International Journal of ChemTech Research
CODEN (USA): IJCRGG, ISSN: 0974-4290, ISSN(Online):2455-9555
Vol.10 No.7, pp 324-329, 2017

Anti-tubercular Evaluation of *Balanites roxburghiana* Linn. Fruit Extracts Against *Mycobacterium tuberculosis* H37Rv Strain

S. P. Soul Shekhar, N. D. Satyanarayan*, Wadhah A. Al-baadani.

Department of Pharmaceutical Chemistry, Kuvempu University, Post Graduate Centre, Kadur-577548, Chikmagalur Dt. Karnataka State, India.

Abstract : The present investigation is to observe the antitubercular activity of different solvent extracts (n-hexane, dichloromethane and methanol) of *Balanites roxburghiana* fruit against *Mycobacterium tuberculosis*H37Rv strain. The antitubercular activity is carried outusing microplate alamar blue assay (MABA) at concentrations of 0.8 µg/ml to 100 µg/ml.The result reveals thatanti-tubercular activity of *B. roxburghiana*n-hexane extract has shown sensitivity at 25µg/ml concentration when compared with the standard pyrazinamide (3.125µg/ml). The other samples viz dichloromethane and methanol extracts have shown less sensitivity compared to n-hexane extract. The anti-tubercular activity might be due to the phytochemicals present in the extracts of *B. roxburghiana* which inhibit the cell wall synthesis or the enzyme responsible for synthesis of cell wall of *M. tuberculosis.*

Keywords : *Mycobacterium tuberculosis, Balanites roxburghiana,* Antitubercular, Pyrazinamide, MABA, H37Rv.

Introduction

Tuberculosis (TB) is a chronic infectious disease caused primarily to lungs by *Mycobacterium tuberculosis*, a leading cause of death worldwide and produces serious health complications[1, 2]. In 2016 World Health Organization (WHO) estimated that TB causes unhealthiness among millions of people every year[3]. Out of 10.4 million new TB cases reported, 5.9 million are men, 3.5 million are women and 1.0 million are children. The quantity of TB deaths can be reduced with a timely diagnosis and correct treatment[3]. Moreover, delay in the treatment give rise to multidrug resistant tuberculosis (MDR-TB), which does not react to first-line drugs. MDR-TB strains are resistant to the regularly used antitubercular drugs like isoniazid and rifampicin[4]. Recently about 5% of new cases of tuberculosis are due to MDR strains, with more than half from China and India. It was also estimated that there are 50,000 cases of extensively drug-resistant tuberculosis (XDR-TB), which does not react to second-line drugs[5].Thus, there is an urgent need in search for new anti-tuberculosis agents, which are safe, effective and affordable. Medicinal plants have always offered a great hope to fulfill these needs, because they are proven template for the development of new drugs[6, 7]. Only a few plant species have been entirely investigated for their medicinal properties[8]. India is one of the countries with unique wealth of medicinal plants and vast traditional knowledge of use of herbal medicine for curing several diseases[9, 10]. Literature survey revealed that only few plants have been tested against *mycobacteria* and few showed anti -TB activity such as *Salvia hypargeia, Euclea natalensis, etc.* In '*Ayurvedic Formulary of India*', less number of medicinal plantshas been reported to be used in different Ayurvedic formulations for TB[11-14].

B. roxburghiana belongs to family Zygophyllaceae; it is furthermore called as Desert date in English. In dry land of Africa and South Asia this plant is treated to be most common but neglected wild species[15]. It is multibranched, thorny shrub or tree grows up to l0 m tall. Crown is spherical, in one or several distinct masses. Stem is short and often branching from near the base. Bark is dark to brown grey, deeply fissured. Branches armed with yellow or green thorns of up to 8 cm long. Leaves are with two separate leaflets; leaflets are obovate, asymmetric, 2.5 to 6 cm long, bright green, leathery, with fine hairs when young. Flowers are fragrant, yellowish green growing in fascicles of the leaf axils. Fruit is rather long, narrow drupe, 2.5 to 7 cm long, 1.5 to 4 cm in diameter. Immature fruits are green and tormentose, turning yellow and glabrous when mature. Pulp of the fruit is bitter-sweet to taste and edible. Seed is the pyrene (stone), measures as 1.5 to 3 cm long, light brown, fibrous, and tremendously hard[16]. The whole plant has been investigated for different pharmacological activities such as cardioprotective[17],anthelmintic[18-23],antibacterial[24-27], antivenin[28],anticancer[29-32], anti-inflammatory, analgesic[33],*in vitro* antioxidant, xanthine oxidase, acetyl cholinesterase inhibitory[34], anti-inflammatory, antinociceptive and antioxidant[35] mosquito larvicidal[36-39], hepatoprotective[40-42], antiviral[43], wound healing[44], hypocholesterolemic[45], diuretic[46], aldose reductase inhibitory[47], antidiabetic effect in streptozotocin-induced diabetic mice[48],inhibit *Escherichia coli* growth in rats[49], lowering the glucose level in alloxan-induced diabetic rats[50], reported as hypoglycemic agent[51] and also as hyperglycemic agent[52], amylase inhibitory activity[53] and antifertility[54].In view of the above facts, the source have revealed that *B. roxburghiana* is one of the medicinally important plant from ancient history and possessmany pharmacological activities.Even though the plant is extensively investigated for wide range of preliminary pharmacological investigation so far no reports nave revealed of its usage as an potential source of anti-TB. Hence, the present study was aimed to determine antitubercular activity by visual MABA method employing *M. tuberculosis*H37Rv strain.

Experimental

Chemicals

The chemicals used were of analytical grade. Diastase, Alamar blue reagent, Tween-80 and other chemicals used for the study are purchased from (HiMedia, Mumbai, India).

Collection of plant material

The fruits of *B. roxburghiana* were collected in the months of February to March-2014 around Kadur town of Chikmagalur District, Karnataka state, India and was authenticated with voucher specimen no KUYLK4410 at the Herbarium, Department of Botany, Kuvempu University, Shankaraghatta, Shimoga Dist. Karnataka State. India. The collected fruits were immediately sprayed with alcohol to cease the enzymatic degradation of secondary metabolites. The fruits were stored in cool, dry place before extraction.

Preparation of extract

The shade dried fruits (80 g) of *B. roxburghiana*was chopped into small fragments of 1-2 inches in length and extracted with different solvent viz n-hexane, dichloromethane and methanol successively in a soxhlet extractor for about 72 h each. The solvent was evaporated under reduced pressure and controlled temperature using a buchi evaporator. The solvent evaporated mass of n-hexane extract (0.68 g), dichloromethane extract (0.76 g) and methanol extract (10.52 g) respectively. The extract were stored in a freezer (- 4^{o}C) until further use.

Antitubercular activity by MABA methods

The antitubercular activity of the extracts was carried out with *M. tuberculosis*H37Rv strain, by microplate alamar blue assay (MABA) method. The advent of visual MABA method has facilitated the simplistic screening of extracts for antitubercular activity making use of a thermally stable and nontoxic reagent. In comparison with the BACTEC and fluorometric MABA methods, visual MABA is an inexpensive, alternative and providing identical and rapid results without the use of specialized equipment. In addition to the above mentioned merits, visual MABA method was adopted for the screening of test extracts. The minimum inhibitory concentration (MIC) was defined as the lowest drug concentration that prevented a colour change from blue (no growth) to pink (growth). The pyrazinamide drug was used as positive standard for comparison. Theprocedure involves by taking 200 µl of sterile deionized water and was introduced into all outer perimeter

wells of sterile 96 well plates to avoid evaporation of medium in the test wells during incubation. The 96 well plate received 100 µl of the Middle brook 7H9 broth and serial dilution of compounds were made directly on plate.The final drug concentrations tested were of 100 to 0.2 µg/ml.Plates were covered and sealed with parafilm and incubated at 37 ºC for five days. After incubation, 25 µl of freshly prepared 1:1 mixture of Alamar blue reagent and 10 % Tween-80 was added to the plate and incubated for 24 h. After 24 h the change in colour was observed[55].

Results

Anti tubercular activity of crude extracts

The result for antitubercular activity by different extract of *B. roxburghiana* fruit on *M. tuberculosis*H37Rv strain exhibit sensitivity of25 µg/mlby n-hexane extract, 50 µg/ml by dichloromethane and methanol extracts when compared with pyrazinamide as standard 3.125 µg/ml concentration. The testing was carried out by visual MABA method as shown in Table l.

Table l: MIC values of various extracts of *B. roxburghiana* fruit.

Sl. No.	ples	Concentration in µg/ml							
		100	**50**	**25**	**12.5**	**6.25**	**3.12**	**1.6**	**0.8**
1	BRH	S	S	S	R	R	R	R	R
2	BRD	S	S	R	R	R	R	R	R
3	BRM	S	S	R	R	R	R	R	R
4	STD	S	S	S	S	S	S	R	R

B. roxburghiana n-hexane extract (BRH), *B. roxburghiana* dichloromethane extract (BRD), *B. roxburghiana* methanol extract (BRM),Pyrazinamide as standard (STD), Resistivity (R),

Sensitivity (S).

*B. roxburghiana*n-hexane extract (BRH), *B. roxburghiana* dichloromethane extract (BRD), *B. roxburghiana* methanol extract (BRM).

Fig. 1: Showing the anti TB result of BRH - *B. roxburghiana* n-hexane extract, BRD - *B. roxburghiana* dichloromethane extract, BRM – *B. roxburghiana* methanol extract on H37Rv strain.

Discussion

The current study is to assess the anti-tubercular potential of different solvent extracts of *B. roxburghiana*fruit in which n-hexane extract (BRH) has shown sensitivity at 25 µg/ml concentration when compared with standard pyrazinamide at 3.125 µg/ml while dichloromethane (BRD) and methanol(BRM) extractshas shown sensitivity at 50 µg/ml concentration each as shown in the Table land Fig 1.The above activity may be due to the phytochemicals present in the extract viz saponins, flavonoids and steroids. The phytochemicals have potential to inhibit *M. tuberculosis* consists of both bacterial and fungal characteristics. In BRH the sensitivity might be because of the presence of steroids, which have the capability to inhibit *M. tuberculosis*[56]. The steroids may enter the cell membrane to react with receptor proteins in the cytoplasm to form a steroid-receptor complex. The complex moves into the nucleus, where it binds to DNA then changes the transcription of messenger RNAacts as template for protein synthesis, these steroids can either stimulate or

inhibit the synthesis of specific proteinsin *mycobacterium tuberculosis*[57]. The BRD and BRM extracts exhibited the activity which may be due to the presence of saponins and flavonoids. Polyphenols effect on microbial metabolism and growth, based on concentration of active compounds[58].Flavonoids show activity by damaging cytoplasmic membrane with the generation of hydrogen peroxide, inhibition of nucleic acid synthesis and inhibition of ATP synthase[59]. Hence the activity of different solvent extracts might be due to the type of phytochemicals present in them respectively.

Conclusion

The present investigation conclude that the n-hexane crude extract of*B. Roxburghiana* fruit have shown better anti-tubercular activity when compared with dichloromethane and methanol extracts at various concentration, which might be due to the presence of phytochemicals in the extracts. Hence, BRH extract can be considered for further fractionation and isolating the active component responsible for anti-tubercular activity.

Acknowledgement

The authors are thankful to the authorities of Kuvempu University, for providing necessary facilities to carry out the present work and Dept of Botany Kuvempu University for authentication of plant material and one of the authors S. P. Soul Shekhar is thankful to OBC Cell, Kuvempu University for awarding OBC fellowship initially. The authors also acknowledge Dr. G R Bhat, Department of Microbiology, Maratha Mandal Dental Collage, Belagavi, Karnataka for anti-TB activity.

Conflict of Interest

The authors confirm that there is no conflict of interest.

References

1. Jordao L, Vieira OV. Tuberculosis: new aspects of an old disease.Int J Cell Biol.,2011,2011;1-13.
2. Thaiss WM, Thaiss CC, Thaiss CA. Recent developments in the epidemiology and management of tuberculosis - new solutions to old problems.Infect Drug Resist., 2012,5;1-8.
3. Mario R.Global tuberculosis report 2016, World Health Organization. (http://www.who.int /tb/publications/ global_report/en/).
4. Lawn SD, Zumla AI. Tuberculosis. Lancet., 2011, 378; 57-72.
5. Jimenez AA, Meckes M, Ramirez R, Torres J, Luna HJ. Activity against multidrug resistant *Mycobacterium tuberculosis* in Mexican plants used to treat respiratory diseases.Phytother Res.,2003, 17; 903-908.
6. David JN, Gordon MC, Kenneth MS. The influence of natural products upon drug discovery.Nat Prod Rep., 2000, 17; 215-234.
7. David JN, Gordon MC, Kenneth MS. Natural products as sources of new drugs over the period 1981-2002. J Nat Prod., 2003, 66; 1022-1037.
8. Michael H, Simon G. Ethnopharmacology in drug discovery: an analysis of its role and potential contribution. J Pharm Pharmacol., 2001, 53; 425-432.
9. John MG, Noel JC. Activity of bromhexine and ambroxol, semi-synthetic derivatives of vasicine from the Indian shrub *Adhatoda vasica*, against *Mycobacterium tuberculosis* in vitro.J Ethnopharmacol.,1996, 50; 49-53.
10. Gupta KC, Chopra IC.Anti-tubercular action of *Adhatoda vasica* (N. O. Aganthacea). Indian JMed Res., 1954, 42; 355-358.
11. Raju G, Arvind S, Sanjay MJ. Indian medicinal plants as a source of antimycobacterial agents. J Ethnopharmacol.,2007, 110; 200-234.
12. Sandra MN, Clara L, Colin WW. A review of antimycobacterial natural products. Phytother Res., 2000, 14; 303-322.
13. Ayhan U, Namik E, Ertan T, Candan J. Diterpenoids from the root of *Salvia hypergeia*.J Nat Prod.,1988, 51; 1178-1183.

14. Lall N, Meyer JJ.Inhibition of drug-sensitive and drug-resistant strains of *Mycobacterium tuberculosis* by diospyrin, isolated from *Euclea natalensis*. J Ethnopharmacol., 2001, 78; 213-216.
15. Hall JB, Waljer DH. *Balanites aegyptiaca* Del. A monograph. School of agricultural and forest science. University of Wales. Banger; 1991.
16. Daya L Chothani, Vaghasiya HU.A review on *Balanites aegyptiaca* Del (desert date): phytochemical constituents, traditional uses, and pharmacological activity. Pharmacogn.,2011, 5; 55-62.
17. El Mastry SM, Ebeed MM, El Sayed IH, Nasr MY, El Halafawy KA. Protective effect of *Balanites aegyptiaca* on antioxidant defense system against adriamycin induced cardiac toxicity in expermental mice. Egypt J Biochem Mol Biol., 2010, 28; 1.
18. Dwivedi A, Joshi V, Barpete PK, Akhtar AK, Kaur A, Kumar S. Anthelmintic activity of root bark of *Balanites aegyptiaca* (L) Del. Ethnobot Leaflets., 2009, 13; 564-567.
19. Koko WS, Galal M, Khalid HS. Fasciolicidal efficacy of *Albizia anthelmintica* and *Balanites aegyptiaca* compared with albendazole. J Ethnopharmacol.,2000, 71; 247-252.
20. Koko WS, Abdalla HS, Galal M, Khalid HS. Evaluation of oral therapy on mansomal schistosomiasis using single dose of *Balanites aegyptiaca* fruits and praziquantel. Fitoter., 2005, 76; 30-34.
21. Gnoula C, Guissou P, Duez P, Frederich M, Dubois J. Nematocidal compounds from the seeds of *Balanites aegyptiaca* isolation and structure elucidation. Int J Pharmacol., 2007, 3; 280-284.
22. Shalaby MA, Moghazy FM, Shalaby HA, Nasr SM. Effect of methanolic extract of *Balanites aegyptiaca* fruits on enteral and parenteral stages of *Trichinella spiralis* in rats. Parasitol Res., 2010, 107; 17-25.
23. Anto F, Aryeetey ME, Anyorigiya T, Asoala V, Kpikpi J. The relative susceptibilities of juvenile and adult *Bulinus globosus* and *Bulinus truncatus* to the molluscicidal activities in the fruit of *Ghanaian Blighia sapida, Blighia unijugata* and *Balanites aegyptiaca*. Ann Trop Med Parasitol., 2005, 99; 211-217.
24. Doughari JH, Pukuma MS, De N. Antibacterial effects of *Balanites aegyptiaca* L. Drel. and *Moringa oleifera* Lam. on *Salmonella typhi*. Afr J Biotechnol., 2007, 6; 2212-2215.
25. Karuppusamy S, Rajasekharan KM, Karmegam N. Antibacterial activity of *Balanites aegyptiaca* (L). Del. J Ecotoxicol Environ Sci Monit., 2002, 12; 67-68.
26. Karmegam N, Karuppusamy S, Mani Prakash M, Jayakumar M, Rajasekar K. Antibacterial potency and synergistic effect of certain plant extracts against food-borne diarrheagenic bacteria. Int J Biomed Pharma Sci., 2008, 2; 88-93.
27. Anani K, Adjrah Y, Ameyapoh Y, Karou SD, Agbonon A, de Souza C et al. Effects of hydroethanolic extracts of *Balanites aegyptiaca* (L) Delile (Balanitaceae) on some resistant pathogens bacteria isolated from wounds. J Ethnopharmacol., 2015, 164; 16-21.
28. Wufen BM, Adamu HM, Cham YA, Kela SL. Preliminary studies on the antivenin potential and phytochemical analysis of the crude extracts of *Balanites aegyptica* (Linn.) Delile on albino rats. Nat Prod Radiance., 2007, 6; 18-21.
29. Gnoula C, Mégalizzi V, De Nève N, Sauvage S, Ribaucour F, Guissou P. Balanitin-6 and -7: Diosgenyl saponins isolated from *Balanites aegyptiaca* Del. display significant anti-tumor activity *in vitro* and *in vivo*. Int J Oncol., 2008, 32; 5-15.
30. Pettit GR, Doubek DL, Herald DL. Isolation and structure of cytostatic steroidal saponins from the african medicinal plant *Balanites aeqyptiaca*. J Nat Prod., 1991, 54; 1491-1502.
31. Issa NM, Mansour FK, El-Safti FA, Nooh HZ, El-Sayed IH. Effect of *Balanites aeqyptiaca* on ehrlich ascitic carcinoma growth and metastasisin swiss mice.Exp Toxicol Pathol., 2015, 67; 435-441.
32. Samir AMZ, Ezzat IAE, Ramadan MA, Sayed B, Ahmed BMM. Anticarcinogenic activity of methanolic extract of *Balanites aegyptiaca* against breast, colon, and liver cancer cells. Int J Adv Res., 2015, 3; 255-266.
33. Gaur K, Nema RK, Kori ML, Sharma CS, Singh V. Anti-inflammatory and analgesic activity of *Balanites aegyptiaca* in experimental animal models. Int J Green Pharm., 2008, 2; 214-217.
34. Meda NT, Lamien-Meda A, Kiendrebeogo M, Lamien CE, Coulibaly AY, Millogo-Rasolodimby J. In vitro antioxidant, xanthine oxidase and acetylcholinesterase inhibitory activities of *Balanites aegyptiaca* (L.) Del. (Balanitaceae). Pak J Biol Sci., 2010, 13; 362-368.
35. Speroni E, Cervellati R, Innocenti G, Costa S, Guerra MC, Dall Acqua S. Anti- inflammatory, anti-nociceptive and antioxidant activities of *Balanites aegyptiaca* (L.) Delile. J Ethnopharmacol., 2005, 98; 117-125.

36. Zarroug IM, Nugud AD, Bashir AK, Mageed AA. *Balanites aegyptiaca* as a mosquito larvicide. Pharma Biol., 1990, 28; 267-271.
37. Wiesman Z, Chapagain BP. Larvacidal activity of saponin containing extracts and fractions of fruits mesocarp of *Balanites aegyptiaca*. Fitoter., 2006, 77; 420-424.
38. Wiesman Z, Chapagain BP. Laboratory evaluation of natural saponin as a bioactive agent against *Aedes aegypti* and *Culex pipiens*. Dengue Bull., 2003, 27; 168-173.
39. Chapagain BP, Saharan V, Wiesman Z. Larvicidal activity of saponins from *Balanites aegyptiaca* callus against *Aedes aegypti* mosquito. Bioresour Technol., 2008, 99; 1165-1168.
40. Mohamed AH, Eltahir KE, Ali MB, Galal M, Ayeed IA, Adam SI. Some pharmacological and toxicological studies on *Balanites aegyptiaca* bark. Phytother Res.,1999, 13; 439-441.
41. Mayba Gnana ST, Parthipan B, Kingston C, Mohan VR, Tresina SP. Hepatoprotective and antioxidant effect of *Balanites aegyptiaca* (l.) Del against CCl_4 induced hepatotoxicity in rats. Int J Pharm Sci Res., 2011, 2; 887-892.
42. Jaiprakash B, Aland R, Karadi RV, Savadi RV, Hukkeri VI. Hepatoprotective activity of fruit pulp of *Balanites aegyptiaca*. Indian Drugs., 2003, 40; 296-297.
43. Hamid OA, Wahab ME, Abdu ZZ, Idris SM. *Balanites aegyptiaca* extracts for treatment of HIV/AIDS and leukemia. Patentscope, WO2001049306 A1, Jul 12, 2001.
44. Annan K, Dickson R. Evaluation of wound healing actions of *Hoslundia opposita* vahl, *Anthocleista nobilis* G. Don and *Balanites aegyptiaca* L. J Sci Technol., 2008, 28; 26-33.
45. Abdel-Rahim EA, El-Saadany SS, Wasif MM. Biochemical dynamics of hypocholesterolemic action of *Balanites aegyptiaca* fruit. Food Chem., 1986, 20; 69-78.
46. Wani NS, Kabade JB, Kabade MV, Joshi SM, Patil AD. Diuretic activity of leaves of *Balanites roxburghii* Linn. Int J Pharma Res Dev., 2010, 2; 4.
47. Amira AM, Heshma EA, Sara C, Olaf K, Basma S, Sharif S et al. Aldose reductase inhibition of a saponin-rich fraction and new furostanol saponin derivatives from *Balanites aegyptiaca*. Phytomed., 2015, 22; 829-836.
48. Mansour HA, Newairy AA. Amelioration of impaired renal function associated with diabetes by *Balanites aegyptiaca* fruits in streptozotocin-induced diabetic rats. J Med Res Inst., 2000, 21; 115-125.
49. George DH, Ali HK, El Abbas OA. Evaluation of the biological activity of *Balanites aegyptiaca* Del Saponin in the control of type 2 diabetes mellitus on rats and the growth of *Escherichia coli*. Ahfad J., 2006, 23; 1.
50. Thirupathi K, Krishna DR, Krishna Mohan G. Antidiabetic effect of kernals of *Balanites roxburghii* in normal and alloxan-induced diabetic rats. Iran J Pharma Ther., 2013, 12; 42-45.
51. El-Saadany SS, Abdel-Rahim EA, Wasif MM. Biochemical action of *Balanites aegyptiaca* fruits as a possible hypoglycemic agent. Food Chem., 1986, 19; 307-315.
52. Al-Malki AL, Barbour EK, Abulnaja KO, Moselhy SS. Management of hyperglycaemia by ethyl acetate extract of *Balanites aegyptiaca* (Desert Date).Molecules., 2015, 20; 14425-14434.
53. Ingrid F, Matthias FM.Traditionally used plants in diabetes therapy - phytotherapeutics as inhibitors of α-amylase activity. Braz J Pharmacogn., 2006, 16; 1-5.
54. Padmashali B, Vaidya VP , Vagdevi HM, Satyanarayana ND. Antifertility efficacy of the plant *Balanites roxburghii* (Balaniteceae) in female rats. Indian J Pharm Sci., 2006, 68; 347-351.
55. Maria CSL, Marcus VND, Alessandra CP, Marcelle de LF, Rasnisb BG, Thais CMN, et al. Evaluation of anti-tubercular activity of nicotinic and isoniazid analogues**.**Arkivoc.,2007, 15; 181-191.
56. Olugbuyiro JAO, Moody JO, Hamann MT. Phytosterols from *Spondias mombin* Linn with antimycobacterial activities. Afr J Biomed Res., 2013, 16; 19–24.
57. Kragballe K. Topical corticosteroids: mechanisms of action. Acta Derm Venereol Suppl., 1989, 151; 7-10.
58. Alberto MR, Farías ME, Manca de Nadra MC. Effect of gallic acid and catechin on Lactobacillus hilgardii 5w growth and metabolism of organic compounds. J Agric Food Chem., 2001, 49; 4359-4363.
59. Antimycobacterial activity of some different *Lamiaceae* plant extracts containing flavonoids and other phenolic compounds: Tulin A, Gulendam T, Fatih S, Seyma M, Onur Y. Understanding tuberculosis - new approaches to fighting against drug resistance, Pere-Joan C, China: InTech; 2012.

*****