Chapter 1

Introduction

1.1. Introduction

Over the decades, there has been increasing evidence of infections caused by viruses and other pathogenic microorganisms. To date, viral infections still remain a major threat to humans and animals. As a metabolically inert particle, viruses reproduce only when they are within the host cell and as such require the metabolic pathway of living cells to replicate. This unique nature of viruses makes it difficult to design a drug that can either attack the virion or stages in the replication cycle, without affecting the host due to poor selective toxicity within host systems. Despite these drawbacks, substantial progress has been made in the development of antiviral agents for some viral infections in humans. On the contrary, despite the outbreaks in recent years of RNA viral infections in the livestock sector, little success has been achieved towards the development of antiviral agents against these diseases and very few, if any, are available for veterinary use.

Viral infections can be controlled either by prophylaxis or therapeutically. Although vaccines are available to protect against certain viral infections and advances are being made in DNA recombinant technology to produce new and safer vaccines, a comprehensive recent review indicates a possible vaccine-induced enhancement of infection in certain viral diseases (Huisman *et al*., 2009). These responses produced in vaccinates may possibly act as a deterrence in the development of vaccines against certain viral infections. Of equal concern is the use of old vaccine viral strains in the formulation of currently available vaccines in the face of emerging virulent strains in the field. Vaccines have also been associated with residual virulence and toxicity, contamination with other pathogens, allergic responses, disease in immunodeficient hosts (modified vaccines), neurological complications, and harmful effects on the foetus and vaccine failure. The situation has become even more complex in the past decade with the rise in viral latency and resistance development to existing antiviral drugs used in humans (Kott *et al*., 1999).

Bacteria and fungi on the other hand, apart from being causative agents in a variety of infections, play a significant role as opportunistic microbes in immunocompromised patients and nosocomial infections. In these infections, the majority of available chemotherapeutic agents rely on the immune competency of the infected host to fight infection. Coupled with this, of concern is the increase in the development of resistance by these pathogens to available antimicrobial agents (Novak *et al.,* 1999; Dessen *et al.,* 2001; White *et al.,* 1998; 2002; Jones *et al.,* 2004). Indeed with the increase in national and international trade as well as travel, resistant organisms can be transported easily across geographical boundaries leading to a global problem.

The structures of bacterial, fungal and viral pathogens differ in significant ways. As such, most chemotherapeutic agents aimed at inhibiting the continuous multiplication of these pathogens in infected hosts target structure or functions relevant for the continued survival of these pathogens within the host. For example, the majority of effective antibacterial agents inhibit steps important for the formation of peptidoglycan, the essential component of the bacterial cell wall. In contrast, most antifungal compounds target the formation or the function of ergosterol, an important component of the fungal cell membrane, while antiviral agents produce their effect either by inhibiting the formation of viral DNA or RNA or inhibiting the activity of viral reproduction (Jassim and Naji, 2003).

Despite these variations, some similarities do occur amongst agents used for treatment. Bauer *et al.* (1963) reported the use of a thiosemicarbazone derivative (morboran), which is effective in the treatment of tuberculosis as well as a prophylaxis in smallpox infection. On the other hand, antifungal nucleoside analogues absent amongst available antibacterial agents are present amongst antiviral agents (Ghannoum and Rice, 1999). Interestingly, the antibacterial RNA polymerase inhibitor rifampin, which demonstrates no intrinsic activity against fungi, appears quite active against several fungal species when used in combination with amphotericin B (Beggs *et al.,* 1976).

Plants provide an unmatched source of chemically diverse constituents (Cos *et al.,* 2006; Maregesi *et al.,* 2008), that may serve as new alternatives in the pursuit towards the development of potentially effective antimicrobial agents to counter the existing threat posed by these pathogenic microorganisms. As a result, some medicinal plants containing substances active against viruses, bacteria and fungi have been identified (Venkateswaran *et al.,* 1987; Hudson, 1990; Lee *et al.,* 1998; Thyagarajan *et al.,* 1990; Yam *et al*., 1998; Chattopadhyay and Naik, 2007; Masoko *et al*., 2008, Rybalchenko *et al.,* 2009). Historical evidence of the use of plants as a source of remedies to treat various disease conditions, coupled with scientific validation and the understanding that monotherapy results in drug resistance, and the growing interest in the use of medicinal plants creates no doubt that plants contain substances with therapeutic relevance. This chapter therefore presents an insight into the understanding of resistance development by microorganisms and the limitations associated with the use of presently available chemotherapeutic agents. It further highlights the economic impact especially of viral infections that pose a threat to food security in under-developed and developing countries.

1.2. Resistance development to antimicrobial agents

1.2.1. Viruses

Development of resistance to antimicrobial agents is a growing problem worldwide that causes difficulties in the treatment of important nosocomial and community-acquired infections. Currently there are available antiviral drugs

for the management of a range of viral infections caused by human immunodeficiency virus 1 (HIV-1), herpes simplex virus (HSV- 1 and HSV-2), cytomegalovirus (CMV), influenza A virus, respiratory syncytial virus (RSV), papilloma viruses and hepatitis B and C viruses in humans. Although considerable progress has been achieved in the past decades in this respect, the understanding of resistance development to antiviral agents is still in rudimentary stages, in large part because of the relatively recent advent of effective antivirals.

To combat the development of antiviral resistance requires knowledge of the mechanism by which these pathogens elude therapeutic agents. Prior to the discovery of antiretrovirals, an extensive and systematic analysis of herpes simplex virus and varicella zoster virus resistance to acyclovir was undertaken and these findings have provided a major insight into antiviral drug resistance (Coen, 1996; Gilbert *et al.,* 2002). With these viruses, the mutations that lead to resistance development to antiviral agents appear to be associated with reduced virulence and ability to cause infection. This phenomenon has served as a positive outcome for the majority of antivirals used in herpes simplex virus (HSV) and varicella-zoster virus (VZV) infections. In contrast, the development of resistance by the human immunodeficiency virus to antiretroviral therapy results from mutations in the genome of the virus coding for structural changes in the target proteins that can affect the binding or activity of currently used antiretroviral drugs (Menéndez-Arias, 2010). Other resistance development mechanisms against effective treatment for influenza virus (Hill *et al.,* 2009), and chronic hepatitis B virus infection (Ghany and Liang, 2007) have been extensively reviewed. That notwithstanding, the processes by which viruses develop resistance to antiviral agents are increasingly being investigated and characterized for the growing number of antiviral agents.

1.2.2. Bacteria

Infectious diseases remain a leading cause of worldwide morbidity and mortality, whether in the general healthy population or in patients who are immunocompromised and are at risk of infection with invasive opportunistic pathogens. Even though antimicrobial drugs have played a major role in keeping these pathogens in check, the development of resistance to antibiotics currently remains one of the biggest challenges facing global health care systems. Available reports indicate that around 90–95% of *Staphylococcus aureus* strains worldwide are resistant to penicillin (Casal *et al.,* 2005) and in most of the Asian countries 70–80% of the same strains are methicillin resistant (Chambers, 2001).

The development of resistance to antibiotics came to light from organisms that were exposed to the first commercially available antibiotics. The antimicrobial drug resistance of staphylococci to penicillin is one such example (Barber, 1947). Resistance develops either passively or actively as a result of attainment of new genetic material by the microbe or pre-existing innate mechanism (Summers, 2006; Wright, 2007). These resistance developments lead to

treatment failure which frequently has fatal consequences. It is worthy of note to recognize that resistance also affects the treatment of individuals with non-resistant organisms in areas with high rates of resistance thereby increasing overall treatment costs (Howard *et al*., 2003). The major mechanisms of antibiotic resistance include prevention of interaction of the drug with the target site, efflux of the antibiotic from the cell, and direct destruction or modification of the compound (Walsh, 2003; Levy and Marshall, 2004; Wright, 2005). Compounding the problem is the continued selective pressure by different drugs, resulting in bacteria acquiring additional kinds of resistance mechanisms that have given rise to multidrug resistance (MDR). Some of these resistance development mechanisms to antibacterial agents in Gram-positive and Gram-negative bacteria as well as molecular mechanisms of multidrug resistance have been extensively documented (Wright, 2005; Tenover, 2006; Rice, 2006; Alekshun and Levy, 2007; Matthew and Bliziotis, 2007).

Poverty, poor access and insufficient health care systems, civil conflicts and lack of commitment on the part of government in developing countries have partly contributed to the rise in treatment failure and have impacted negatively on efforts to control infectious diseases. Other factors within established clinical settings include inappropriate use of broad-spectrum antibiotics, lack of prudent judgement in instituting treatment (Hancock, 2005), colonisation pressure amongst infected patients as a source of the spread of methicillin-resistant *Staphylococcus aureus* (MRSA) (Merrer *et al.,* 2000) and vancomycin resistant *Enterococcus faecalis* (VREF) (Bontem *et al.,* 1998). Prolonged intensive care unit (ICU) stay leads to exposure to hospital-acquired infections (Bontem *et al.,* 1998; Rahal *et al.,* 1998). The use of invasive devices such as endotracheal tubes has also been incriminated (Richards *et al.,* 1999; Kollef *et al.,* 1997).

Furthermore, antibiotics are not only used in human medicine but also for the treatment, mass prophylaxis and growth promotion in animals, thereby presenting a possible risk of resistant bacteria passed to humans via the food chain. Antibiotics used in both veterinary and human medicine are: penicillins, cephalosporins, tetracyclines, chloramphenicols, aminoglycosides, spectinomycin, lincosamide, macrolides, nitrofuranes, nitroimidazoles, sulfonamides, trimethoprim, polymyxins and quinolones (Prescott, 2000). It is considered that one way to prevent the transfer of antibiotic resistant strains from animals to the human population is to withdraw the use of antibiotics as production enhancers in veterinary practice (Hancock, 2005). This initiative has been recommended by the World Health Organisation (WHO) because of risk factors associated with their continued use.

The increased development of resistance is accompanied by medical and economic implications (Paladino, 2002; Cosgrove and Carmeli, 2003). The costs to bring a new drug onto the market are estimated at a minimum of US$300 million. Hence, the inability of developing economies to manage the spread of resistant strains through globalisation increases the demand for resources and services.

1.2.3. Fungi

Despite the increase in the prevalence of resistance to antibacterial and antifungal agents, not much attention has been devoted to the study of antibiotic resistance. Studies of resistance development specifically to antifungal agents have lagged even further behind. Resistance development to antifungal agents is a broad concept that describes failure of a fungal infection to respond to antifungal therapy (Sheehan *et al*., 1999). Traditionally, this resistance development is classified as either primary (intrinsic), where the organism is resistant prior to exposure to the antifungal, or secondary (acquired), due to a stable transient genotypic modification following exposure to an antifungal agent. A third type of antifungal resistance can be described as "clinical resistance", which arises from progression or relapse of an infection caused by a susceptible isolate in *in vitro* testing to an antifungal agent recommended for the treatment of the given infection. Such resistance development is common amongst immunocompromised patients or in patients where prosthetic materials have been used (Sheehan *et al*., 1999). In some cases, suboptimum drug concentrations in the blood might contribute to the development of clinical resistance.

The exposure of fungal pathogens to antifungal agents stimulates different responses in the metabolism of the organism. As a survival instinct, the fungal pathogen will strive to overcome the growth inhibitory effect of the antifungal agent by development of various mechanisms to counteract the inhibitory effect of the antifungal agent. These mechanisms will permit the growth of the pathogen at higher drug concentrations than is the case for normal susceptible pathogens, while in others where higher drug concentration results in growth inhibition, the fungal pathogen can alter the therapeutic potency of a given antifungal agent, which will determine if the agent will produce a static or a cidal effect (Sanglard, 2003). This property exhibited by fungal pathogens is termed antifungal drug tolerance. Prior to the validation by the United States National Committee for Clinical Laboratory Standards (NCCLS) now known as the Clinical and Laboratory Standard Institute (CLSI) in 1997, there was no widely accepted method for *in vitro* susceptibility testing of fungal pathogens. The method describes the determination of minimum inhibitory concentrations (MICs) of widely used antifungals against one species, which helps to evaluate with greater confidence whether *in vitro* susceptibilities are correlated with clinical response to therapy. Interpretive breakpoints of resistance with this standard method currently exist only for fluconazole, itraconazole, and flucytosine.

For many years, amphotericin B was the only drug available to control fungal infections until the advent in the 1980s and 1990s of the imidazoles and the triazoles. The introduction of these agents led to their widespread usage and to the development of drug resistant strains (Rex *et al.,* 1995). Resistance development mechanisms to the azoles have been most extensively investigated in recent years, as a large number of yeast isolates were available to research laboratories. Several reviews are available that describe in detail the different mechanisms resulting in resistance of

fungal pathogens to the azoles and other classes of antifungal agents including the molecular bases for such resistance (Sanglard *et al.,* 1998; Ghannoum and Rice, 1999; White *et al.,* 1998; Sanglard, 2002; Kontoyiannis and Lewis, 2002; Chamilos and Kontoyiannis, 2005; Prasad and Kapoor, 2005; Kanafani and Perfect, 2008).

With the increase in the incidence of systemic fungal infections, the choice of suitable antifungal agents remains relatively limited. The use of highly active antiretroviral therapy (HAART) has decreased the occurrence of mucosal candidiasis among acquired immune deficiency syndrome (AIDS) patients in the USA (Martins *et al.,* 1998). In under-developed and developing countries, the increase in the number and spectrum of fungal infections boosted by the AIDS pandemic coupled with poor access to HAART and problems associated with adherence to medication and toxicity, implies that resistance to antifungal agents still remains a major threat. Furthermore, advances in anticancer chemotherapy and organ transplants have attracted new interest in the development of new compounds with antifungal activity. Over the next decade, antifungal resistance may become an increasingly crucial determinant of the outcome of antifungal treatment.

1.3. Adverse effects associated with the use of antimicrobial agents

1.3.1. Antiviral agents

An adverse drug reaction is defined by the WHO as any response to a drug 'which is noxious and unintended and which occurs at doses used in man for prophylaxis, diagnosis or therapy' (WHO, 1984). As the incidence of drug resistance increases, the situation has become more alarming with the toxic effects associated with currently used antimicrobial agents. Presently, there is no cure for HIV infection and patients using therapies recommended either as immune boosters or for the reduction of viral load are expected to undergo treatment for life. The introduction of highly active antiretroviral therapy (HAART), which consists of a cocktail of drugs, has led to substantial reductions in morbidity and mortality associated with HIV-1 infection. Although considerable improvement has been made in the management of HIV infection (HIV/AIDS, 2008), drug related toxicity is increasingly being recognized because of the reduced incidence of HIV-1-associated opportunistic infections (Carr and Cooper, 2000). The present threat posed however does not seem to be life–threatening but can affect the quality of life and patient's compliance to treatment regimens and as a consequence will result in the development of resistant strains in the near future. Conversely, long-term toxic effects associated with prolonged therapy can also lead to changes in treatment regimens or discontinuation due to adverse effects that could not be foreseen in the short-term (Esté and Cihlar, 2010).

Numerous antiretrovirals that are currently used for the treatment of HIV infection have adverse effects, ranging from

nausea, headache, nail pigmentation, diarrhoea, mouth ulcers, central nervous stimulation, hypersensitivity, perioral paraesthesiae, renal calculi, hyperbilirubinaemia, reflux oesophagitis, retinoid effects and haemolytic anaemia, which are associated in some patients with the use of either nucleoside analogues, non-nucleoside analogues or HIV protease inhibitors. These effects and many more associated with the use of available therapies for HIV treatment have been documented (Carr and Cooper, 2000; Izzedine *et al.,* 2005; Hawkins, 2010).

In the management of hepatitis C viral infection, the side effect profile of combination therapy using standard interferon and ribavirin have been reported to be associated with fatigue, influenza-like symptoms, gastrointestinal disturbances, neuropsychiatric symptoms, and hematologic abnormalities which may call for the reduction in the dose of the treatment regimen or a total discontinuation of treatment depending on the severity of the effect (Poynard *et al.*, 1998; Maddrey, 1999; McHutchison and Poynard, 1999). In the case of chronic hepatitis B (CHB) virus infection, where patients are required to take treatment for a prolonged time period, it is important to strike a balance between long-term benefits and potential adverse effects. For instance, long-term lamivudine treatment in CHB virus infection in patients undergoing therapy is associated with an increased rate of drug resistant mutations (Guan *et al.,* 2001; Leung *et al.,* 2001) coupled with serious hepatitis flares and hepatic decompensation in isolated cases of individuals (Tipples *et al.,* 1996; Bartholomew *et al.,* 1997). Such risk may however be higher in older patients who are immunosuppressed and suffering from advanced liver diseases.

Furthermore, although foscamet is an effective treatment for acyclovir-resistant herpes simplex virus and acyclovir-resistant varicella-zoster virus, it significantly decreases the circulating levels of HIV antigens in AIDS patients with cytomegalovirus disease. The major adverse effect associated with the use of foscamet is renal dysfunction (Akesson *et al.,* 1986; Jacobson *et al.,* 1988) and acute renal failure when used alone or in combination with certain drugs (Cacoub *et al.,* 1988). The nephrotoxicity induced by this drug has limited its more widespread utilization, especially in transplant patients.

1.3.2. Antibacterial agents

Quinolones are widely used antibacterial agents with excellent activity against Gram-negative bacteria. Nausea, vomiting, diarrhoea and other reactions of the gastrointestinal tract are among the most common side effects associated with the use of quinolones. Compared to other groups of antibacterial agents with a broad spectrum (e.g. penicillins or cephalosporins), the incidence of diarrhoea is low and has been associated with the newly introduced quinolones such as levofloxacin, moxifloxacin and gatifloxacin. Depending on the type of quinolones, adverse effects on the cardiovascular system, phototoxicity (photocarcinogenicity) and connective tissue structures (chondrotoxicity

and tendinopathies) have been reported (Anderson *et al.,* 2001; Bailey *et al.,* 1983; Stahlmann, 2002).

With the β-lactams (comprising over 40 derivatives within the penicillin and cephalosporin families), paramount to their popularity has been their impressive safety profile. Although severe life threatening reactions occur with the use of these agents, most reported toxicities are mild and reversible in nature. The most common adverse effect associated with the use of these classes of drugs is hypersensitivity reactions, which can vary in severity from skin rashes to life-threatening anaphylaxis. The reported frequency of reactions ranges from 0.7% to 10% (Petz, 1978; Norrby, 1986). While anaphylactic reactions are exceedingly rare, death may result due to a combination of symptoms, which includes nausea, vomiting, abdominal pain, pallor, tachycardia, severe dyspnoea due to bronchospasm, rigors, loss of consciousness and peripheral circulatory failure due to vasodilatation and loss of plasma volume within minutes if not properly managed. In addition, mild to severe cases of haematological reactions, hepatotoxicity, nephrotoxicity, gastrointestinal reactions and neurotoxicity have been reported in some patients (Moake *et al.,* 1978; Yust *et al.,* 1982; Lurie *et al.,* 1970; Ruley and Lisi, 1974; Milman, 1978; Gardner *et al.,* 1978; Enat *et al.,* 1980; Onorato, 1978).

Due to a relatively low potential for Gram-positive organisms to develop resistance to aminoglycosides, their use in combination with the β-lactam antibacterials continues to be relevant for the treatment of serious Gram-negative infections despite the undesirable effects that are associated with their use. Both nephrotoxicity and ototoxicity are well described adverse effects associated with the use of aminoglycosides (Davey *et al.,* 1991; Kaloyanides and Pastoriza-Munoz, 1980). However, the damage caused to the tubes of the kidney as a result of aminoglycoside use is usually reversible, resolving soon after the drug is discontinued, while that due to damage to the inner ear may be permanent.

Toxic effects associated with the use of vancomycins have limited their use in the past decade, however, the increase in the incidence of infections involving methicillin-resistant staphylococci has once again led to an increase in the use of this drug in methicillin-resistance staphylococci infections. Reported adverse effects associated with vancomycin include: nephrotoxicity, ototoxicity, anaphylactoid reactions and phlebitis (Rybak *et al.,* 1990; Brummett and Fox, 1989; Farber and Moellering, 1983). Purification of the commercial product in the early 1980s was thought to have decreased the frequency of adverse effects; however, information evaluating the adverse-effect profile of the reformulated product is scanty. Additional risk factors include: increased age (i.e. geriatric population), liver disease, peritonitis, neutropenia, male gender and concurrent use of nephrotoxic agents (Pauly *et al.,* 1990).

The macrolides have been considered to be among the safest class of antimicrobials in clinical use due to the low incidence of severe toxic reactions. However, with the macrolides, gastrointestinal (GI) adverse effects were reported

to be responsible for the high rates of intolerance among treated patients (Itoh *et al.,* 1984; Zara *et al.,* 1985). The use of the newer macrolides has been associated with a lower incidence of the above adverse effects compared with erythromycin (Periti *et al.,* 1993). Apart from GI adverse effects, thrombophlebitis has been associated with intravenous administration of erythromycin. Other notable macrolide-induced adverse effects include transient sensorineural ototoxicity (Brummett and Fox, 1989). Clinical evidence indicates that ototoxicity is more likely to occur in patients who are elderly, renally or hepatically impaired or those receiving an erythromycin dosage exceeding 4 g/day. Furthermore, cardiovascular effects have been associated with intravenous infusion of erythromycin in critically ill patients (Tschida *et al.,* 1996).

1.3.3. Antifungal agents

Antifungal agents are mostly used in patients with severe underlying diseases, which make detection of adverse drug effects and that due to the underlying disease difficult. Amphotericin B deoxycholate has been the gold standard for the treatment of patients with invasive mycoses. Although impairment of liver function has not been considered to be a typical adverse effect associated with the use of amphotericin B, nephrotoxicity resulting from the cumulative effect of the drug leading to renal impairment, hypokalemia, hypomagnesemia, and acidosis have been reported (Gallis *et al*., 1990). As such, the use of the drug in combination with nephrotoxic drugs such as cyclosporine, vancomycin, aminoglycosides and cisplatin warrants regular monitoring of patients during and after treatment. Compared with conventional amphotericin B deoxycholate, the new formulations, namely lipid-based amphotericin B colloidal dispersion, amphotericin B lipid complexes, and liposomal amphotericin B, are less nephrotoxic.

Currently, newer classes of the third-generation triazoles have been introduced for clinical use, with less toxic effects, and in some cases are more effective than amphotericin B. One such drug, voriconazole, has been approved for first-line treatment of invasive aspergillosis. However, voriconazole is associated with mild elevation of the transaminase enzymes and visual disturbances (photophobia, blurred vision and altered colour discrimination), rash and gastrointestinal symptoms (Hoffman and Rathbun, 2002). Similarly, posaconazole has few side effects, the most common of which are nausea, vomiting, headache, abdominal pain and diarrhoea (Raad *et al.,* 2006) while in some cases, elevated liver enzymes may be observed. Another group of drugs, the echinocandins, have a mode of action that is different from all the other antifungals. These drugs produce their antifungal effect by inhibition of β-1, 3-D-glucan synthase, which forms an important component of the cell wall of many pathogenic fungi such as *Candida* and *Aspergillus* species. Drugs belonging to this group comprise caspofungin, micafungin, and anidulafungin, with fewer side effects ranging from headache, nausea, phlebitis, fever, rash, diarrhoea, leukopenia, anaphylaxis and haemolysis in isolated cases to hypokalaemia and elevated hepatic enzymes. In general this class of drugs is well tolerated.

1.4. Virus

Viruses are obligate intracellular parasites, which contain little more than bundles of gene strands of either RNA or DNA, and may be surrounded by a lipid-containing envelope (Wagner and Hewlett, 1999). This gene strands, whether DNA or RNA, may be single or double stranded. Single stranded viral nucleic acid may be of positive or negative polarity (Duguid *et al.,* 1978). The molecular weight and type of nucleic acid are characteristic for each group of viruses. Depending on the type of virus, the nucleic acid can be linear or circular (Duguid *et al.,* 1978).

Although extremely simple in structure and composition, viruses are masters of camouflage and deception. Devoid of any means of independent locomotion, they disseminate by exploiting host cell organelles and metabolic pathways to propagate new viruses. They use the reproductive machinery of invaded cells, causing various ailments. Each strain of virus has its own unique configuration of surface molecules (Wagner and Hewlett, 1999). These surface molecules work like keys in a lock, enabling viruses to infect their hosts by precisely fitting the molecules on their surfaces to those on the membranes of target cells. Viral particles mediate the transfer of the viral genome and accessory proteins from an infected host cell to a non-infected host cell. This involves packaging the viral genome (RNA or DNA) and accessory proteins, releasing the package from the infected cell, protecting the essential components during extracellular transmission, and delivering them into a new host cell. Many viruses with a DNA genome must enter the nucleus, whereas RNA viruses, with a few exceptions, replicate in the cytosol.

The success of viruses over time has been established by four general attributes: genetic variation, variety in means of transmission, efficient replication within host cells, and the ability to persist in the host (Wagner and Hewlett, 1999). Consequently, viruses have adapted to all forms of life and have occupied numerous ecological niches resulting in widespread diseases in humans, livestock and plants alike. Unlike bacteria and fungi, viruses are the only pathogens in nature that use RNA as a store of genetic information (Duguid *et al.,* 1978). The body responsible for the classification of viruses is the International Committee on Taxonomy of Viruses (ICTV). Viruses have been classified into two broad groups based on the nature of the genome and the structure of the virion.

Viral diseases are still fatal and new viral diseases continue to emerge. Although some viral diseases can be kept under control by the use of vaccines or antiviral agents, the development of resistance in recent years to available antiviral drugs and the need to develop new therapies for the majority of viral infections, makes the search for new antivirals a virgin area for continued drug discovery. Furthermore, delays in the availability of vaccines and in the onset of protection (immunity gap) imply that during the critical initial stage of an outbreak, livestock will remain highly susceptible to infection. Many viruses have unique features in their structure or in their replication cycles, that can act as potential targets for antiviral drug testing. If antiviral agents were available to counter diseases in livestock this

could be a valuable adjunct to available disease control measures both in disease-free and disease-enzootic settings. In this study, greater attention will be given to literature on the antiviral activity of plants, as there are relatively few studies on the antiviral activity of plant compounds. Coupled with the fact that presently available antiviral drugs are still far beyond the means of rural populations in most developing countries, poverty, in terms of hunger eradication, child mortality reduction and improved human health, can be reduced by improved animal health (Perry and Sones, 2007).

1.4.1. Impact of viral disease outbreak on agriculture and livestock

"Livestock" is a collective term used for any breed or population of animals kept by humans for subsistence or commercial purpose. These sectors contribute to the livelihood of approximately 70% of the world's poor (DFID, 2000). Globally, the sector accounts for about 40% of the agricultural gross domestic product (Steinfeld *et al.,* 2006). Highly pathogenic viruses of livestock can be defined as those viruses that cause highly contagious or transmissible animal diseases and have the potential for very severe and rapid spread, irrespective of national borders, which are considered to be of serious socio-economic and/or public health significance, and which are of major importance in international trade of animals and their products (Domenech *et al.,* 2006). Such diseases have been listed by The Office International des Epizooties (OIE), which highlights the threat these agents pose to the livestock industry (OIE, 2004).

Foot and Mouth Disease Virus (FMDV) is an example of one such highly contagious virus that affects several species of animals and has an exceptionally high mutation rate (Domingo *et al.,* 2003), with high economic impact during an outbreak. The economic impact of the 2001 FMD epidemic in the United Kingdom (UK) rose to the tune of £8–9 billion, of which £3.1 billion represented direct losses to agriculture and the food chain while indirect losses to tourism were estimated to be as high as £5 billion (Thompson *et al.,* 2002; Campbell and Lee, 2003). Similarly, the direct economic consequences of the 1997 outbreak in Taiwan totalled US$ 3.31 billion (Yang *et al.,* 1999). As such, high revenue impact figures were predicted in case of FMD incursions in Australia, New Zealand and the United States of America (Belton, 2004; Garner *et al.,* 2002; Paarlberg *et al.,* 2002). The situation in Africa is of particular interest as five serotypes of the viruses are in circulation (Domenech *et al.,* 2006).

Rift valley fever (RVF) is a viral zoonosis that affects sheep, goat, buffalo and cattle (Van Tongeren, 1979). Human diseases due to infection with the virus also occur, especially during periods of intense epizootic activity, which occur after heavy rainfall, when there is an increase in the vector (mosquitoe) population (Arthur, 2000). The burden of human morbidity and mortality due to an outbreak often has a direct impact on economic loss of livestock, which can be as high as 70% of all affected animals. Many sub-Saharan tropical and sub-tropical countries in Africa have

reported outbreaks of RVF and the disease is encountered in an enzootic or epizootic form along the east and south coast of Africa and also in Madagascar. The 1970s saw the most severe outbreaks in South Africa in 1975 and in Egypt in 1977 (Gear, 1979; Hoogstraal *et al.,* 1979). Outbreaks of the disease in East Africa in the late 1970s and in 2000 caused livestock losses and human deaths and seriously affected international trade in livestock to the Middle East (Otte *et al.,* 2004). Other such outbreaks in past decades include rinderpest (RP) in Africa in the 1980s (Rweyemamu *et al.,* 2000), lumpy skin disease (Hunter and Wallace, 2001), African swine fever (Rweyemamu *et al.,* 2000), and Peste des petits ruminants (PPR) in India and Bangladesh (Roeder and Obi, 1999).

Of an equal severe consequence was the avian influenza epidemic in Asia caused by the highly pathogenic H5N1 strain in poultry in 2003. The virus has a high potential for rapid spread and is characterized by high mortality in chickens of between 75% and 100%. The outbreak of the disease resulted in the deaths and culling of about 40 million birds resulting in an economic loss to South East Asia to the tune of more than US $60 billion (Lokuge *et al.,* 2005). In Africa, routine vaccination of livestock has been prohibitively expensive to the common farmer, making the continent a refuge for endemism of some of these diseases.

The economic impact of animal diseases is complex and can go beyond the immediate impact on the directly affected animal producers. The most direct impact is the loss or reduced efficiency of production, which reduces farm income. These effects can create a negative impact on variation of prices determined by supply and demand, which in turn can pose a heavy burden on international trade. Of concern is the effect of these diseases and their negative impact on food security and nutrition in developing countries amidst associated health and environmental concerns where the majority of livestock production occurs in areas close to human populations (Otte *et al.,* 2004). A further limitation is the fact that most of these diseases occur in areas where resources are minimal, and where routine vaccination is seldom practiced.

Local remedies used in the treatment of various ailments have a long history in rural Africa and traditional medicines that have antimicrobial activity could have important benefits in communities where they are widely used. As traditional knowledge of disease treatment erodes in the face of socioeconomic change, the urgency to document indigenous remedies increases (Farnsworth, 1993). In the next chapter, a literature review of the antimicrobial activity of plants will be presented, followed by the aims and objectives of this study.

Chapter 2

Ethnomedicine in infections disease treatment and the relevance of cell culture in toxicity studies of medicinal plants

2.1. The value of ethnomedicine in drug discovery

In broad terms, ethnomedicine can be defined as the use of plants by humans as medicines (Farnsworth, 1990; 1994), while traditional medicine refers to any non-Western medical practice (Bannerman *et al.,* 1983). On the other hand, ethnopharmacology is a highly diversified approach to drug discovery involving the observation, description, and experimental investigation of indigenous drugs and their biological activities. It encompasses several disciplines that contribute to the discovery of natural products with biological activity (Rivier and Bruhn, 1979). The purpose of using plants as natural sources in drug development is multifaceted. These include a) the isolation of bioactive compounds for direct use as drugs, b) the production of bioactive compounds of novel or known structures as lead compounds to synthesize entities of higher activity and/or lower toxicity, c) to use agents as pharmacologic tools, and d) to use an extract of the whole plant or part of it as a herbal remedy.

Different approaches have been employed when selecting higher plants in the drug discovery process. These selections can be done either randomly, or by chemical screening, or ethnomedical claims involving the targeted disease. In either case, there are shortfalls associated with the selection process since plants, as biological systems, inherently vary in their chemistry and resulting biological activity.

There are advantages and disadvantages of using plants as the starting point in any drug development program. If the selection process is based on the ethnomedicinal approach, one can presume that any extract of the plant is likely to be safer than extracts or active compounds from plants with no history of human use. However, this presumption should be treated with caution as long-term toxic effects are often not linked to the use of the plant. If the active principle derived from such an investigation yields novel structures with useful biological activity, or novel biological activity of known compounds, patent protection may be possible.

Other problems encountered when plants are used as starting materials in the drug discovery process are mix-ups in labelling of plant samples or inaccurate taxonomic identification and the choice of inappropriate bioassay systems. Selection of plants based on ethnomedical use in conjunction with rational biological assays that correlate with the ethnomedical uses would be most appropriate in the drug discovery process (Fabricant and Farnsworth, 2001). Figure 2.1 illustrates a flow chart of sequence for the study of plants used in traditional medicine.

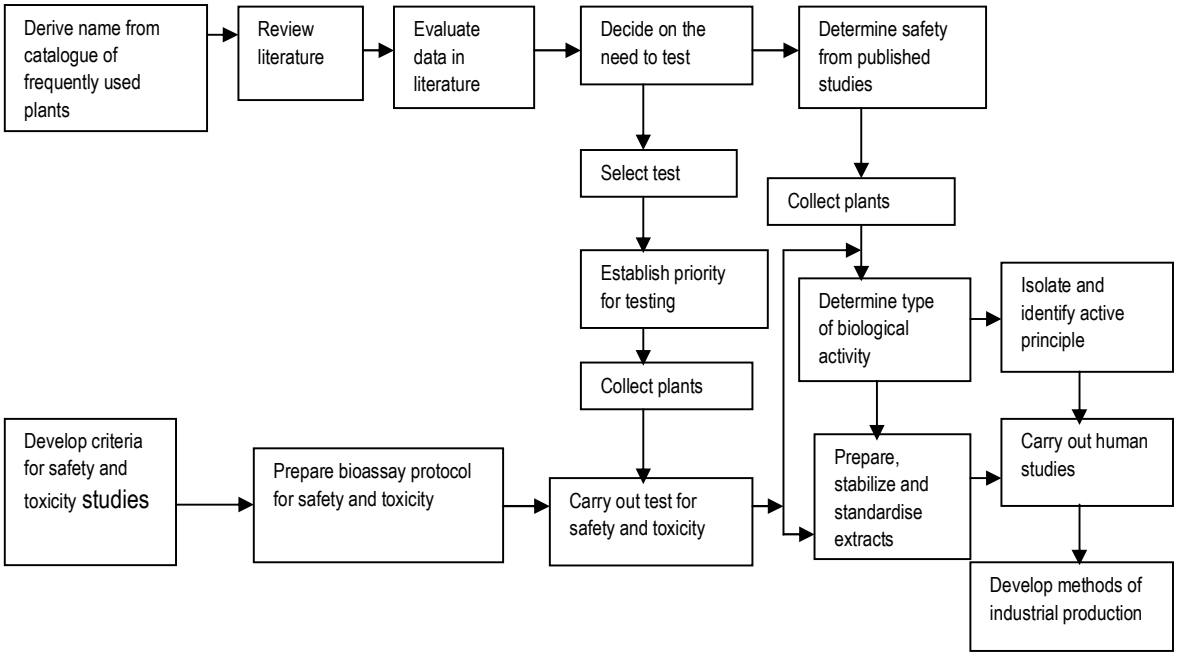

Figure 2.1: Flow chart of sequence for the study of plants used in traditional medicine (adapted from Farnsworth *et al.* (1985).

Several molecules of great interest because of their therapeutic usefulness, novel biological activity, or potential as pharmacologic probes have already been introduced in recent years following the approaches outlined above. One such is the antimalarial artemisinin from the Chinese medicinal herb Qing hao su (*Artemisia annua*), which is poorly soluble in water, making it unsuitable for administration. However, a derivative, sodium artesunate, has been developed, which retains the antimalarial activity but has much improved water solubility (Haynes and Vonwiller, 1994). More importantly, at least 119 compounds derived from plants have been considered as important drugs currently in use in one or more countries, with 77% of these derived from ethnomedical sources (Farnsworth *et al.,* 1985).

2.2. Use of plants as antimicrobial agents

Plants have been used as medicines for thousands of years (Samuelsson, 2004) and that they indeed contain pharmacologically active constituents is widely acknowledged. To ensure continued use, the specific plants to be used and the methods of application for particular ailments were passed down from generation to generation through oral history. These medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders, and

other herbal formulations (Balick and Cox, 1997; Samuelsson, 2004).

It is estimated that today, plant materials are present in, or have provided the models for, 50% of Western drugs (Robbers, 1996). The role of plant-derived medicine is two fold in the development of new drugs. Firstly, they may serve as a natural blueprint for the development of new drugs, or secondly, as a phytomedicine to be used for the treatment of disease. Many commercially proven drugs used in modern medicine were initially used in crude form in traditional or folk healing practices, leading to the early development of drugs from natural sources before the advent of their synthetic counterparts (Table 2.1).

Table 2.1. Some drugs of natural origin (adapted from Tulp and Bohlin, 2002)

Natural compound	Mode of action	(Semi) synthetic drug
Amphotericin B	Cell membrane permeability	Not yet described
Artemisinin	Interaction with haem	Arthemether
Atropine	Muscarinic acetylcholine receptor antagonist	Butylscopolamine
Avermectin	Cl– channel activator	Selamectin
Camptothecin	Topoisomerase I inhibitor	Irinotecan
Dicoumarol	Vitamin K antagonist	Acenocoumarol
Digoxin	Na+–K+-ATPase inhibitor	Acetyldigoxin
Ephedrine	β-Adrenoceptor agonist	Isoprenaline
Ergocornine	Dopamine receptor agonist	Bromocryptine
Gentamicin	Protein synthesis inhibitor	Metamycin
Griseofulvin	Mitosis inhibitor	Not yet described
Morphine	Opioid receptor agonist	Pethidine, fentanyl
Papaverin	Phosphodiesterase inhibitor	Sildenafil
Penicillin G	Transpeptidase inhibitor	Ampicillin, cyclacillin
Physostigmine	Cholinesterase inhibitor	Neostigmine
Podophyllotoxin	Topoisomerase II inhibitor	Etoposide
Vincristine	DNA polymerase inhibitor	Vindesine
Tetracyclines	Protein synthesis inhibitor	Doxycycline

Plant-based antimicrobials have huge therapeutic potential. They are effective in the treatment of infectious diseases and may mitigate side effects that are often associated with synthetic antimicrobials. An important issue with regard to drug discovery research is the role of unexpected observations. Herbal remedies usually have multiple effects on the body with activities that often act beyond the symptomatic treatment of disease. Such an effect can be observed with *Hydrastis canadensis*. Hydrastis not only has antimicrobial activity, but also increases blood supply to the spleen, promoting optimal activity of the spleen to release mediating compounds (Murray, 1995).

A great deal of the exploration and utilization of natural products as antimicrobials began from microbial sources. The discovery of penicillin created inroads for the later discoveries of streptomycin, aureomycin and chloromycetin (Trease and Evans, 1972). Although most of the antibiotics in clinical use were discovered from microorganisms in

the soil or fungi, plants have also been a source of antimicrobial agents (Table 2).

Table 2.2. Plants containing antimicrobial activity (adapted from Cowan, 1999)

Common name	Scientific name	Compound	Class	Activity
Oregon grape	*Mahonia aquifolia*	Berberine	Alkaloid	Bacteria, fungi
Onion	*Allium cepa*	Allicin	Sulfoxide	Bacteria, fungi
Olive oil	*Olea europaea*	Hexanal	Aldehyde	Bacteria, fungi
Lavender-cotton	*Santolina Chamaecyparissus*			Bacteria, fungi
Hemp	*Cannabis sativa*	b-Resercyclic acid	Organic acid	Bacteria and viruses
Garlic	*Allium sativum*	Allicin, ajoene	Sulfoxide Sulfated terpenoids	Bacteria, fungi
Echinacea	*Echinacea Angustifolia*			Bacteria, fungi
Cascara sagrada	*Rhamnus purshiana*	Tannins	Polyphenols Anthraquinone	Viruses, bacteria, fungi
Apple	*Malus sylvestris*	Phloretin	Flavonoid derivative	Bacteria, fungi
Woodruff	*Galium odoratum*		Coumarin	Viruses, bacteria, fungi
Purple prairie clover	*Petalostemum*	Petalostemumol	Flavonol	Bacteria, fungi
Papaya	*Carica papaya*	Latex	Mix of terpenoids, organic acids, alkaloids	Bacteria, fungi
Caraway	*Carum carvi*		Coumarins	Bacteria, fungi, viruses
Thyme	*Thymus vulgaris*	Caffeic acid Thymol Tannins	Terpenoid Phenolic alcohol Polyphenols Flavones	Viruses, bacteria, fungi
Burdock	*Arctium lappa*	Polyacetylene, tannins, terpenoids		Bacteria, fungi, viruses

The antimicrobial properties of plants have been associated with the presence of secondary metabolites produced by these plants that act as a deterrence against predation by microorganisms, insects, and herbivores. These secondary metabolites play a huge role as antimicrobial agents. Useful antimicrobial phytochemicals can be divided into several categories, summarized in Table 2.3.

Of these, the phenolics represent an extensive metabolic family given that this group of molecules is involved in lignin synthesis, making them common to all higher plants. However, other compounds such as alkaloids are sparsely distributed in the plant kingdom and are much more specific to defined plant genera and species. This narrower distribution of secondary compounds constitutes the basis for chemotaxonomy and chemical ecology

Table 2.3. Major classes of antimicrobial compounds from plants (adapted from Cowan, 1999)

Class	Subclass	Examples	Mechanism of action
Phenolics	Simple phenols	Catechol	Substrate deprivation
		Epicatechin	Membrane disruption
	Phenolic acids	Cinnamic acid	
	Quinones	Hypericin	Bind to adhesins, complex with cell wall, inactivate enzymes
	Flavonoids	Chrysin	Bind to adhesins
	Flavones		Complex with cell wall
		Abyssinone	Inactivate enzymes
			Inhibit HIV reverse transcriptase
	Flavonols	Totarol	
	Tannins	Ellagitannin	Bind to proteins
			Bind to adhesins
			Enzyme inhibition
			Substrate deprivation
			Complex with cell wall
			Membrane disruption
			Metal ion complexation
Terpenoids, essential oils		Capsaicin	Membrane disruption
Alkaloids		Berberine	Intercalate into cell wall and/or DNA
		Piperine	
Lectins and polypeptides		Mannose-specific agglutinin	Block viral fusion or adsorption
		Fabatin	Form disulfide bridges
Polyacetylenes		8S-Heptadeca-2(Z),9(Z)-diene-4,6-diyne-1,8-diol	-

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2.3. Common classes of antiviral compounds present in medicinal plants

Presently, it is doubtful that available mainstream antiviral drugs can solve the problems posed by viruses and emerging viral infections. Historically, scores of traditional medicinal plants have been used to treat viral infections the world over. Depending on the mode of application, different combinations of medicinal plants that have been used for this purpose may cause variations in therapeutic effect. Some of these variations have resulted in the therapeutic success of many medicinal plant extracts in several unrelated syndromes by virtue of their synergistic effects. Hence, several medicinal plants have been reported to have strong antiviral activity, some of which have been used to treat disease in humans and animals that suffer from viral infection (Hudson, 1990; Venkateswaran *et al.,* 1987; Thyagarajan *et al.,* 1988; 1990).

With an ever-growing potential, compounds isolated from natural sources are increasingly investigated for their ability to inhibit the replication cycle of various types of DNA or RNA viruses. A wide variety of active phytochemicals, including the flavonoids, terpenoids, organosulfur compounds, limonoids, lignans, sulfides, polyphenolics, coumarins, saponins, chlorophyllins, furyl compounds, alkaloids, polyenes, thiophenes, proteins and peptides have therapeutic applications against different genetically and functionally diverse viruses (Chattopadhyay and Naik, 2007; Chattopadhyay and Bhattacharya, 2008; Naithani *et al.,* 2008). The antiviral mechanism of these agents may be related to their antioxidant activities, scavenging capacities, inhibition of DNA or RNA synthesis, inhibition of viral entry, or inhibiting viral reproduction (Christopher and Wong, 2006; Chattopadhyay and Naik, 2007; Naithani *et al.,* 2008). As a result, large numbers of candidate substances, such as phytochemicals and their synthetic derivatives, have been identified by a combination of *in vitro* and *in vivo* studies in different biological assays (Christopher and Wong, 2006; Naithani *et al*., 2008). Representatives of some active antiviral compounds from medicinal plants are shown in Table 2.4.

Table 2.4. Mechanism of action of the most active antiviral compounds from medicinal plants (adapted from Jassim and Naji, 2003)

Class of compound	Mechanism of antiviral activity / target
Furyl compounds: furocoumarins, furanochromones	DNA and RNA genomes: Interaction require long wave ultraviolet light
Alkaloids:B-carbolines, furanoquinolines, camptothecin, atropine, caffeine, indolizindine, swainsonine, castanospermine, colchicine, vinblastine	DNA and other polynucleotides and virion proteins. Some interactions are enhanced by ultraviolet light
Polyacetylenes (polyines)	membrane interaction. Phototoxic activity frequently required ultraviolet light
Polysaccharides	blocking viral binding
Thiophenes	membrane interaction. Phototoxic activity frequently required ultraviolet light
Flavonoids: amentoflavone, theaflavine, iridoids, Phenylpropanoid, agathisflavone, robustaflavone succedaneflavanone, chrysosplenol C, morin, coumarins galangin, baicalin	blocking RNA synthesis. Exhibits HIV inhibitory activity
Terpenoids: sesquiterpines, triterpeniods (moronic acid, ursolic acid, maslinic acid and saponin)	membrane mediated mechanisms. Inhibition of viral DNA synthesis
Lignans: podophyllotoxin and related lignans (schizarin-B, rhinacanthin E and F)	blocking virus replication: hepatitis B and influenza A virus
Miscellaneous phenolic compounds: caffeic acid, tannins, eugeniin, hypericin, quinine, salicylate etc	inhibition of virus DNA and RNA replication
Proteins and peptides:	
a) Single chain ribosome inactivation protein	interacts with ribosome function in infected cells thereby inhibiting viral protein synthesis
Pokeweed antiviral protein	inactivate infective HIV and HIV infected cells
Panaxagin	inhibits HIV-1 reverse transcriptase
Alpha and beta antifungal proteins	inhibits HIV-1 reverse transcriptase
b) Dimeric cytotoxins	interacts with ribosome function in infected cells thereby inhibiting viral protein synthesis
c) Lectins	viral membrane interaction
d) Antiviral factor	mechanism not clear
e) Meliacin	affects viral replication cycle

2.4. Cell cultures and toxicity studies

The development of a novel drug requires the evaluation of three major areas in drug design i.e. the efficacy, bioavailability and safety of the drug. About 30% of failures in the development of drugs have been associated with toxicity and safety issues (Kola and Landis, 2004). Among these, toxic effects imposed on the liver by these substances are one of the major issues encountered. Moreover, off-target or idiosyncratic toxicity, resulting in the post market withdrawal of drugs, is of increasing concern. This is to a certain extent due to the unavailability of adequate *in vitro* screening design that can effectively correlate with animal studies and its application to humans. However, Ekwall and co-workers have shown that a test battery of *in vitro* methods can predict human toxicity and that *in vitro* IC_{50} values correlate with *in vivo* LD_{50} data (Ekwall *et al.,* 1998; Clemedson *et al.,* 2000).

The rationale behind using cytotoxicity assays to predict *in vivo* toxicity stems from the concept of 'basal cell cytotoxicity'. It was suggested that for most chemicals, toxicity is an end result of non-specific change in cellular functions. In light of this, assessing the cytotoxic potential of compounds may possibly give an indication of their toxic potential *in vivo*. Cytotoxicity has been defined as the adverse effects resulting from interference of agents with structures and processes essential for cell survival, proliferation and function (Ekwall, 1983). One major factor that warrants consideration in a drug discovery programme is the toxic potential of new chemical entities (NCEs). At this stage, the rationale behind the screening for toxicity would not be directed towards predicting the extent and nature of all possible toxic effects *in vivo* but at the assessment of the risk of failure in *in vivo* studies.

The value of vertebrate cells in testing the toxic effect of substances came to light in the late 1960s/1970s when mammalian cell cultures were used to investigate potential effects posed to humans by chemicals in general (Rees, 1980). The term *in vitro* (literally in glass) refers to keeping entities, such as isolated cells of an organism, outside the living body in an artificial environment, in contrast to *in vivo*, i.e. in the organism. As such, cell cultures are used as *in vitro* models to mimic possible effects in the live animal. According to the use of *in vitro* terminology suggested by Schaeffer (1990), vertebrate cell lines are derived from primary cultures, taken directly from cells, tissues or organs of an organism. When a primary culture is successfully propagated into new culture vessels, it becomes a cell line and may be propagated a limited number of times (finite), or indefinitely, in which case it becomes an immortal or continuous (or permanent) cell line.

The use of vertebrate cells for predicting the toxicity of a substance in whole animals is based on the understanding that any interaction of a substance with an organism is initiated at the level of the cells. Toxic effects on cells by substances can translate to changes in tissue or organ function resulting in a detrimental effect on the whole organisms. Drug regulatory authorities in the USA and Europe emphasize the need for better non-animal alternative

test methods (Collins *et al.,* 2008; Xia *et al.,* 2008). Based on the central role of cells in the expression of toxicity, several mammalian *in vitro* models have received regulatory acceptance by the Organisation for Economic Cooperation and Development (OECD) as alternatives to whole animal tests. The European Commission also encourages the application and development of alternative models to the use of live animals in line with the new European legislation on the Registration, Evaluation and Authorisation of Chemicals (REACH) to be executed in an ethically and financially acceptable manner (Castaño *et al.,* 2003).

Besides their potential to replace or reduce animals in toxicity tests, cell cultures have several advantages compared to whole animal tests. For instance, small amounts of potentially toxic substances can be rapidly screened and analyzed in large numbers. With the quantity of test substance involved, less toxic waste is produced. The use of cells can also help identify the mechanisms underlying a toxic response. For instance, Noor *et al.* (2009), in a study using frequently used cytotoxicity assays, compared the toxic effect of different hepatotoxins on the human hepatoma cell line (HepG2) and rat hepatocytes. The study revealed that careful selection of assay parameters and inclusion of a kinetic time based assay improved prediction for non-metabolism mediated toxicity using HepG2 cells. With a better characterization of a wider range of cell culture models for use in *in vitro* tests, a more selective approach can be adapted in the choice of cells most suitable for the type of test to be conducted and the substance to be tested. Thus, to study the effect of medicinal plants (MPs) in cell culture, testing of extracts with a wide range of cell types should be undertaken, since cytotoxicity to a certain degree is cell-type-specific (Tang *et al.,* 2004). One should however, keep in mind that changes in the animal gut or pharmacokinetic issues such as absorption, distribution, metabolism and excretion may break the link between cellular and whole animal toxicity.

2.5. Antioxidants and cell culture

An antioxidant is defined as "any substance that delays, prevents or removes oxidative damage to a target molecule" (Halliwell and Gutteridge, 2007). Certain groups of compounds like flavonoids and other polyphenols have powerful antioxidant activities *in vitro*, by scavenging a wide range of reactive species, including hydroxyl radicals, peroxyl radicals, hypochlorous acid and superoxide radical (Rice-Evans, 2000). Flavonoids can also inhibit biomolecular damage by peroxynitrite *in vitro* (Pannala *et al.,* 1997; Heijnen *et al.,* 2001; Santos and Mira, 2004). However, this biomolecular damage is said to be less effective in the presence of physiological levels of HCO_3^-/CO_2 (Ketsawatsakul *et al.,* 2000; Santos and Mira, 2004). Figure 2.2. illustrates how cells respond to oxidative stress. Oxidative stress is an end result of a serious imbalance between reactive species (RS) production and antioxidant defence. Under such circumstances, cells elicit a wide range of responses as a result of this imbalance, ranging from increased proliferation, prevention of cell division, senescence, necrosis, apoptosis, or cell death mechanisms.

As such, antioxidants can be helpful to control the levels of free radicals and other RS to minimize oxidative damage, especially where imbalance between stress and protective elements *in vivo* is considered to play a role in disease development (Halliwell and Gutteridge, 2007). The beneficial effects of many medicinal plants may be via antioxidant activity due to high polyphenolic contents of these plants.

Cell culture is one of the most popular and commonly used methods to study the cellular effects of medicinal plants and chemical constituents isolated from them. In the human body, with the exception of the cells lining the corneal, skin and respiratory tract, most of the cells are exposed to O_2 concentrations in the range of 1–10 mm Hg while cells cultured under laboratory conditions of 95% air or 5% CO_2 experience about 150 mm Hg of O_2. Therefore, cells cultured under laboratory conditions are constantly under oxidative stress because the rate of reactive oxygen species (ROS) production from cellular enzymes increases when O_2 levels are increased (De Groot and Littauer, 1989; Halliwell, 2003). This phenomenon is most likely to increase their rates of ROS formation.

Due to the rich phenolic content of MPs, their oxidation in cell culture media can provoke biological effects in cultured cells. Such effects can make results of cell culture studies often confusing because of the oxidation process in the medium, especially when iron and other transition metals are present in the medium (Halliwell, 2003). Oxidation in culture media has also led to uncertain results in at least some studies of the effects of ascorbate, phenolic compounds and other antioxidants on cells. The observed effects due to oxidation following the addition of these compounds to culture media resulted in the production of H_2O_2 and other oxidation products (e.g. quinines and semiquinones from polyphenols) that were the true mediators of the effects observed (Halliwell, 2003; Long *et al.,* 2000; Lapidot *et al.,* 2002; Clement *et al.,* 2001; Chai *et al.,* 2003). Also H_2O_2 generation rates can be affected in complex ways when two or more antioxidants are present (Wee *et al.,* 2003). Some studies have shown that phenolic compounds are more stable in culture media such as F-10 and F-12 and can help minimize artefacts in cell culture associated with the use of DMEM (Long *et al.,* 2007).

To prevent oxidation in culture media, the addition of catalase or presence of pyruvate in some culture media can help scavenge H_2O_2 when generated. However, the absence of H_2O_2 in such media does not necessarily imply the lack of oxidation by medicinal plants in cell culture media (Long and Halliwell, 2009).

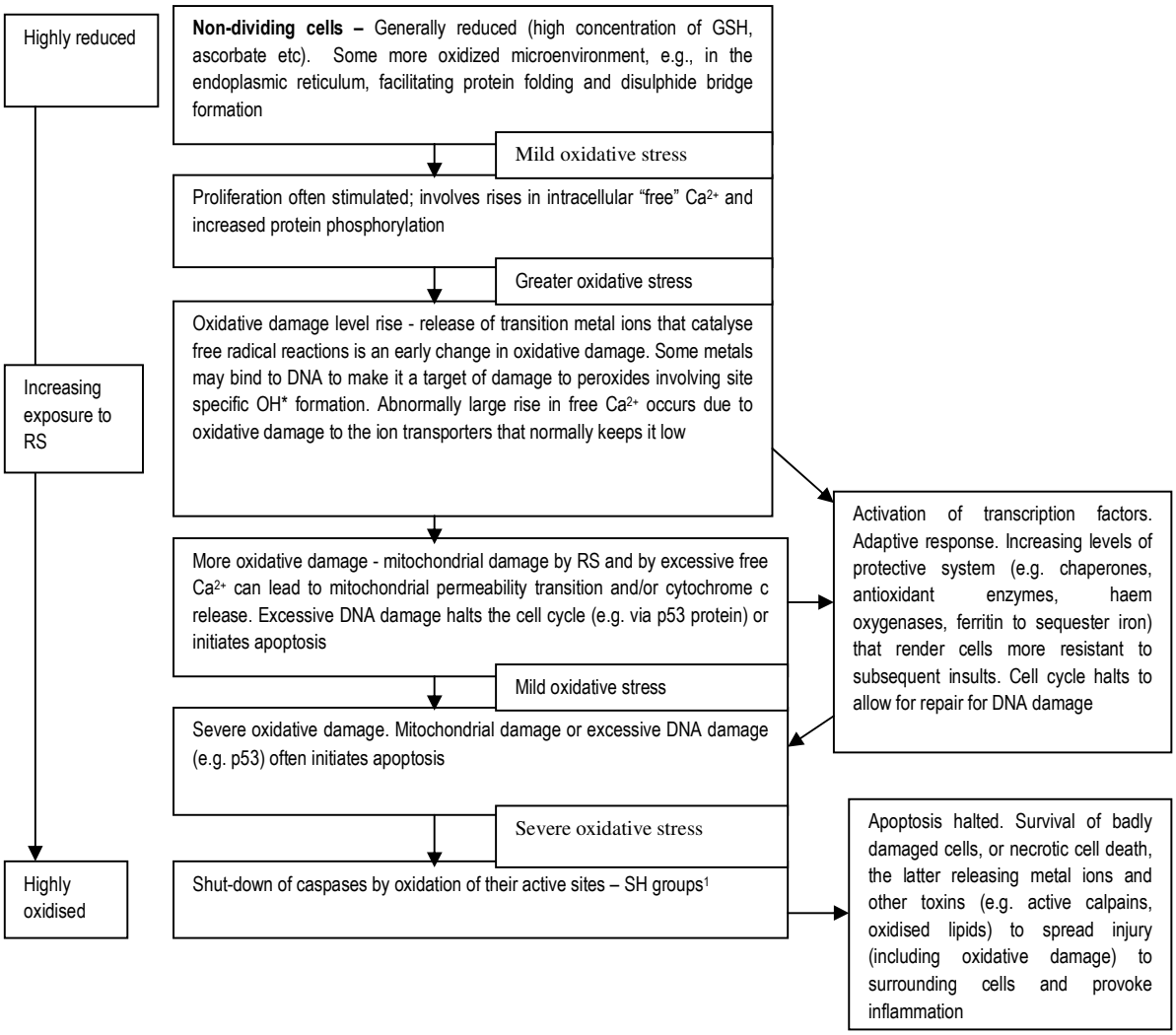

Figure 2.2: Response of cells to oxidative stress (Adapted from Halliwell and Gutteridge, 2007)

2.6. Hypothesis

1. Antibacterial and antifungal compounds can be isolated using bioassay-guided fractionation since activity against these pathogens is much easier to determine than antiviral activity.

2. The antibacterial and antifungal compounds isolated may have antiviral activity with relatively low toxicity. Some plant species have been shown to have antibacterial, antifungal and antiviral activity and an antibiotic has been reported as an effective prophylaxis in smallpox infection (Bauer *et al*., 1963).

2.7. Aim of the study

The aim is to develop a low toxicity plant extract or isolated compound that is effective against bacteria, fungi or viruses.

2.8. Objective

The aim of this study will be reached by addressing the following:

1. Determining antibacterial and antifungal activity of different leaf extracts of selected plant species
2. Determining the cytotoxic effect of different extracts on selected cell types compatible for the group of viruses chosen for later testing
3. Determining the antiviral activity of the different extracts on selected viruses
4. Selection of plant species to be investigated
5. Selection of the best extractant for high activity of the plant material
6. Isolation of antibacterial and antifungal compounds
7. Determining the chemical structure of isolated compounds
8. Determining the cytotoxic and genotoxic effects of isolated compounds
9. Determining the antibacterial, antifungal and antiviral activity of isolated compounds
10. Evaluating correlations between antiviral and antimicrobial activity