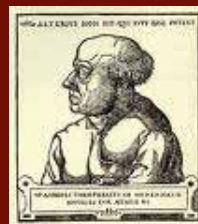


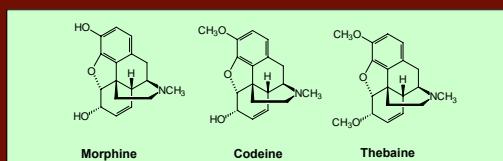
Together we're stronger: Extracts vs isolated compounds in phytotherapy

Peter Houghton
Emeritus Professor in
Pharmacognosy
Pharmaceutical Sciences Division
King's College London

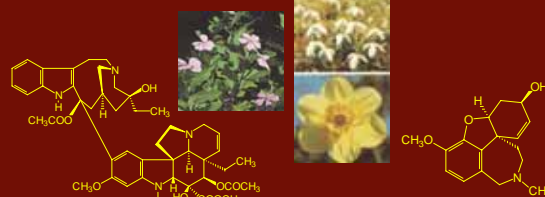


Paracelsus (1493-1541)
– the concept of the 'active ingredient'

Activity of an extract due to one compound



Naturally-occurring compounds in current therapeutic use



Vincristine from *Catharanthus roseus*
for leukemia

Galantamine from *Galanthus*
and *Narcissus* spp. for
Alzheimer's disease

'One active constituent' - is this the only paradigm for explaining activity of medicinal plants?

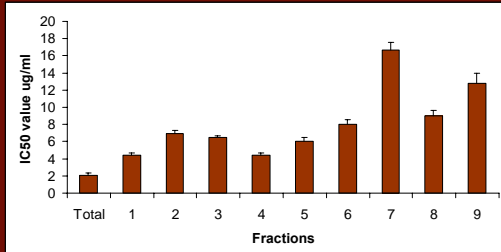
- If no compound easily isolated – plant largely neglected in modern therapeutics
- Most active compounds from 'poisonous', rather than 'medicinal', plants
- Continuing and increasing use of 'herbs' in recent years in Western society
- Recent validation of usefulness of some of these in clinical studies
- Advances in isolation and structural characterisation techniques has advanced knowledge of chemicals present
- In vitro bioassays have enabled bioassay-guided isolation of active compounds

Bioassay-guided isolation of active compounds

- Devise bioassay related to traditional disease state
- Test extract made in similar way to tradition
- If active, fractionate extract e.g. by chromatography
- Test fractions, select most active, refractionate etc until pure compounds isolated
- Determine structure of pure compounds
- Test activity of pure compounds
- Determine mode of action, SAR studies, synthesis of analogues

Bioassay-guided isolation of active compounds

Which fraction contains the 'active'?



'Active ingredient'

- it all depends

'Active ingredient'

- it all depends on the concentration

ANTI-INFLAMMATORY THAI MEDICINAL PLANTS

from ethnobotanical survey

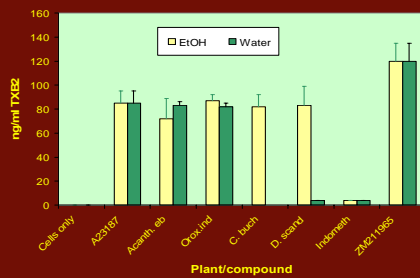
Acanthus ebracteatus Vahl. -leaves used for joint pain, roots for asthma

Oroxylum indicum (L.) Vent. - root or stem extract used on inflamed joints

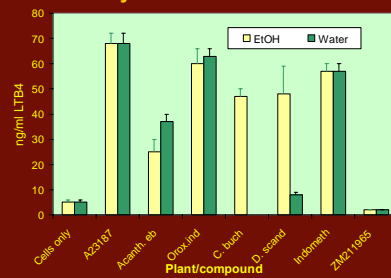
Cryptolepis buchanani Roem. & Schult - leaf or stem applied or taken for muscle pain

Derris scandens Benth. - stem used for inflammation and fever

ANTI-INFLAMMATORY THAI MEDICINAL PLANTS Extracts (100ug/ml) tested for TXB₂ synthesis inhibition



ANTI-INFLAMMATORY THAI MEDICINAL PLANTS Extracts (100ug/ml) tested for LTB₄ synthesis inhibition



Bioassay guided fractionation of *D. scandens* aqueous extract

Freeze-dried aqueous extract of *D. scandens* stem

VLC on C-18 silica
Water-methanol gradient
TLC analysis

A1 12±1
A2 20±2
A3 49±4
A4 88±4

TXB₂ Inhib (%) 125ug/ml

Rhamnose-Glucose

B

Hydrolysis
Prep TLC

Sugars

AC1

AC2

Silica CC

ANTI-INFLAMMATORY THAI MEDICINAL PLANTS

IC₅₀ values (µg/ml) for compounds on eicosanoid synthesis

Compound	TXB ₂	LTB ₄
B	1500	2500
AG1	100	80
AC1	3	6
AC2	8	1.6

% inhibition

concn AC2 ug/ml

1 5 25 50

10µmeth

ZM219055

□ TXB₂ ■ LTB₄

ANTI-INFLAMMATORY THAI MEDICINAL PLANTS

Content of compounds and their activity

	<i>D. scandens</i> extract			
	B	AC1	AC2	
IC ₅₀ TXB ₂ generation	188	875	30.4	3.64
Amount *(ng) /1µg extract		160.8	2.7	0.8
Amount (µg) in 188µg extract	30.2	0.5	0.15	

* From HPLC measurement

Compound	TXB ₂	LTB ₄
B	1500	2500
AG1	100	80
AC1	3	6
AC2	8	1.6

IC₅₀ µg/ml

'Active ingredient'

– it all depends on the compounds present

Cholinergic effects in 'Paan'

'Paan'

Betel nut *Areca catechu*

- Paan - chewed regularly by over 500 million people, mainly in South Asia
- Shredded fruits of *Areca catechu* mixed with crude lime and wrapped in leaf of *Piper betle*
- 'Kathi'– extract of *Acacia catechu*, is often added
- Arecoline from *Areca catechu* is a cholinergic alkaloid

Acetylcholine

Arecoline

'Paan'

Betel nut *Areca catechu*

- Extracts of *Areca catechu* also shown to have AChE inhibitory activity.
- Bioassay-guided fractionation led to identification of (+)-catechin and (-)-epicatechin as active compounds
- These also occur in *Acacia catechu* extract
- Paan therefore possesses a high cholinergic activity 1) due to direct cholinergic agonist by arecoline and 2) by AChE inhibition by catechins from *Areca catechu* and *Acacia catechu*

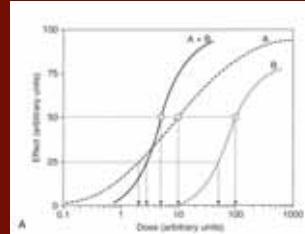
(+)-Catechin
IC₅₀ 12.5 µM

(-)-Epicatechin
IC₅₀ 6.1 µM

Mukherjee & Houghton (2006) *J Pharm Pharmacol Suppl.* A83

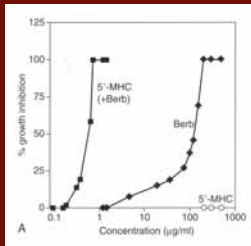
'Active ingredient' – it all depends on synergy

SYNERGY - different compounds with same activity combined produce a greater effect than expected



Dose-response curves
for two compounds A
and B,
- alone and combined

SYNERGY - different compounds with same activity: combined produce a greater effect than expected

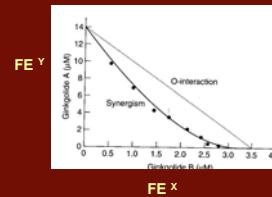


Antimicrobial effect of
berberine (berb)
and
5'-methoxyhydrnocarpin (5'- MHC)

Stermitz et al. (2000) *Proc.Nat
Academy of Sciences of USA*
97:1433-1437.

SYNERGY

FE^x = MIC X in combination with Y
MIC X independently
FE^y = MIC Y in combination with X
MIC Y independently

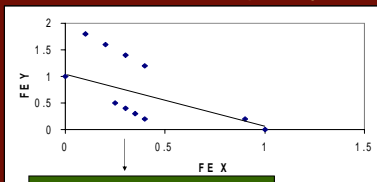


Isobologram to
demonstrate synergy
between platelet-
aggregation inhibition
of ginkgolides A (X)
and B (Y).

Williamson, E.M. (2009) Synergy in relation to the pharmacological action of phytomedicinals. In *Trease and Evans Pharmacognosy, 16th edn* Ed. W.C. Evans. W.B.Saunders, Edinburgh, UK, pp 53-61.

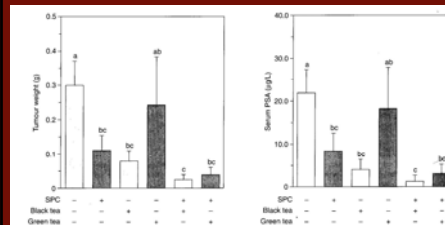
SYNERGY

FE^x = MIC X in combination with Y
MIC X independently
FE^y = MIC Y in combination with X
MIC Y independently



Isobologram to demonstrate synergy, additive and antagonistic antibacterial effects with different ratios of volatile and non-volatile fractions of the South African herb *Tarchonanthus camphoratus* L. (Kamatou et al. (2008) *Nat. Prod. Comm.* 3: 1111-1115.)

SYNERGY - extracts from different plant species – combined produce a greater antitumour effect than expected



SPC = soya
phytochemicals
Black tea
Green tea

Mice containing
human prostate
cancer cells

Williamson, E.M. (2009) Synergy in relation to the pharmacological action of phytomedicinals. In *Trease and Evans Pharmacognosy, 16th edn* Ed. W.C. Evans. W.B.Saunders, Edinburgh, UK, pp 53-61.

'Active ingredient' – it all depends on polyvalence

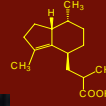
POLYVALENCE - For a plant extract, the overall effect is greater or more complex than might be predicted from considering the individual components

A variety of compounds with a variety of activities contributing to the overall effect

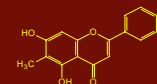
Valerian - anxiolytic and to help sleep



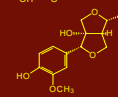
Valeriana officinalis



Valerenic acid – acts on GABA receptor, inhibit breakdown of GABA



Flavonoids – bind to benzodiazepine receptors



Lignans – inhibit 5-HT binding

Valepotriates - relax smooth muscle

Which is the 'active ingredient'?

Polyvalence shown by in vitro tests for wound healing properties

Wound healing - a complex process

- Inflammation
- Cell (mainly fibroblast) proliferation
- Collagen formation and contraction of collagen lattice

Impediments to wound healing

- Microbial infection
- Oxygen free radicals

WOUND-HEALING PLANTS FROM ASHANTI REGION, GHANA

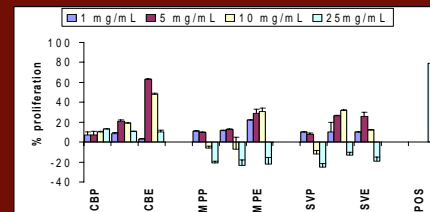
Material investigated

Plant species	Part used	Mode of application
<i>Caesalpinia benthamiana</i> (Baill.) Herend. & Zarucchi	Root bark	Paste made with shea butter
<i>Microglossa pyrifolia</i> Lam.	Root bark	Paste made with water
<i>Paullinia pinnata</i> L.	Root	Decoction of roots in water
<i>Securinega virosa</i> Roxb. & Willd.	Root bark	Paste made with water

TESTS FOR WOUND HEALING FIBROBLAST GROWTH STIMULATION

- Human dermal fibroblasts cultured and passaged
- Cells from 5th to 9th passage used, grown in microtitre well plates
- Treated with compound or extract for specified period
- Positive (2% FCS) and negative controls used
- Medium removed, cells washed with phosphate buffer, Neutral Red solution added and incubated for 2h.
- Neutral Red removed, cells washed and then the cells were lysed to release absorbed Neutral Red
- Absorbance measured at 540nm, the greater the absorbance, the greater number of viable cells present

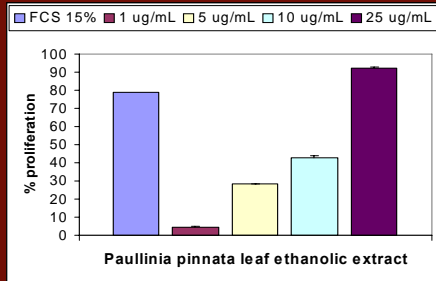
FIBROBLAST GROWTH STIMULATION



CB = *C. benthamianum*; MP = *M. pyrifolia*; SV = *S. virosa*
P = petroleum spirit C = chloroform E = ethanol
POS = 15% Foetal calf Serum
***C. benthamianum* ethanol extract gives the greatest effect**

FIBROBLAST GROWTH STIMULATION

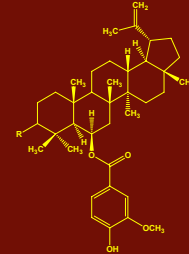
King's College LONDON
Founded 1829



FIBROBLAST GROWTH STIMULATION

King's College LONDON
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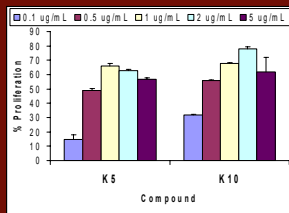
- *Paullinia pinnata* root extract shows greatest fibroblast growth stimulatory effect
- Bioassay-guided fractionation led to the isolation of two active compounds K5, K10



K5 R = O
K10 R = H α , OH β

FIBROBLAST GROWTH STIMULATION

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K5 R = O
K10 R = H α , OH β

ANTIMICROBIAL ASSAYS serial dilution for Minimum Inhibitory Concentration (MIC $\mu\text{g/mL}$)

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	SA	MRSA	BS	MF	MS	EC	PA	KA
CBP	125	64	125	250	500	>1000	1000	>1000
CBC	250	128	250	500	>1000	>1000	>1000	>1000
CBE	125	64	63	31	>1000	>1000	500	>1000
MPE	>1000	>1000	500	>1000	>1000	>1000	>1000	>1000
SVC	125	64	250	250	250	500	>1000	250
SVE	500	>1000	500	500	>1000	>1000	>1000	>1000
PPE	32	>1000	256	32		128	256	

CB = *C. benthamianum*; MP = *M. pyriformis*; SV = *S. virosa*; PP = *P. pinnata*
P = petroleum spirit C = chloroform E = ethanol
SA = *Staph. Aureus*; MRSA = Methicillin-resistant SA; BS = *B. subtilis*; MF = *Micrococcus flavus*; MS = *Mycobacterium smegmatis*; Ec = *E. coli*; PA = *Ps. aeruginosa*; KA = *K. aerogenes*

TESTS FOR WOUND HEALING - ANTIMICROBIAL ASSAYS

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- *C. benthamianum* ethanol extract gave the greatest antibacterial activity
- Several compounds isolated, including 4 diterpenoids
- Compounds tested against bacteria

TESTS FOR WOUND HEALING ANTIMICROBIAL ASSAYS ON DITERPENOIDS FROM *C. benthamianum*

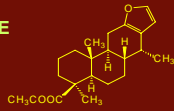
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	SA	MRSA	BS	MF	MS	PA	SF
R1	64	64	16	32	>1000	250	>1000
R2	16	32	32	16	>1000	125	125
R3	32	32	64	34	32	>1000	>1000
R4	>1000	>1000	>1000	>1000	>1000	>1000	>1000

SA = *Staph. Aureus*; MRSA = Methicillin-resistant SA; BS = *B. subtilis*;
MF = *Micrococcus flavus*; MS = *Mycobacterium smegmatis*;
PA = *Ps. aeruginosa*; SF = *Streptococcus faecalis*

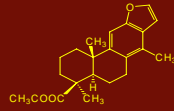
**TESTS FOR WOUND HEALING
COMPOUNDS ISOLATED FROM
*C. benthamianum***

ACTIVE



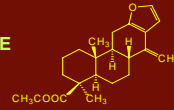
R1 Deoxycsaesaldekarin C

ACTIVE



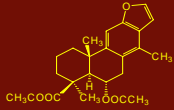
R2 Benthamin 1

ACTIVE



R3 Benthamin 2

INACTIVE



R4 Benthamin 3

**TESTS FOR WOUND HEALING
Antioxidant assays on cultured cells**

Human dermal fibroblasts cultured and passaged
Suitable concentration of hydrogen peroxide (H_2O_2) to give recoverable damage determined ($1 \times 10^{-4}M$)

Three protocols:-

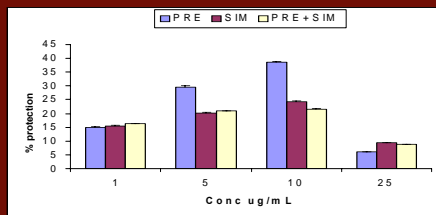
- extract added to cells simultaneously with H_2O_2 PRE
- cells pretreated with extract before H_2O_2 SIM
- cells pretreated with extract then extract added simultaneously with H_2O_2 PRE+SIM

Catalase used as positive control, no antioxidant as negative control

Neutral Red assay used to assess growth/survival of cells
Protection expressed as percentage survival cf. no peroxide

**TESTS FOR WOUND HEALING
Antioxidant assays on cultured cells**

C. benthamianum chloroform extract



c. 35% protection PRE at 10 µg/mL

IC₅₀ (µg/mL) DPPH 20 TBA 30

**Results for activities related to
wound-healing by various
Ghanaian plant extracts**

Plant	Fibroblast stimulation	Antibacterial	Antioxidant
<i>C. benthamianum</i>	++	+++	++
<i>M. pyrifolia</i>	+	-	+
<i>S. virosa</i>	+	++	+
<i>P. pinnata</i>	++++	++	+++

'Active ingredient'

– it all depends on the disease being treated, and the extract

***Salvia miltiorrhiza* root 'Dan shen'**

Used extensively in traditional Chinese medicine



- For cardiovascular complaints
- For improvement of memory in old age

Constituents of *S. miltiorrhiza* root

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Diterpenes – lipophilic

Inhibit acetylcholinesterase
Anti-inflammatory
Prevent neurodegeneration

IMPROVE COGNITION AND MEMORY

Caffeic acid derivatives – water soluble

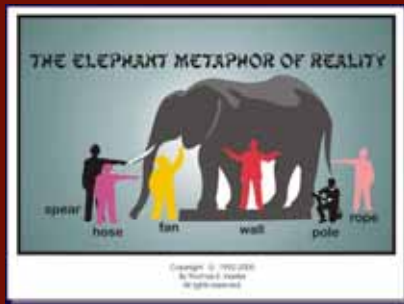
Antioxidant
Anticoagulant

PREVENT ATHEROSCLEROSIS

Together we're stronger!

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- Most active not necessarily major contributor if present in small amounts only
- Different compound types present have different effects but same net effect
- Synergy might occur
- Polyvalence may exist
- Different extracts for different conditions



BEWARE OF OVER-SIMPLIFICATION!

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ACKNOWLEDGEMENTS

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Workers

- Thai plants – Pisamai Laupattarakasem
- Ghanaian plants - Kofi Annan, Rita Dickson
- *Salvia miltiorrhiza* – Melanie Howes, Yuhao Ren

Funding

- Royal Thai Government
- Overseas Research Awards Scheme
- Commonwealth Scholarships
- Government of Ghana