Screening of antimycobacterial natural products with anti-inflammatory and anticancer activities

Origin of research problem

The intracellular pathogens, *Mycobacterium* species can lead to the death in animals and humans. In the case of tuberculosis (TB), current epidemiological evidence suggests that one-third of worlds population are infected with *Mycobacterium tuberculosis*, that 8 million new cases emerge annually and that 3 million death per year. TB is particularly prevalent in individuals with compromised immune system, such as those that are HIV positive. Most *M. tuberculosis* infections are asymptomatic but can be reactivated under certain debilitating circumstances that impair the immune system such as malnutrition, diabetes, malignancy and especially AIDS (WHO, 2003).

India is contributing nearly one-third of the worlds TB cases and has the highest rate of new TB cases. Prevalence of multi-drug resistance tuberculosis (MDR-TB) cases is on the rise in our country. An increasing trend of MDR-TB cases in different parts of India alarming the seriousness of the problem (Mondal and Jain, 2007).

Significance of study

MDR-TB cases threaten the effectiveness of chemotherapy for both treatment and control TB by existing drugs. The anti-TB drugs like isoniazid, ethambutol, rifampicin, streptomycin and fluoroquinolines have serious side effects and many pathogenic isolates of *M. tuberculosis* are getting resistance to these drugs. *Mycobacterium* resistance to the antibiotics currently used to treat some of these conditions is increasing and there is an urgent need to search compounds against MDR-TB with minimizing side effects. Research work has been carried out on antimycobacterial natural products around the
world. But there is lack of proper investigation of natural products against Indian isolates of *M. tuberculosis*. Study of anti-inflammatory and anticancer properties of molecules will be useful to establish as significant drugs in chemotherapy of AIDS. Hence an investigation being taken to screen better antimycobacterial natural products against MDR-TB and for anti-inflammatory and anticancer activities.

**Objectives**

- Selection of multi-drug resistant *M. tuberculosis* isolates
- Screening of potential antimycobacterial natural products
- Determination of cytotoxicity and anti-inflammatory activity of antimycobacterial products
- Chemical characterization of antimycobacterial products
- Screening of antimycobacterial products against mycolic acid and its synthesis

**Review of literature**

Naturally occurring pure compounds as well as extracts from higher and lower plants, microorganisms and marine organisms have indicated the inhibitory activity against *M. tuberculosis*. Mitscher and Baker (1998) detected anti-TB activity among different medicinal plant extracts. Cantrell *et al.* (2001) investigated the antimycobacterial activities of plant derived terpenoids. Okunade *et al.* (2004) reviewed antimycobacterial compounds from different chemical classes, such as alkaloids, terpenoids, coumarins, peptides and phenolics from plants, marine organisms, fungi and bacteria. Calanolide A isolated from the rainforest tree *Calophyllum lanigerum* as an anti-HIV agent has been found to exhibit activity against drug resistant *M. tuberculosis* strains.

In previous studies, we investigated bioactive metabolites from different actinobacterial strains (Narayana et al. 2007). Three bioactive compounds, 3-phenylpropionic acid, anthraquinone and 8-hydroxyquinolines isolated from a terrestrial Streptomyces sp. ANU 6277 exhibited activity against drug resistant pathogenic bacteria like Staphylococcus aureus (Narayana et al. 2008). An investigation is being taken to test these compounds against different isolates of M. tuberculosis. Along with these compounds, selective medicinal plant extracts and actinobacterial extracts would be screened for antimycobacterial activities.

**Methodology**

**Cultivation and maintenance of isolates**

Different isolates of M. tuberculosis would be procured from MTCC and local general hospital and cultivated on Middlebrook 7H9 agar medium (Himedia code M 197, India)
Antimycobacterial activity

Different concentrations of test substances would be prepared by using dimethyl sulfoxide and antimycobacterial activity is determined by agar proportion method (Kent and Kubica, 1985). In the agar proportion method, an isolate will be classified as susceptible to a drug, if the number of colonies that grew on the drug containing plate is < 1% of the number of colonies that grew on a control plate without drug, partially resistant if the number was between 1 and 10%, and resistant if the number is > 10%.

Inocula will be prepared by growing strains of *M. tuberculosis* in Middlebrook 7H9 broth supplemented with 0.5 ml of tween 80 and 10% ADC (albumin-dextrose-catalase) enrichment to turbidity equal to ~ 3x10^7 colony forming units (CFU)/ml and diluting the culture 1:5 in broth. The final concentration of mycobacterium in the broth is ~ 6x10^5 CFU/ml. Middlebrook 7H9 agar medium will be prepared and amended with known concentration of test natural products. Before pouring into plates, 7H9 agar medium was inoculated with 0.1 ml of 1:5 diluted culture broth. After solidification the plates were incubated up to 3 weeks and number of CFU per plate would be counted to assess the susceptibility of *M. tuberculosis* isolates to the test substances.

Anticancer assay

Anticancer activity of test substances will be tested on different cancer cell lines using MTT assay (Plumb et al. 1989). Cell lines which would be obtained from National Centre for Cell Science, Pune (India) will be cultured at 37°C with 5% CO₂ using RPMI-1640 (Himedia®, India) media containing fetal bovine serum. Cell lines (2x10⁴ cells per well) seeded in a 96-well plate containing 100 µl of RPMI medium and incubated for 24 h. The cells will then be treated with different concentration of test substances. After 48 h
incubation, 100 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (Sigma Chemicals, USA) will be added to each well, and the plates would be incubated in a CO₂ incubator at 37°C for 4 h. Thereafter, the supernatant is removed from each well. Then 100 µl DMSO is added to dissolve the colored formazan crystals produced by the MTT. Subsequently, the optical density will be measured at 570 nm using an ELISA reader (Molecular Devices Corp., USA).

**In vitro study of anti-inflammatory activity**

Natural products are tested for anti-inflammatory activity by detecting 5-lipoxygenase inhibitory activity using colorimetric method of Gay and Gebicki (2002). The assay mixture would be contained 50 mM phosphate buffer (pH 6.3), 5-lipoxygenase, various concentrations of test substances and linoleic acid (80 mM) in total volume of 0.5 ml. Incubate the mixture for 5 min and add 0.5 ml of ferric-xylinol orange reagent, color intensity of reaction mixture will be measured after 2 min at 585 nm using a spectrophotometer.

**Chemical characterization of antimycobacterial products**

The natural products with antimycobacterial activity are selected and partially purified by column chromatography and chemical constituents will be analyzed by GC-MS and LC-MS studies.

**Screening of antimycobacterial products against mycolic acid and its synthesis**

All mycobacterial species contain complex lipids that have subunits of mycolic acid, which control the permeability of the antimycobacterial agents. Hence antimycobacterial natural products will be screened for inhibition of mycolic acid and its synthesis.
Facilities required

- 96-well microtiter plates
- CO₂ incubator
- ELISA reader
- Haemocytometer
- Phase-contrast microscope
- Basic microbiology lab equipments
- LC-MS and GC-MS

References


