937	200	> 200	> 200	> 200	> 200	> 200
<i>Pseudomonas aeruginosa</i> ATCC 25619	200	200	10	10	200	> 200
<i>Vibrio cholerae</i> 2	100	150	100	150	200	200
<i>Vibrio cholerae</i> 785	100	150	100	150	200	> 200
<i>Vibrio cholerae</i> 1037	100	200	100	150	200	> 200
Fungal strains						
<i>Candida albicans</i> ATCC 10231	800	900	200	300	800	1000
<i>Aspergillus niger</i> ATCC 6275	1500	1800	800	1000	1000	1200
<i>Penicillium notatum</i> ATCC 11625	1500	1800	1500	1500	1500	2000
<i>Penicillium funiculosum</i> NCTC 287	1500	2000	1500	2000	1500	2000

Key: Mean values from three replicates are recorded, MIC – Minimum Inhibitory Concentration, MBC – Minimum Bactericidal Concentration, MFC – Minimum fungicidal Concentration

Determination of Minimum Inhibitory Concentration (MIC), and Minimum Bactericidal Concentration (MBC) and Minimum fungicidal Concentration (MFC) MIC was determined by tube dilution method for each of the test organism in triplicates²⁰. To 0.5 ml of varying concentrations of the extracts (0 – 200 $\mu\text{g/ml}$ for bacterial strains and 0 - 2000 $\mu\text{g/ml}$ for fungal strains), 2ml of nutrient broth was added and then a loopful of test organism previously diluted to 0.5 McFarland turbidity standard for (Bacterial

isolates) and 10^6 cfu/ml (for fungal strains) was introduced to the tubes. The procedures were repeated on the test organisms using standard antibiotics ciprofloxacin (for bacteria) and griseofulvin (for fungi). A tube containing nutrient broth only seeded with the test organisms was served as control. Tubes containing bacterial cultures were then incubated at 37 °C for 24 hours for bacteria and 30 °C for 3 days for fungal spores. After incubation the tubes were

examined for microbial growth by observing the turbidity.

To determine the MBC and MFC, for each set of test tubes in the MIC determination, a loopful of broth was collected from those tubes which did not show any growth and inoculated on sterile nutrient agar (for bacteria) and Saboraud glucose agar (for fungi) by streaking. Plates inoculated with bacteria and fungi were then incubated at 37 °C for 24 hours and 30 °C for 3 days respectively. After incubation the concentration at which no visible growth was seen was noted as MBC (for bacteria) and MFC (For fungi).

RESULTS

The *in vitro* antimicrobial activity of MEDP, MCEG and MESM were shown in table 1. The MEDP and MESM have shown maximum zone of inhibition against *Escherichia coli* K88 of 12.56 and 10.67 mm respectively whilst MCEG produced maximum zone diameter of 14.10 mm against *Pseudomonas aeruginosa* ATCC 25619. The activity of MEDP, MCEG and MESM among fungi strains was found highest with *Candida albicans* ATCC 10231 (10.7, 16.5 and 11.2 mm respectively) and lowest with *Penicillium funiculosum* (7.33, 7.03 and 7.63 mm respectively). In this preliminary antimicrobial assay ciprofloxacin (200 µg/ml), griseofulvin (2000 µg/ml) were taken as standard antibacterial and antifungal agents. The results of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were shown in table 2. The results showed that MEDP is highly sensitive against *Escherichia coli* strains (MIC and MBC 10 - 25 µg/ml), moderately sensitive (MIC 100 µg/ml and MBC 100 - 150 µg/ml) to *Staphylococcus aureus* and *Vibrio cholerae* strains, less sensitive (MIC 200 µg/ml) to *Shigella* spp. and *Pseudomonas aeruginosa* whilst resistant (MIC and MBC > 200 µg/ml) to *Sarcina luteus* and *Bacillus* spp. MCEG has shown maximum activity against gram-positive organism including *Staphylococcus aureus* (MIC 10 µg/ml and MBC 25 µg/ml) and gram negative cultures including *Escherichia coli* (MIC 10 - 25 µg/ml and MBC 25 - 50 µg/ml), *Shigella dysenteriae* (MIC and MBC

10 µg/ml), *Shigella sonnei* (MIC 10 µg/ml and MBC 25 µg/ml) and *Pseudomonas aeruginosa* (MIC and MBC 10 µg/ml); moderately sensitive (MIC 100 µg/ml and MBC 150 µg/ml) to *Vibrio cholerae*, *Sarcina luteus*, less sensitive (MIC 200 µg/ml and MBC > 200 µg/ml) to *Bacillus* spp., whilst resistant (MIC and MBC > 200 µg/ml) to *Shigella flexneri* and *Shigella boydii*. MESM was found maximum sensitive (MIC 50 µg/ml and MBC 50 - 75 µg/ml) to *Escherichia coli* strains; less sensitive (MIC 200 µg/ml) to *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Sarcina luteus* and *Bacillus* spp. and resistant (MIC and MBC > 200 µg/ml) to all *Shigella* spp. Against fungi strains all extracts were found effective at higher concentrations. *Candida albicans* has shown highest sensitivity with MIC values of 800, 200, 800 µg/ml and MFC values of 900, 300, 1000 µg/ml with MEDP, MCEG and MESM respectively whilst *Penicillium* spp. were found least effective with MIC and MFC values of 1500 µg/ml and 2000 µg/ml respectively with all three extracts.

DISCUSSION

The antimicrobial activities of various plants have been reported by many researchers^{21, 22}. Phytoconstituents present in plants namely flavonoids, alkaloids, tannins and triterpenoids are producing exciting opportunity for the expansion of modern chemotherapies against wide range of microorganisms^{23, 24}. In present study a variety of gram positive, gram negative bacteria and fungal stains were selected for the screening of antimicrobial effect of three selected plant extracts to perceive the antimicrobial spectrum as well to authenticate ethnomedicinal claims. The results of this study showed that the MEDP, MCEG and MESM have varied antimicrobial activities against the tested organisms. Among these three extracts MCEG was found most effective against selected strains followed by MEDP and MESM in order effectiveness. Thus in search of novel broad spectrum antimicrobial agent, the formulation comprising different proportions of these extracts may be proven good. This study has not only shown the scientific basis for some of the therapeutic uses of traditional

plants, but also confirmed the ethnomedicinal claims for the selected plants.

CONCLUSION

In conclusion, the results of this investigation revealed that methanol extracts of all three plants possess differentiating antimicrobial activity against selected bacterial and fungal strains. The differentiating activities against variety of microorganisms of these three extracts encourage developing a novel broad spectrum antimicrobial formulation in future. Now our research will be directed to develop a broad spectrum antimicrobial herbal formulation with these plants.

ACKNOWLEDGEMENTS

The authors are thankful to All India Council for Technical Education, New Delhi, India for financial assistance.

REFERENCES

- Weisser R, Asscher AW, Winpenny J. *In vitro* reversal of antibiotic resistance by DTA. *Nature* 1966; 219: 1365-1366
- Balandrin MF, Klocke JA, Wutule ES, Bollinger WH. *Natural plant chemicals: Sources of industrial and medicinal materials*. *Science* 1985; 228: 1154-1160.
- Satish S, Raveesha KA, Janardhana GR. Antibacterial activity of plant extracts of phytopathogenic *Xanthomonas campestris* pathovars. *Lett Appl Microbiol* 1999; 28: 145-147.
- Jones FA. *Herbs-useful plants. Their role in history and today*. *Eur J Gastroenterology Hepatol* 1996; 8: 1227-1231.
- Anjaria J, Parabia M, Bhatt G, Khamar R. *A Glossary of selected indigenous medicinal plants of India, 2nd Ed, SRISTI Innovations, Ahmedabad, India, 2002; pp 26.*
- Kirtikar KR, Basu BD. *Indian Medicinal Plants. Reprint edition, Vol-2, Bishen Singh Mahendra Pal Singh, Deharadun, 1975; pp. 1502-1504.*
- Chopra RN, Nayar SL, Chopra IC. *Glossary of Indian Medicinal Plants. (3rd reprint). CSIR, New Delhi, 1992; pp. 99.*
- Chauhan JS, Saraswat M, Kumari G. 1982. Structure of a new flavanone glycoside from *Doiospyros peregrina* roots. *Ind J Chem* 21: 169-170.
- Jain N, Yadav RN. 1994. Peregrinol, a lupane type triterpene from the fruits of *Diospyros peregrina*. *Phytochemistry* 35: 1070-1072.
- Misra PS, Misra G, Nigam SK, Mitra CR. 1971. Constituents of *Diospyros peregrina* fruit and seed. *Phytochemistry* 10: 904-905.
- Asolkar LV, Kakkar KK, Chakre OJ. *Second supplement to Glossary of Indian Medicinal Plants with Active Principles Part-I (A-K). Council of Scientific and Industrial Research (PID) (Part- I), New Delhi, 1992; pp. 217-218.*
- Nadkarni KM, Nadkarni AK. *Indian Materia Medica 3rd Ed. (Vol-I), Popular Prakashan Pvt. Ltd., Mumbai, 1992; pp. 300-302.*
- Rastogi RP, Mehrotra BN. *Compendium of Indian Medicinal Plants. (Vol-I), New Delhi, 1990; pp. 115.*
- Munoz V, Sauvain M, Bourdy G, Callapa J, Rojas I, Vargas L, Tae A, Deharo E. *The search for natural bioactive compounds through a multidisciplinary approach in Bolivia. Part II. Antimalarial activity of some plants used by Mosetene indians. J Ethnopharmacol* 2000; 69(2):139-155.
- Guha Sircar SSG, Chakraborty T. *Tetranortriterpenoid from Swietenia macrophylla. J Ind Chem Soc* 1951; 28: 207.
- Mootoo BS, Allisha A, Motilal R, Pingal R, Ramlal A, Khan A, Reynolds WF, McLean S. *Limonoids from Swietenia macrophylla and S. aubrevilleana. J Nat Prod* 1999; 62(11): 1514-1517.
- National Committee for Clinical Laboratory Standards (NCCLS), 3rd Ed. approved standard M7-A3, NCCLS, Villanova, PA, 1993.
- Mandal SC, Nandy A, Pal MP, Saha BP. *Evaluation of antimicrobial activity of Asperagus recemosus Willd. root. Phyto Res* 2000; 14: 118-119.
- Mandal SC, Majumdar A, Majumdar R. *Antibacterial activity of Litsea glutinosa bark. Fitoterapia* 2000; 71: 439.
- Doughari JH. *Antimicrobial activity of Tamarindus indica Linn. Trop J Pharm Res* 2006; 5(2): 597-603.
- Cowan MM. *Plant products as antimicrobial agents. Clin Microbiol Rev* 1999; 22: 564-582.
- Shariff ZU. *Modern Herbal Therapy for common Ailments. Nature Pharmacy Series (Volume 1), Spectrum Books Limited, Ibadan, Nigeria in Association with Safari Books (Export) Limited, United Kingdom, 2001; pp 9-88.*
- Lutterodt GD, Ismail A, Basheer RH, Baharudin HM. *Antimicrobial effects of Psidium guajava extracts as one mechanism of its antiarrhoeal action. Malaysian J Med Sci* 1999; 6 (2):17-20.
- Marjorie MC. *Plant products as antimicrobial agents. Clin Microbiol Rev* 1999; 12(4): 564-582.