CHAPTER 7

7. BIOLOGICAL ASSAYS

7.1 ANTIMICROBIAL ACTIVITY

The MIC values were calculated using the well dilution method as described in chapter 4. All experiments were repeated at least in duplicate, with some compounds repeated at least six times for more accurate results. For all organisms a standard antibiotic, either gentamicin or ampicillin, was included as a positive control. Amphotericin B was included for the fungus. Since all compounds were solubilized in DMSO and made up to final concentration of 200 μg/ml with distilled water, the same quantity of DMSO to water was included as a negative control.

Figures 7.1 and 7.2 show the activity of test compounds against four common pathogens, namely *E. coli*, *S. sonei*, *V. cholerae* and *E. faecalis*. MIC values were calculated by comparing the size of the pellet of test compound dilutions with those of the growth control (Medium + test organism) (Table 7.1).

Table 7.1: MIC values (μg/ml) of isolated compounds using 11 different bacterial species and a fungus.

	CE36	CE46	CE51	IIIa90	IIIa150
S. typhimurium	>100	>100	>100	>100	>100
K. pneumonia	>100	>100	>100	>100	>100
S. faecalis	**50**	50-100	>100	50-100	50-100
M. luteus	100	**25-100**	>100	**25**	**50**
E. coli	50-100	50-100	100	50-100	100
S. aureus	>100	>100	>100	**50**	>100
S. sonei	**50**	**25-50**	>100	**25-100**	**25-50**
V. cholerae	**25-50**	**50**	**50**	**25-50**	**50**
P. aeruginosa	100	100	100	100	100
E. faecalis	**50**	**25-50**	**25**	**25-50**	50-100
B. subtilis	>100	>100	>100	>100	>100
A. niger	>100	>100	>100	>100	>100

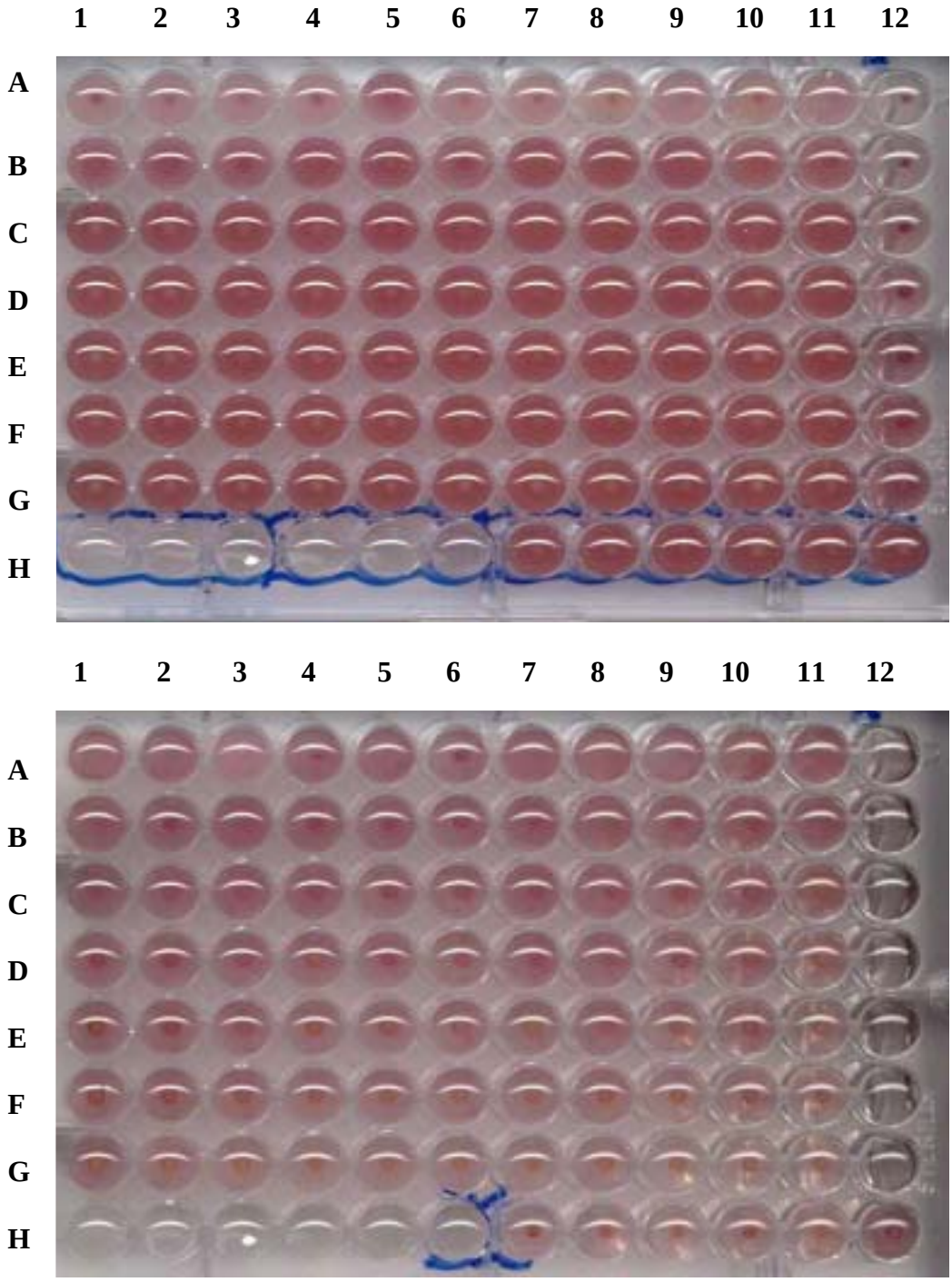

Figure 7.1: MIC plate of *E. coli* (top) and *S. sonei* (bottom) with the concentration in well A = 100µg/ml, B = 50 µg/ml etc. H1-6 represents the sterility control and H7-9 is the growth control with which the compounds were compared. Row 12 is the antibiotic controls, gentamicin (10mg/ml)and ampicillin (250 µg/ml)respectively. The compounds are represented as follows: 1 = IIIa142; 2 = CE37; 3 = CE32; 4 = IIIa150; 5 = IV33; 6 = IIIa105; 7 = CE46; 8 = CE139; 9 = CE36; 10 = IIIa90 and 11 = CE43.

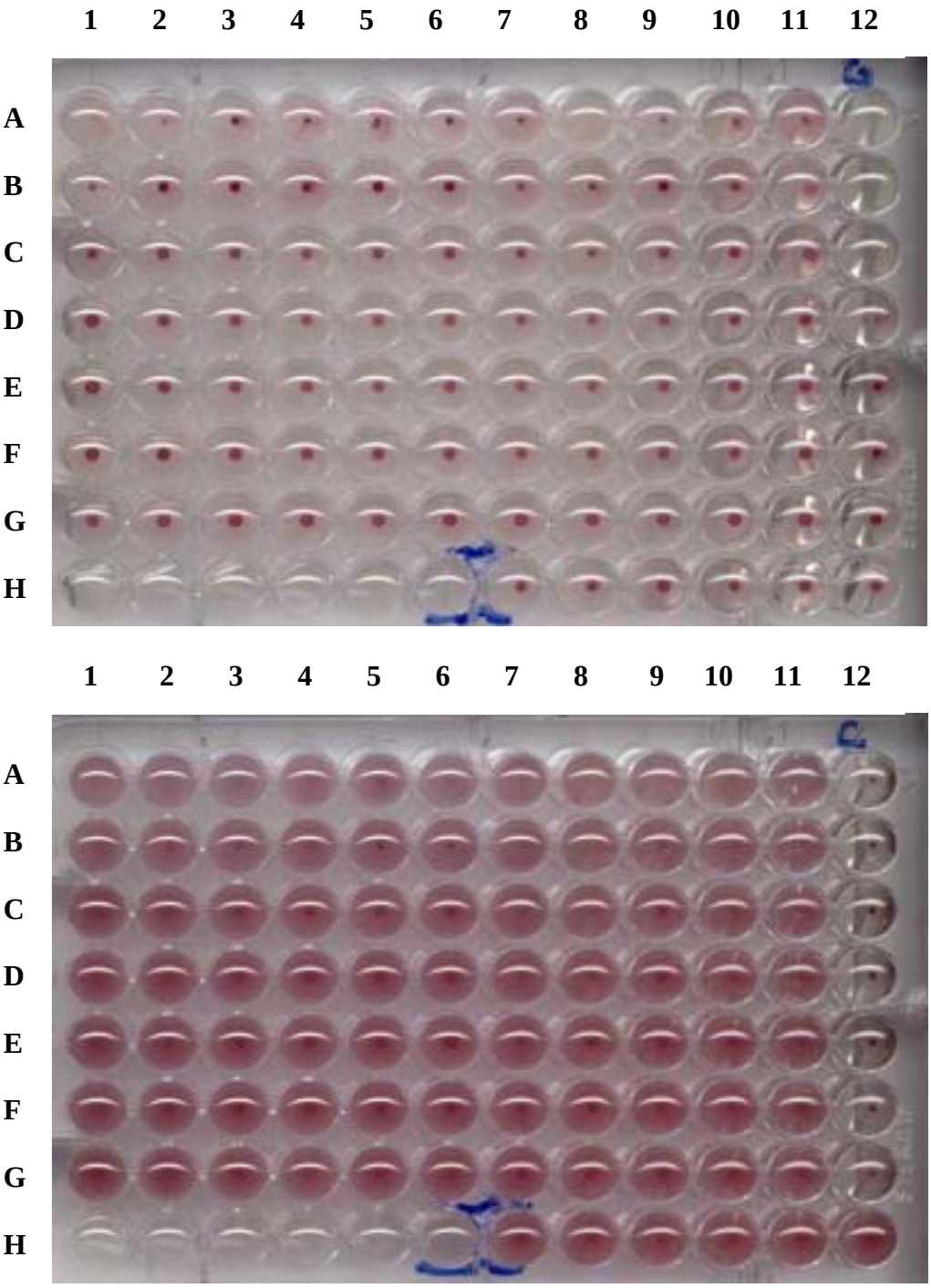

Figure 7.2: MIC plate of *E. faecalis* (top) and *V. cholerae* (bottom) with the concentration in well A = 100µg/ml. H1-6 represents the sterility control and H7-9 is the growth control. Row 12 is the antibiotic positive control, which is gentamicin (10mg/ml) and chloramphenicol (250µg/ml) respectively. The compounds are represented as follows: 1 = IIIa142; 2 = CE37; 3 = CE32; 4 = IIIa150; 5 = IV33; 6 = IIIa105; 7 = CE46; 8 = CE139; 9 = CE36; 10 = IIIa90 and 11 = CE43.

Some of the compounds were identical e.g. CE32, CE36 and CE37 and the MIC range of inhibition is presented (Table 7.1). Other identical compounds were CE43 and CE46; IIIa90, IIIa105 and CE139. Although in some cases no inhibition was apparent, those results showing inhibition were used as these indicated a possibility of antimicrobial activity. DMSO and water did not inhibit growth of organisms.

A. niger gave unpredictable and unreliable results. Some compounds seemed to enhance the growth of the fungus, whereas in other wells, the same compound showed inhibition. Also no growth was observed in wells with very diluted test compounds. For this reason, a member of the same family, *A. fumigatus*, was attempted, which produced clearer results, possibly due to experience developed with the technique procedure. There was no apparent inhibition at the concentrations used (100 mg/ml) and the size and colour of the wells were the same as those of the growth control. Amphoteracin B exhibited an MIC value below 0.78 mg/ml.

Test compounds exhibited similar activities against microorganisms, possibly due to similarities between structures and hence structure activity relationships. There does not appear to be a vast difference in inhibitory activity between Gram-positive and Gram-negative organisms, and it is therefore unclear how these compounds target microbial invasion but is most probably not due to inhibition of cell wall synthesis. With some compounds there appears to be a bacteriostatic rather than bactericidal activity as wells previously showing inhibition had become infected after prolonged incubation periods.

SUMMARY OF RESULTS:

7.1.1 CE36 (5-hydroxy-7,4'-dimethoxyflavone)

This compound exhibited good activity against *V. cholerae* (25-50µg/ml), *S. sonei* (50 µg/ml), *E. faecalis* (50 µg/ml) and *S. faecalis* (50 µg/ml). Not much data regarding the antimicrobial activity of this compound has been recorded but complexes of CuII, NiII, CoII, ZnII, FeII, CrII, CdII and MnII have been synthesized with 5-OH-7,4'-dimethoxyflavone and activity of both the ligand and the complexes have been determined on Gram positive and negative organisms [Wang, 1992].

7.1.2 CE46 (Quercetin-5,3'-dimethylether)

Activity against *V. cholerae* (50µg/ml), *S. sonei* (25-50 µg/ml), *E. faecalis* (25-50 µg/ml) and *M. luteus* (25-100 µg/ml) was recorded. No antimicrobial data regarding this compound could be sourced in the literature.

7.1.3 CE51 (Rhamnazin)

Thic compound did not appear to be very active except in *V. cholerae* (50µg/ml) and *E. faecalis* (25 µg/ml). No literature relating to this compound's antimicrobial activity was sourced.

7.1.4 IIIa90 (Rhamnocitrin)

Activity was recorded against *V. cholerae* (25-50µg/ml), *S. sonei* (25-100 µg/ml), *E. faecalis* (25-50 µg/ml), *S. faecalis* (50-100 µg/ml) and *M. luteus* (25 µg/ml), making this compound quite active. This was the only flavonoid to show activity against *S. aureus* at 50 µg/ml.

Antibacterial activity has been tested against *Streptococcus mutans, Actinomyces viscosus, Porphyromonas gingivalis* and *Prevotella intermedia* [Cai, 1996]

7.1.5 IIIa150 (Genkwanin)

Genkwanin is found to accumulate in barley leaves in response to attack [Christensen, 1998]. This phytoalexin is active against *V. cholerae* (50μg/ml), *S. sonei* (25-50 μg/ml), *E. faecalis* (50-100 μg/ml), *S. faecalis* (50-100 μg/ml) and *M. luteus* (50 μg/ml).

7.1.6 Seph51 (Kaempferol)

Although not included in this testing due to widespread studies, kaempferol-3-O-rutinoside has been found to exhibit low antibacterial and antimycotic activities 125 μg/ml against *S. aureus* and 250-500 μg/ml (*C. albicans*) [Bisignano, 2000]. It has also potent growth inhibitory activity against periodontal pathogens *Porphyromonas gingivalis* and *Prevotella intermedia* [Cai, 1996].

7.1.7 CE144 (Apigenin)

Also not included in the testing due to widespread studies, apigenin has been found to be selectively toxic to *S. aureus* including MRSA and methicillin sensitive strains with MICs ranging from 3.9-15.6 μg/ml. In general Gram-negative bacteria are less sensitive to inhibitory action of this phytoalexin than Gram-positive bacteria [Kurosaki and Nishi, 1983] and some studies have demonstrated that apigenin exhibited no activity against some Gram-positive coccal strains, including S*treptococcus epidermidis* and no appreciable activity against bacteria other than *S. aureus*. All MICs were greater than 250 μg /ml [Sato, 2000].

Other studies showed activity against *P. mirabilis* (16 µg/ml), *P.aeruginosa* (8 µg/ml). *S. typi* (128 µg/ml), *E.coli* (128 µg/ml), *E. aerogenes* (4 µg/ml), *E. cloaceae* (4 µg/ml) and *K. pneumoniae* (128 µg/ml). Apigenin has been found to exert a higher activity than apigenin-7-*O*-triglycoside. Glycosilation of apigenin causes a reduction in antibacterial power due to reduction in lipophilia and a diminished ability to penetrate bacterial membrane [Basile, 1999].

7.2. TOXICITY ASSAY

Lactate dehydrogenase (LDH) is a cytosolic enzyme present within all mammalian cells. The normal plasma membrane is impermeable to LDH, but damage to the cell membrane results in a change in the membrane permeability and subsequent leakage of LDH into the extracellular fluid. *In vitro* release of LDH from cells provides an accurate measure of cell membrane integrity and cell viability. As a result, the release of LDH is a popular and reliable test for cytotoxicity in immunological as well as biocompatibility studies (Allen, 1994).
This assay is based upon the ability of LDH to catalyse the reaction:

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{Lactate}^{+} + \text{NAD}^{+}$$

Changes in optical absorbance, measured at 340 nm, reflect changes in NAD^+ and therefore the level of LDH in the test compound.

For comparative purposes, a positive control is required which causes substantial cell membrane damage. The compound included in this study was LPC (lysophosphatidylcholine), which destroys the cell membrane causing maximum release of LDH. Negative controls included a water control and DMSO + water control. The concentration of the test compounds used in this assay was 100 µg/ml, the highest concentration used to test antimicrobial activity and chosen for this reason.

The main aim was to show whether these compounds could be selectively toxic against micro-organisms and spare the host cells.

The percentage LDH release was calculated by subtracting the time after 1, 5 and 10 minutes from time 0, dividing by time 0 x 100.

i.e. Time 0 = 0.775 Time after 1 min = 0.675
Therefore 0.775-0.675 = 0.1/0.775 x 100 = 12.9%

These values were plotted on a graph (Figure 7.3) for comparative purposes.

Table 7.2: The average percentage LDH released from test compounds after 1, 5 and 10 minutes.

	1	5	10
Water	8	33	48
Water + DMSO	5	19	27
LPC	35	76	77
CE36	11	39	47
CE46	4	17	25
CE51	8	34	47
IIIa90	5	20	30
IIIa150	7	34	43

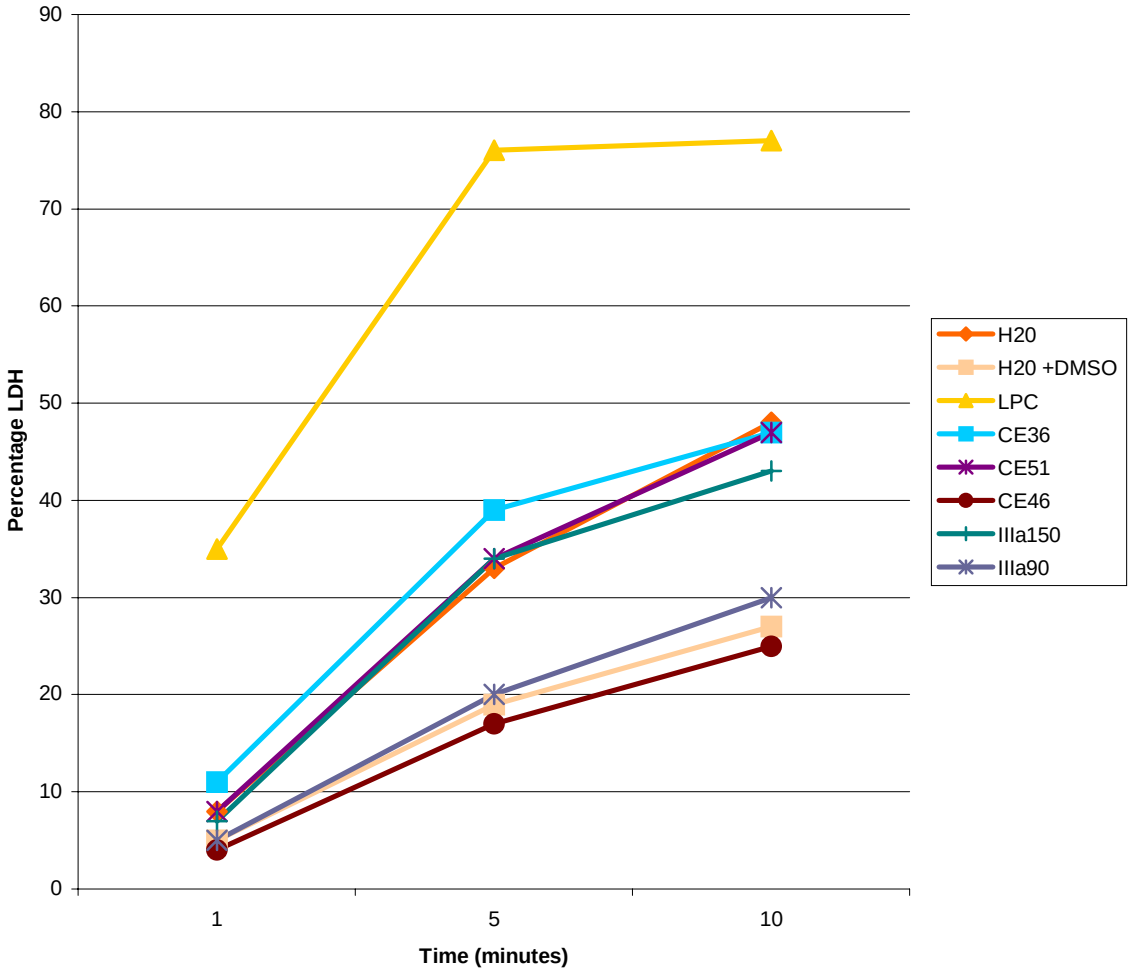

Figure 7.3: The average percentage LDH released by LPC, water, water + DMSO and 5 test compounds

According to these results it is apparent that most of the compounds have similar toxicity profiles to that of water. Since water in large volumes can cause lysis of cells, this could possibly explain the increase in LDH levels. DMSO + water was used as solvent for the compounds and did not appear to influence the cells to any large extent. Rhamnocitrin (IIIa90) and quercetin-5,3'-dimethylether (CE46) had similar profiles to that of the solvent, whereas genkwanin (IIIa150) and rhamnazin (CE51) were close to that of water.

By assaying LDH leakage from LLC-PK1 cells in culture, a study was carried out to clarify whether flavonoid compounds ameliorate renal cellular injury. Genkwanin had virtually no effect. Yokozawa [1999] suggested that the difference in activity is largely decided by the number and position of phenolic hydroxyl groups linked to structural backbone.

Compound CE36 had in some cases shown values very close to those obtained with LPC and since its influence on cell lysis was greater than that of water, therefore it is presumed to be potentially toxic.

7.3 ANTIOXIDANT / ANTI-INFLAMMATORY RESPONSES

Inflammation is a series of events that occur in response to various injuries to living tissues. It is characterised by local vascular changes and infiltration of leukocytes, especially granulocytes in acute inflammation. Granulocytes exhibit an increase in chemotaxis, degranulation and phagocytosis, and the activity is controlled in time and intensity so that healing takes place as soon as possible. Luminol-dependant chemiluminescence (CL) has been recognised as a useful tool for evaluating the phagocytic activity of granulocytes and lymphocytes. Changes in counts of luminol-dependant CL can represent the effects of various drugs on the functions of granulocytes [Ozaki, Y, 1984].

Luminol is converted to an excited amonophthalate ion in the presence of oxidising compound and this reaction emits blue light measured by the chemilumninometer. The chemical basis of the chemiluminescence reaction is not known in every detail but superoxide anion and the myeloperoxidase product hypochlorite (HOCl) are necessary for generating luminol amplified chemiluminescence [Dahlgren as quoted by Wiik, 1996].

Lymphocytes were used primarily due to ease of isolation and greater stability in comparison to neutrophils whose life-span is considerably shorter. A blank (cells + luminol + zymosan) was included as negative control and mefenamic acid (100 mg/ml), a non-steroidal anti-inflammatory drug, as positive control. Cells were stimulated with opsonised zymosan as described in Chapter 4.

Results (Figure 7.4) show, as expected, the blank control emitting the greatest percentage of light (in millivolts) due to superoxide production. The solvent (DMSO + water) had virtually no effect on the free radical production and therefore did not interfere with activity of the test compounds. Mefenamic acid is a commercially available anti-inflammatory and analgesic preparation with moderate anti-inflammatory activity. It is expected to reduce inflammatory components suppressing the reaction. All compound were compared to this positive control.

Genkwanin (IIIa 150) showed greater suppression of the curve than mefenamic acid and was assayed as an *in vitro* antioxidant by its ability to inhibit tert-butyl hydroperoxide initiated chemiluminescence of mouse liver homogenates (IC50 > 1 microM) [Fraga, 1987]. It makes a promising substance to be investigated as a water-soluble protector against lipid peroxidation and other free radical-mediated cell injury. CE46 had a similar activity to genkwanin but no information regarding its activity could be sourced in the literature.

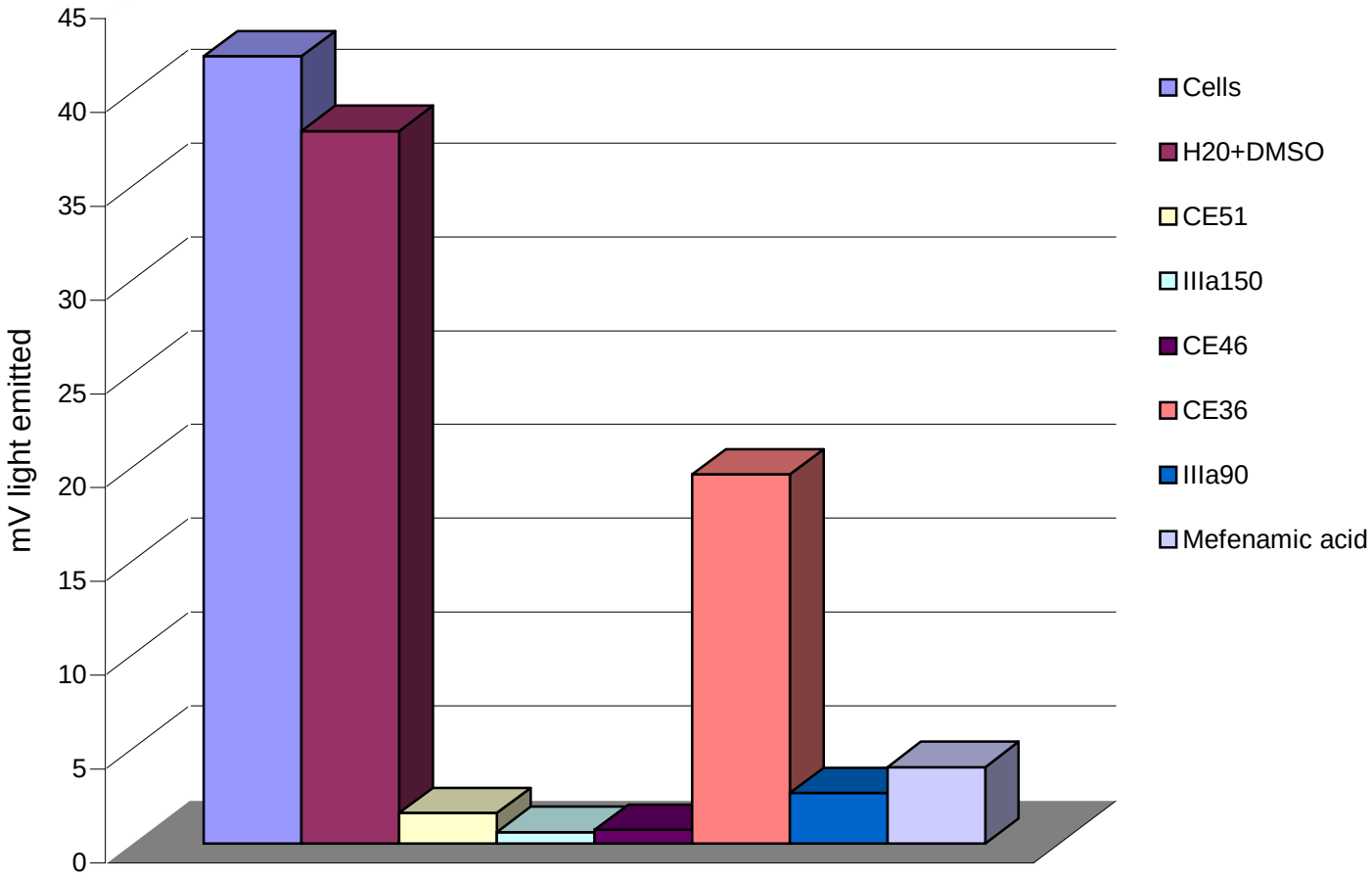

Figure 7.4: Free radical production (measured in millivolts light emitted) after 30 minutes produced by lymphocytes after stimulation with zymosan and test compounds

Both IIIa90 (rhamnocitrin) and CE51 (rhamnazin) showed strong antioxidant effects confirming the results of Manez [1999] and Yun [2000]. Rhamnocitrin was found to exert anti-inflammatory action and exhibit effectivity against 12-O-tetradecanoylphorbol-13-acetate-induced ear oedema in mice, although lacking activity against arachidonic acid-induced oedema [Manez, 1999]. Rhamnazin significantly inhibited lipid peroxidation in rat liver microsome [Yun, 2000].

Although radical production was inhibited by CE36 it had the weakest activity and is therefore not regarded as having good antioxidant activity. A compound not tested was Seph51 (kaempferol) which is already known to have a remarkably high ROO(*)-scavenging activity. It is suggested that a diet rich in these radical scavengers would reduce the cancer-promoting action of ROO(*) and consequently the carcinogenic potentials of oxygen-related radicals may be suppressed [Sawa, 1999].