増加してくると想定される．CFE を安全かつ有効に利用するためには，CFE の安全性を確保することが重要になる．Forskolin の経口投与における LD_{50} は，ラットで 2,550 mg/kg，マウスでは 3,100 mg/kg である．また，CFE の腹腔内投与による LD_{50} はマウスで 375 mg/kg と報告されている (de Souza et al., 1983)．現時点で CFE の経口摂取によるヒトでの有害事象の報告は見当たらない．しかし，CFE 自体に有害性がなくても，医薬品等の併用による相互作用が，安全性に影響する可能性が考えられる．例えば，セントジョーンズワートは，CYP3A 誘導と P 糖タンパク活性化により，併用医薬品の薬効を減弱させることが明らかにされている (Mannel, 2004)．そこで著者らは CFE と医薬品の相互作用を検討する目的で，先ず CFE の薬物代謝酵素の誘導の有無を検討した．その結果，CFE は肝シトクローム P450 (CYP) を強く誘導すること，その誘導作用は CYP2B とともに医薬品代謝の 70%を占める CYP2C と CYP3A (Rendic, et al., 1997; Tompkins, et al., 2007) で著しいことを認めた (Virgona, et al., 2011)．CFE の CYP 誘導は，投与量 60 mg/kg から認められ，この量は Regan-Shaw ら (Reagan-Shaw, et al., 2008) の方法によるヒト摂取量への換算では約 5 mg/kg となり，一般的なサプリメントからの摂取量の範囲内であった．また，CFE の活性成分である forskolin をマウスに投与しても CYP の誘導はほとんど認められなかったことから，forskolin 以外の成分が CYP 誘導に関与していることが示唆された (Virgona et al., 2011)．Ding ら (Ding, et al., 2005) は，肝細胞を用いた *in vitro* 系において CFE による CYP 誘導を報告しているが，彼らは forskolin が CYP 誘導作用を持つことを示しており，著者らの *in vivo* の結果とは異なっていた．これまでヒトを含めて *in vivo* において CFE と医薬品の相互作用を示した報告は認められない．

CFE 中の CYP 誘導成分，すなわち医薬品との相互作用に影響する成分が特定できれば，その成分の製品からの除去，あるいは減量などによって，医薬品との相互作用の影響を回避する方策を立てることができる．ちなみに，セントジョーンズワート中の相互作用に関連する成分は hyperforin と考えられ，hyperforin 含量の少ないセントジョーンズワート製品では，経口避妊薬との相互作用を認めないことがヒト試験において報告されている (Will-Shahab, et al., 2009)．

そこで本研究では，CFE 中に存在する CYP 誘導成分を明らかにする目的で，CFE を溶媒により 4 分画し，それらの画分の CYP 誘導作用を，マウスの *in vivo* 評価系および肝マイクロソームを用いた *in vitro* 評価系において検討し，CFE 中に含まれる CYP 誘導物質を推定した．

方 法

1．試薬と飼料

実験に利用した CFE は，既報 (Virgona et al., 2011) と同様に（株）常磐植物化学研究所より提供を受けた．その CFE はインドのバンガロールで採取した *Coleus forskohlii* 乾燥根を粉砕し，超臨界抽出法により forskolin が豊富に含まれる画分 (20–30%) を調製し，その後に 10% forskolin になるようにデキストリンを添加したものである．一般成分は，水分 5.6%，タンパク質 0.3%，脂質 22.7%，灰分 2.2%，炭水化物 69.2%であった． CYP2B，CYP2C，CYP3A の測定試薬は，シグマ・アルドリッチ （株）(St Louis, MO, USA) より，NADPH はオリエンタル酵母工業（株）より購入した．*In vitro* の CYP 阻害実験に利用した P450-Glo™ CYP3A4 測定キット (Luciferin-PPXE) はプロメガ（株）(Madison, WI, USA)，その他の試薬は全て和光純薬工業（株）より購入した．

飼料は Table 1 に示した AIN93G を基本とした Patten ら (Patten, et al., 2004) の組成のものを利用し，これに CFE ならびに CFE の各画分を添加した．飼料に添加した CFE ならびに CFE の各画分は，CFE として 1% (w/w) 相当として調製した．飼料の原材料となるコーンスターチ，カゼイン，α-セルロース，ミネラル (AIN93G)，ビタミン (AIN93G) 等はオリエンタル酵母工業（株）より購入した．

Table 1 Composition of experimental diets.

Ingredient	Diet
	g
Cornstarch	690.686
Sucrose	40
Casein	140
Sunflower oil	30
Cellulose	50
Vitamin mixture (AIN93G)	10
Mineral mixture (AIN93G)	35
L-Cystine	1.8
Choline hydrogen tartrate	2.5
Tertiary butylhydroquinone	0.014
Total	1000

2．CFE の分画

CFE は既報 (Umegaki, et al., 2007) の方法に準じて 4 分画した (Fig. 1)．具体的には，CFE 粉末 100 g を水 2,000 mL に溶解し，ジエチルエーテル 2,000 mL を加えて分液ロートにて十分に振盪し，ジエチルエーテル層を減圧濃縮乾固して画分 1 を調製した (17.11 g)．一方，水層には酢酸エチル 2,000 mL を加え，同様に振盪して酢酸エチル層を減圧濃縮乾固して画分 2 を調製した (4.57 g)．残った水層を減圧濃縮乾固し，アセトン 1,000 mL を加えて 35℃で 4 時間撹拌し，吸引濾過した．このアセトン層を減圧濃縮乾固して画分 3

を調製した (1.03 g). またアセトン不溶部の残渣を乾固して画分 4 とした (76.05 g). 各画分中に含まれていた forskolin の含有率は，画分 1 が 41.8%，画分 2 が 39.7%，画分 3 が 18.8%，画分 4 が 0.001%であった．

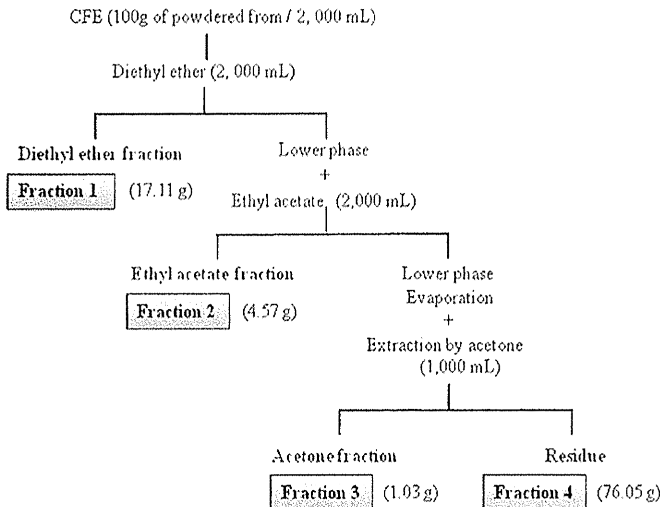


Fig. 1 Fractionation of *C. forskohlii* extract

3．動物実験

ICR 系雄マウス 4 週齢（日本クレア（株））を利用し，室温 23±1℃，12 時間の明暗サイクルの環境で飼育した． 5 日間の予備飼育の後に，対照食，CFE 食，CFE の各画分食，全画分混合物食を 2 週間摂取させた．前述のように飼料に添加した CFE ならびに CFE の各画分は CFE として 1% (w/w) に調製し，飼料と水は自由摂取させた．飼料中 1 kg の被検物質の添加量は，画分 1 が 1.73 g，画分 2 が 0.48 g，画分 3 が 0.10 g，画分 4 が 7.7 g，画分 1-4 の混合物と未分画 CFE が 10 g/kg であり，これらの添加量に相当するコーンスターチ量を飼料から減量した．マウスは一夜絶食させた後，ペントバルビタール麻酔下で開腹し，直ちに肝臓を摘出して重量を測定し，CYP 測定まで-80℃にて保存した．以上の動物実験は（独）国立健康・栄養研究所実験動物倫理委員会の承認を得，同委員会のガイドラインに準じて行った．

4．酵素活性測定法

肝マイクロソームは既報 (Umegaki, et al., 2002) に従って調製し，CYP 含量は Omura ら (Omura, et al., 1964) の方法，CYP2B 活性 (Pentoxyresorufin *O*-dealkylase)，CYP2C 活性 ((*S*)-warfarin 7-hydroxylase)，CYP3A 活性 (testosterone 6β-hydroxylase) はそれぞれ HPLC 法 (Umegaki et al., 2002) により測定した．被検物質の CYP3A 活性に対する *in vitro* での影響は，CFE を 2 週間投与したマウス肝マイクロソームを CYP 酵素として用い，CYP3A 測定用キット (P450-Glo™ CYP3A4) により評価した．CYP3A の阻害活性は，酵素反応により生成した発光シグナルをルミノメーター (GloMax™96, Promega Co.) で測定し，コントロールに対する割合 (%) で算出した．この測定系の CYP3A に対する特異性は，ketoconazole の阻害により確認した．

タンパク量は，Pierce™ BCA タンパク測定キット (Thermo Fisher Scientific Inc., Rockford, IL, USA) により定量した．

5．統計処理

各データは平均±標準誤差で示した．統計処理は Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) を用い，一元配置分散分析を実施後，Tukey の多重比較検定により各群間の有意差を検定した．肝臓重量と CYP の関係については，Pearson の相関係数を求めた．$P<0.05$ 以下を有意とした．

結　果

1．*In vivo* における CFE 画分の肝 CYP の誘導作用

1% CFE 添加食またはそれに相当する CFE の各画分の添加食を 2 週間混餌投与したとき，マウスの体重と摂餌量は，対照群と各 CFE 添加飼料群で差異はなかった (Table 1)．肝重量/体重比は，CFE 画分群の中で画分 1 群が最も高値であった．この画分 1 群の肝重量/体重比は，全画分混合物群（画分 1 から 4 の混合）と有意な差異はなかったが，未分画 CFE 群よりも有意に低かった．肝 CYP の総量，CYP2B，CYP2C，CYP3A の活性は，肝重量/体重比とほぼ同様の変化を示し，4 つの画分群の中では画分 1 群が最も高く，全画分混合物群が画分 1 群よりも高い傾向を示した (Fig. 2)．全画分混合物群と未分画 CFE 群を比べると，CYP2B 活性と CYP2C 活性では大きな違いが認められたが，CYP 含量と CYP3A 活性の違いは比較的小さかった．Table 1 の肝重量/体重比と Fig. 2 の CYP 含量や各 CYP 活性には，ほぼ同様の変化が認められ，有意な正相関を示した (Fig. 3)．

2．*In vitro* における CFE 画分の肝 CYP3A 活性の阻害作用

CFE ならびに各画分の CYP 阻害作用を *in vitro* において検討した (Fig. 4)．CYP サブタイプは高い活性が認められた CYP3A とし，測定系への被検物質の添加量は一定濃度とした (2.5，7.5，25 μg/mL)．その結果，分画していない CFE ならびに画分 1，2，3 は，それぞれ濃度依存的に CYP3A 活性を阻害し，画分 4 は阻害作用を示さなかった．画分 4 において CYP 阻害活性がなかったことは，Fig. 2 の *in vivo* において CYP 誘導作用を認めなかった結果とよく一致した．この被検物質濃度を一定にした *in vitro* 条件では，分画していない CFE に比べて，画分 1，画分 2，画分 3 の CYP 阻害濃度は低い値となった．

考　察

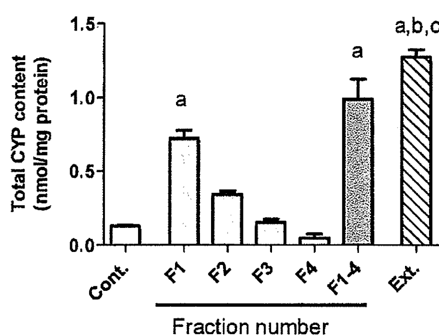
著者らのこれまでの研究から，CFE による肝 CYP 誘導には，CFE の活性成分である forskolin 以外の成分が関与していると考えられた (Virgona et al., 2011)．そこで，本研究では CFE をジエチルエーテル，酢酸エチル，アセトンを用いて 4 つに分画し，マウスの *in vivo* 実験系と肝マイクロソー

Table 2 Body weight, liver weight and food intake of mice fed diets containing either fractionated or unfractionated *C. forskohlii* extract (CFE).

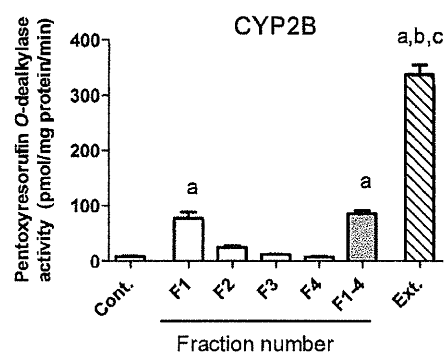
	Control	Fractionated extracts F 1	F 2	F3	F4	Sum of F1-4	Unfractionated Extract
Final body weight (g)	32.7 ± 0.8	34.9 ± 1.3 [1.1]	32.6 ± 1.0 [1.0]	33.3 ± 0.9 [1.0]	32.9 ± 0.8 [1.0]	34.9 ± 0.8 [1.1]	31.8 ± 0.6 [0.97]
Liver weight (% /body weight)	4.30 ± 0.11	5.80 ± 0.12 [1.3][a]	5.11 ± 0.022 [1.2]	4.48 ± 0.086 [1.0]	4.27 ± 0.043 [0.99]	6.57 ± 0.27 [1.5][a]	9.92 ± 0.65 [2.3][abc]
Average dairy food intake (g)	4.5 ± 0.12	4.5 ± 0.22 [0.99]	4.5 ± 0.14 [1.0]	4.5 ± 0.063 [1.0]	4.5 ± 0.067 [0.99]	4.5 ± 0.10 [0.99]	4.5 ± 0.20 [1.0]

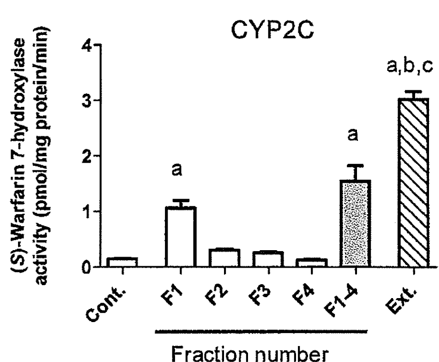
Male ICR mice were fed diets containing either fractionated or unfractionated *C. forskohlii* extract (CFE) at the dose equivalent of 1% (w/w) CFE for 2 weeks.
Values are expressed as means and SE for 5 mice.
[a]: Significant difference from control group at $p<0.05$.
[b]: Significant difference from fraction 1 group at $p<0.05$.
[c]: Significant difference from fraction 1-4 mixture group at $p<0.05$.

a) CYP content



b) CYP activities





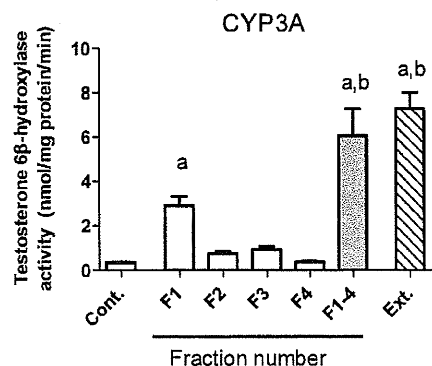


Fig. 2 Hepatic CYP in mice fed diets containing either fractionated or unfractionated *C. forskohlii* extract (CFE).
Each value is the means and SE for 5 mice. Cont., control; F, fraction; Ext., unfractionated extract.
[a]: Significant difference from control group at $p<0.05$.
[b]: Significant difference from fraction 1 group at $p<0.05$.
[c]:Significant difference from fraction 1-4 mixture group at $p<0.05$.

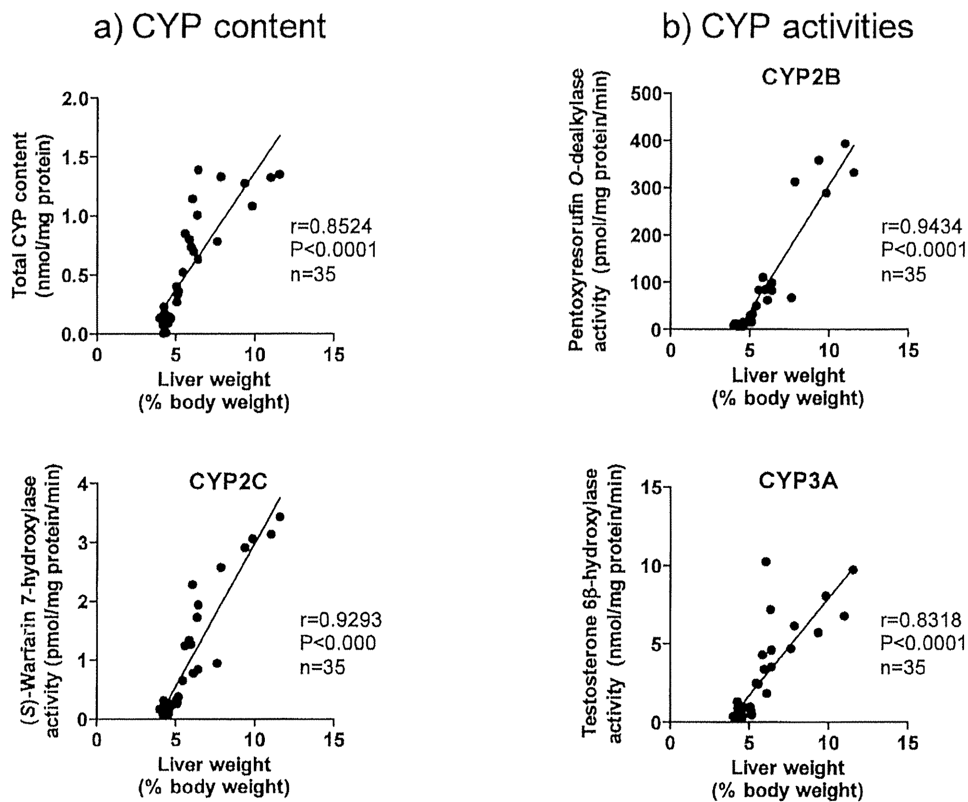


Fig. 3　Correlation between the liver weight/body weight and hepatic CYP content or activities.
Data was from Table 1 and Fig. 2.

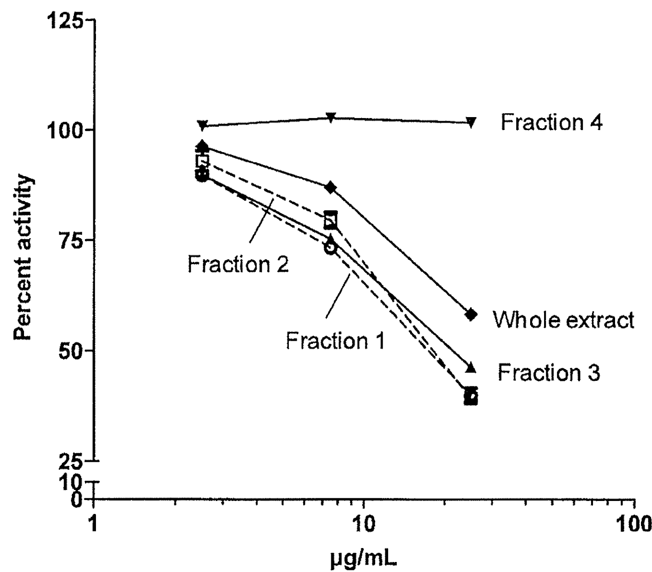


Fig. 4　Inhibition of CYP3A activity by CFE whole extract or its fractionated samples.
CFE (whole extract) or fractionated samples were added into CYP3A assay medium at the concentration of 2.5-25 μg/mL. CFE-treated mouse liver microsome was used as CYP3A enzyme.
Values are expressed as means and SE of duplicate determinations.

ムを用いた *in vitro* 実験系においてCYP誘導成分の検索を試みた．*In vivo* の試験結果から，CYP誘導成分は画分1(エーテル画分)に多く含まれ，画分2，3になるほどその活性が低くなり，画分4にはほとんど含まれていないことが明らかとなった．また，*in vitro* のCYP3Aに対する直接作用を検討した結果では，CFEならびに画分1から3がCYPの阻害活性を示し，画分4には阻害活性は認められなかった．以上の結果から，CFEの誘導成分はエーテル可溶で水に難溶の特性を有していることが推定された．画分4に活性がないことは，*in vitro* のCYPに対する直接作用でも確認された．CFEで認めた *in vivo* におけるCYP誘導と *in vitro* におけるCYP活性阻害は，*in vitro* のCYP活性の測定に利用した基質とCYP誘導成分が，競合阻害，非競合阻害，不競合阻害のいずれかの阻害様式を示した結果と考えられる．本研究では酵素の阻害様式については検討していないが，競合阻害と考えると，*in vivo* と *in vitro* で認めた現象は解釈しやすい．

マウスにforskolinを投与した前報の実験結果から，CFE中のCYP誘導成分はforskolinではないことが示されている(Virgona et al., 2011)．本実験において，各画分中のforskolinの含有率は，画分1が41.8%，画分2が39.7%，画分3が18.8%，画分4が0.001%であった．画分1と2ではforskolinの含有率はほぼ同程度であったが，CYP含量と活性から示されたCYP誘導作用は，画分1に対して画分2でおよそ1/2であった．この各画分中のforskolin含量とCYP誘導作用の関係が一致しない結果からも，CFE中に含まれるCYP誘導成分はforskolin以外の成分と考えられる．

CYPの誘導において，各画分を合わせた群が画分1群よりも高かったことは，検索しているCYP誘導成分が画分2や3にもある程度分散していることから概ね説明することができる．しかし，各画分を合わせた群が，未分画群よりも有意に低いこと，また，その影響の程度がCYP含量やCYP3Aに比べてCYP2BやCYP2Cで著しいことは説明できない．これはCYP誘導成分が，分画操作において一部除去されている可能性やCYP誘導作用を有する成分が複数存在している可能性を示している．例えば，CFE中にはforskolinの類縁体である1,9-dideoxyforskolin, 7-deacetyl-1,9-dideoxyforskolinの存在が明らかにされている(Inamdar, et al., 1989)．また，1,9-dideoxyforskolinについては，肝CYP誘導作用を有することが報告されている(Ding et al., 2005)．今回は検討できなかったが，これらがCYP誘導物質であるかもしれない．

CFE中のCYP誘導成分の検索において，今回示した *in vitro* のCYP活性阻害系は微量の試料で迅速に検討できる利点がある．しかし，*in vitro* でCYP活性を阻害する成分であっても，それが *in vivo* においてCYPを誘導するとは限らない．実際，イチョウ葉エキス中に含まれるプロアントシアニジンは *in vitro* においてCYP2B活性を阻害したが，*in vivo* ではCYP2Bの誘導は示さなかったケースもあった(Sugiyama, et al., 2004)．このようなことから，*in vitro* における阻害作用を認めた成分について，*in vivo* におけるCYP誘導を必ず確認する必要がある．*In vivo* でCYP含量や活性を測定することが確実であるが，肝CYP含量や活性は，食餌条件や栄養状態によって変動し(Guengerich, 1995)，試料の保存状態によっても低下する可能性がある．従って，より簡単なCYP誘導の評価指標が求められる．本研究で示したようにCFEによるCYP誘導は，肝重量/体重比の増加と極めて強い相関を示した．この肝重量/体重比の増加は肝臓重量の増加に由来するものであり，肝重量の測定は極めて簡単で，CYP活性測定のように，マイクロソームの調製や酵素活性の測定における特殊な技術は必要でない．つまり，肝重量/体重比を測定すれば，CFEによる肝CYPの誘導レベルを容易に把握することが可能である．今後のCYP誘導成分の検索実験では，微量で検討できる *in vitro* のCYP活性阻害系，ならびに簡単で安定したCYP誘導の評価指標となる肝重量/体重比を測定する *in vivo* の評価系を組み合わせて実施することが効率的と考えられる．

ハーブ類は複数の成分から構成される天然物である．そのために収穫時期や地域，抽出方法によって素材中の個別成分の含有量が変動すると考えられる．CFEについては，forskolinが薬理活性を示す成分として同定されていることから，市場に流通しているCFEは，10% forskolinを含有した素材が主流である(Virgona, et al., 2010)．しかし，これまでの著者らの検討からCFEによるCYP誘導には，forskolin以外の成分が関連することが明らかとなっている．CFE中のforskolinの薬理活性は明確である．従って，CFEを安全に利用するためには，今後のCYPを誘導する未知成分の同定，ならびにその成分の原材料からの除去あるいは低減，またその成分を含めたCFEの規格基準の作成が必要であろう．本実験で示したCYP誘導成分はエーテル画分に多く存在する事実や，*in vivo* における肝重量の増加によるCYP誘導の評価法，ならびに *in vitro* におけるCYP阻害による評価法は，今後の検討において役立つと考えられる．

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引用文献

Allen, D. O., Ahmed, B., Naseer, K., (1986): Relationships between cyclic AMP levels and lipolysis in fat cells after isoproterenol and forskolin stimulation, *J Pharmacol Exp Ther*, 238(2), 659-64

Bauer, K., Dietersdorfer, F., Sertl, K., Kaik, B., Kaik, G., (1993): Pharmacodynamic effects of inhaled dry powder formulations of fenoterol and colforsin in asthma, *Clin Pharmacol Ther*, 53(1), 76-83

Baumann, G., Felix, S., Sattelberger, U., Klein, G., (1990): Cardiovascular effects of forskolin (HL 362) in patients

with idiopathic congestive cardiomyopathy--a comparative study with dobutamine and sodium nitroprusside, *J Cardiovasc Pharmacol,* 16(1), 93-100

de Souza, N. J., Dohadwalla, A. N., Reden, J., (1983): Forskolin: a labdane diterpenoid with antihypertensive, positive inotropic, platelet aggregation inhibitory, and adenylate cyclase activating properties, *Med Res Rev,* 3(2), 201-19

Ding, X., Staudinger, J. L., (2005): Induction of drug metabolism by forskolin: the role of the pregnane X receptor and the protein kinase a signal transduction pathway, *J Pharmacol Exp Ther,* 312(2), 849-56

Godard, M. P., Johnson, B. A., Richmond, S. R., (2005): Body composition and hormonal adaptations associated with forskolin consumption in overweight and obese men, *Obes Res,* 13(8), 1335-43

Guengerich, F. P., (1995): Influence of nutrients and other dietary materials on cytochrome P-450 enzymes, *Am J Clin Nutr,* 61(3 Suppl), 651S-58S

Inamdar, P. K., Khandelwal, Y., Garkhedkar, M., Rupp, R. H., de Souza, N. J., (1989): Identification of Microbial Transformation Products of 1, 9-Dideoxyforskolin and 7-Deacetyl-1, 9-dideoxyforskolin, *Planta Med,* 55(4), 386-7

Mannel, M., (2004): Drug interactions with St John's wort : mechanisms and clinical implications, *Drug Saf,* 27(11), 773-97

Omura, T., Sato, R., (1964): The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature, *J Biol Chem,* 239, 2370-8

Patten, G. S., Adams, M. J., Dallimore, J. A., Abeywardena, M. Y., (2004): Depressed prostanoid-induced contractility of the gut in spontaneously hypertensive rats (SHR) is not affected by the level of dietary fat, *J Nutr,* 134(11), 2924-9

Reagan-Shaw, S., Nihal, M., Ahmad, N., (2008): Dose translation from animal to human studies revisited, *FASEB J,* 22(3), 659-61

Rendic, S., Di Carlo, F. J., (1997): Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors, *Drug Metab Rev,* 29(1-2), 413-580

Sugiyama, T., Shinozuka, K., Sano, A., Yamada, S., Endoh, K., Yamada, K., Umegaki, K., (2004): [Effects of various ginkgo biloba extracts and proanthocyanidin on hepatic cytochrome P450 activity in rats], *Shokuhin Eiseigaku Zasshi,* 45(6), 295-301

Tompkins, L. M., Wallace, A. D., (2007): Mechanisms of cytochrome P450 induction, *J Biochem Mol Toxicol,* 21(4), 176-81

Umegaki, K., Saito, K., Kubota, Y., Sanada, H., Yamada, K., Shinozuka, K., (2002): Ginkgo biloba extract markedly induces pentoxyresorufin O-dealkylase activity in rats, *Jpn J Pharmacol,* 90(4), 345-51

Umegaki, K., Taki, Y., Endoh, K., Taku, K., Tanabe, H., Shinozuka, K., Sugiyama, T., (2007): Bilobalide in Ginkgo biloba extract is a major substance inducing hepatic CYPs, *J Pharm Pharmacol,* 59(6), 871-7

Virgona, N., Taki, Y., Umegaki, K., (2010): A rapid HPLC with evaporative light scattering method for quantification of forskolin in multi-herbal weight-loss solid oral dosage forms, *Pharmazie,* 65(5), 322-6

Virgona, N., Yokotani, K., Yamazaki, Y., Shimura, F., Chiba, T., Taki, Y., Yamada, S., Shinozuka, K., Murata, M., Umegaki, K., (2011): Coleus forskohlii extract induces hepatic cytochrome P450 enzymes in mice, *Food Chem Toxicol,* 50(3-4), 750-55

Will-Shahab, L., Bauer, S., Kunter, U., Roots, I., Brattstrom, A., (2009): St John's wort extract (Ze 117) does not alter the pharmacokinetics of a low-dose oral contraceptive, *Eur J Clin Pharmacol,* 65(3), 287-94

健康食品の摂取に伴う有害事象の因果関係評価のための樹枝状アルゴリズムの改変

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Modification of a Dendritic Algorithm for Evaluation of Causal Relationships of Adverse Events with Health Food

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Information of adverse events associated with dietary supplements or health food, which is collected by manufacturers or healthcare facilities, is inconsistent. Therefore, a method to collect essential information from patients or consumers and evaluate the causal relationship of adverse events is necessary. We previously modified a dendritic algorithm for evaluating medication-related adverse events (Jones JK. Fam Community Health 1982; 5: 58-67) for use in dietary supplements or health food. In this study, we improved the dendritic algorithm, especially in the temporal relation between taking dietary supplements or health food and onset of adverse events, and compared to a different algorithm based on the scoring scale developed by Naranjo et al (Naranjo CA, et al. Clin Pharmacol Ther 1981; 30: 239-45). Using both algorithms, eight raters (pharmaceutical science students) assessed 200 cases of adverse events provided by the manufacturer's customer inquiry center. The κ coefficient of multi-rater reliability was 0.51 for the modified dendritic algorithm and 0.35 for the scoring scale. The time required to complete the evaluation tended to be shorter using the dendritic algorithm. In conclusion, the present results indicate that the improved dendritic algorithm may be reliable and suitable for universal usage. Pilot studies using the modified algorithm during history taking of consumers or patients to collect information on adverse events are needed to assess the utility of the algorithm in clinical practice.
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Key words: health food, adverse event, algorithm, causal relationship

緒　論

保健機能食品（特定保健用食品，栄養機能食品）およびそれ以外のいわゆる健康食品（以下，両者を総称して健康食品と略す）の摂取が原因と疑われる有害事象の発生が近年問題となっている[1~5]．この背景には，多種多様な製品が流通し消費される一方，製品に対する法的規制が十分になされていないということに加え，有害事象が発生した場合の評価法が確立されていないという問題がある[6]．健康食品の摂取による有害事象の報告は，販売店や製造販売元への問い合わせ，医療機関での診療記録などを基に保健所を介して厚生

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労働省に集約される．また，消費者庁を介した情報の集積も行われている．このように，多様な場で異なる専門性を持つ集団により収集された事例が集積されるという特性から，健康食品の摂取と有害事象の因果関係を正確かつ効率的に判断するための評価法が必要とされている．

健康食品の摂取に伴う有害事象の評価法が確立されていない反面，医薬品の投与に伴う有害事象の評価法として，Naranjoらの評価票[7]やJonesの樹枝状アルゴリズム[8]といった評価法が報告されている[9]．健康食品と医薬品は共に生体分子に働きかけることでその作用を発現するという共通点を持ち，薬物代謝酵素の誘導や阻害，受容体に対する結合などを介して作用を示す．我々はこれまでに，薬理学的・薬力学的アプローチを基盤としたNaranjoらの評価票やJonesの樹枝状アルゴリズムを改変し，最適化することで健康食品の摂取に伴う有害事象の因果関係評価法の確立を試みてきた[10~12]．その過程で，Jonesの樹枝状アルゴリズムの内容を検討し，質問項目や分岐形式，カテゴリー分類を改変したアルゴリズムを構築した．構築したアルゴリズムを用いて事例を評価し，分析した結果，1）健康食品の摂取と有害事象の発生の時間的関連，2）パッチテストなどによる客観的根拠の取り扱いに関するカテゴリー分類上の課題が残った[12]．そこで，Jonesの樹枝状アルゴリズムにこれらの課題を反映した質問項目，分岐形式，カテゴリー分類などの改変を加え，臨床応用可能な樹枝状アルゴリズムの最適化を試みた．さらに，これまでにNaranjoらの評価票を基に改変を重ねることで構築した評価票による評価を行い，両者を比較した．

方　法

先行研究で作成したJonesの樹枝状アルゴリズムの改変を健康食品の有害事象として報告される情報の特性とその重み付けを考慮して再検討し，カテゴリー分類，分岐形式を改変し，質問項目を追加した（Fig. 1）．改変は以下の2項目について行った（変更点1はカテゴリー分類における変更点，2は分岐形式における変更点である）．

1）評価開始時の質問項目において，(a) 有害事象と健康食品の摂取との時間的関連が否定された事例と (b) 時間的関連がみられるが他の要因に起因する可能性が高い事例が同一のカテゴリー「関連なし（Remote, Doubtful）」に分類されていたため，(a) を「関連なし（Highly unlikely）」，(b) を「ほぼ関連なし（Unlikely）」として分類を細分化した．

2）有害事象の再現性が確認されていない事例である場合，客観的証拠（DLST，パッチテスト等）の有無が問われる質問が存在していなかった．客観的証拠は，因果関係を判断するうえで重要な要素であるため，再現性が確認されていない事例であっても，客観的証拠の有無が問われるように分岐形式を改変し，質問項目を追加した．

次いで今回改変を加えた樹枝状アルゴリズム（以下，改変樹枝状アルゴリズムと略す）およびこれまでにNaranjoらの評価票を基に改変を重ね構築した評価票（以下，改変評価票と略す）を用い健康食品販売業者のお客様相談センターに寄せられた保健機能食品（特定保健用食品，栄養機能食品）および保健機能食品以外の健康食品の摂取に伴う有害事象相談事例200例に対して8名（薬学部6年生2名，5年生2名，4年生3名，大学院博士課程1名）が因果関係を評価した．8名をランダムに2群に割り付け，一方の群（A群）は1. 改変評価票，2. 改変樹枝状アルゴリズムの順に，他方の群（B群）は1. 改変樹枝状アルゴリズム，2. 改変評価票の順に評価を行った．評価はそれぞれ独立して行い，1と2の評価の間には6カ月の期間を設けた．改変樹枝状アルゴリズムにおける評価判定は，因果関係が強い順に，非常に確からしい（Highly probable），確からしい（Probable），可能性がより強くある（Highly possible），可能性がある（Possible），ほぼ関連なし（Unlikely），関連なし（Highly unlikely），情報不足・評価不能（Lack of information）の7段階にカテゴリー分類した．改変評価票に関しては，合計点をスコア化し，因果関係の強い順に，非常に確からしい（Highly probable），確からしい（Probable），可能性がより強くある（Highly possible），可能性がある（Possible），関連なし（Doubtful）の5段階にカテゴリー分類した．次いで，評価者間信頼性の指標としてFleissの多評価者間κ係数を算出した．加えて，臨床における汎用性の観点から200事例の評価に要した時間を計測した．統計解析にはR ver. 2.15.2（R Development Core Team, 2012）を用いた．

なお，本研究で利用した有害事象事例の個別内容については，機微情報を含むことから提示しないこととした．

結　果

改変評価票，改変樹枝状アルゴリズムのいずれにおいても200事例の評価結果は，「可能性がある（Pos-

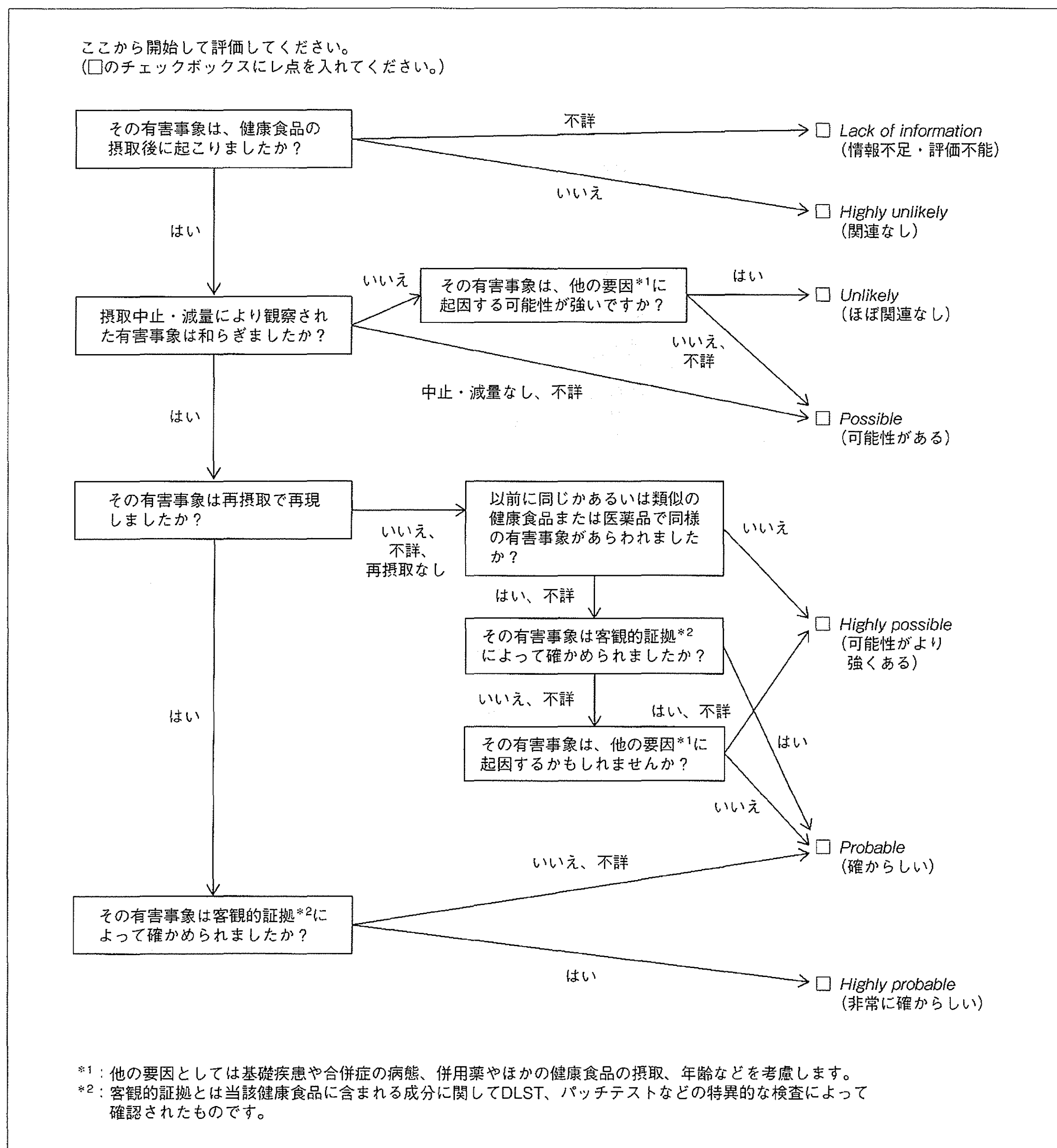


Fig. 1 改変樹枝状アルゴリズム

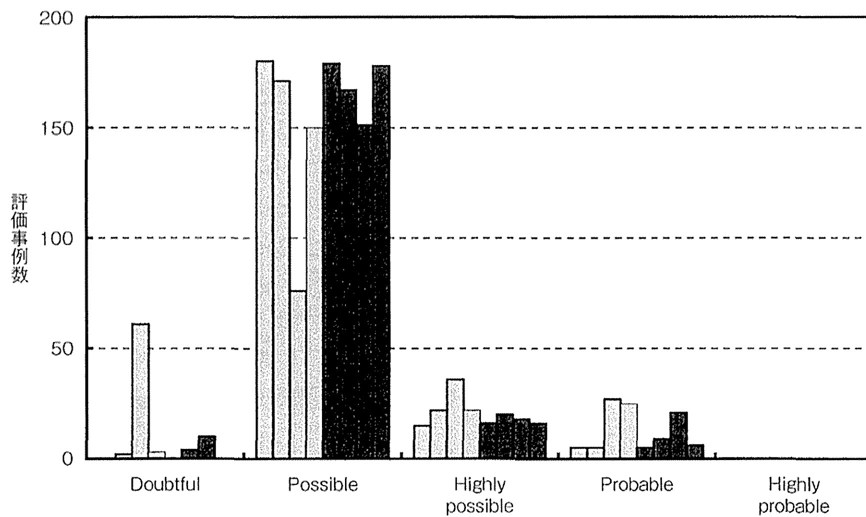
Jonesの樹枝状アルゴリズムを基に，改変を重ねることで構築したアルゴリズム．

sible)」に集中し，「非常に確からしい（Highly probable)」に分類された事例はなかった（Fig. 2）．1. 改変評価票，2. 改変樹枝状アルゴリズムの順に評価を行った群（A群）の κ 係数は，改変評価票0.21，改変樹枝状アルゴリズム0.53であった．また，1. 改変樹枝状アルゴリズム，2. 改変評価票の順に評価を行った群（B群）の κ 係数は，改変樹枝状アルゴリズム0.50，改変評価票0.54であった．両群全体の κ 係数は，改変評価票0.35，改変樹枝状アルゴリズム0.51であった．

評価時間は改変評価票97±63分，改変樹枝状アルゴリズム79±27分であった（Table）．

考　察

本研究では，Jonesの樹枝状アルゴリズムに更なる改変を加え，健康食品の摂取に伴う有害事象の因果関係評価法の最適化を試みた．また，κ 係数，評価時間



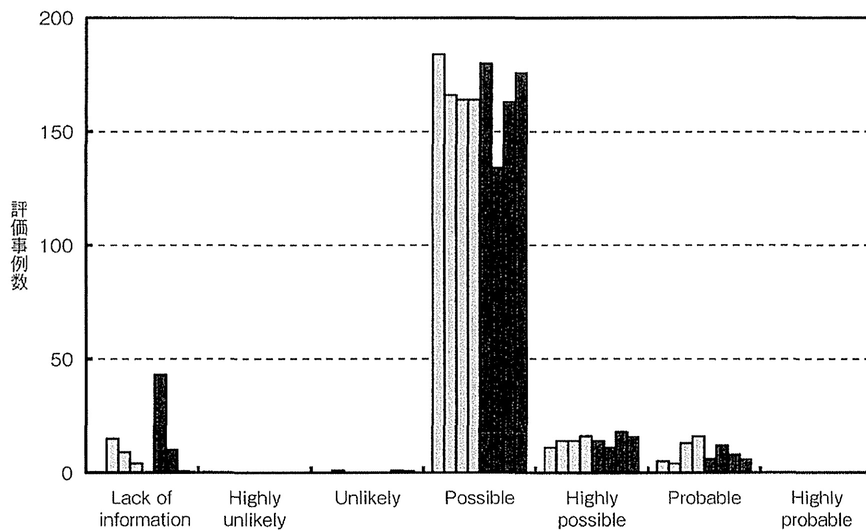


Fig. 2 改変評価票および改変樹枝状アルゴリズムによる有害事象評価結果の分布
改変評価票，改変樹枝状アルゴリズムの順に実施した群（A 群）：灰色，改変樹枝状アルゴリズム，改変評価票の順に実施した群（B 群）：黒色，評価した有害事象事例：200 例．

を指標として改変評価票との比較を行った．樹枝状アルゴリズムの改変前後での相違は，1）健康食品の摂取と有害事象の時間的関係から「関連なし（Remote, Doubtful）」を「関連なし（Highly unlikely）」，「ほぼ関連なし（Unlikely）」の 2 つのカテゴリーに細分化し，2）客観的証拠に対する重み付けを加えた点にある．このようにして構築した改変樹枝状アルゴリズムとこれまでに構築した改変評価票をランダムに 2 群に割り付けた 8 名の薬学生を評価者としてクロスオーバー方式で実施し，信頼性と臨床的な汎用性を評価した．

結果，両群全体における κ 係数は改変樹枝状アルゴリズムにおいて良好な値を示し，評価時間においても短い傾向がみられた．一方，改変評価票においては，先に改変評価票を実施した群で κ 係数が低く，改変樹枝状アルゴリズムでの評価後に改変評価票を実施した群では改変樹枝状アルゴリズムと同程度の κ 係数を示した．評価時間については個々のばらつきが大きく，今後より大規模な検討において比較する必要があると考えられた．また，改変評価票を先に実施した群において κ 係数が低い値を示した理由として，先行研究において示された改変評価票の使用におけるトレーニングの必要性が影響している可能性がある．評価者が健

Table 各評価法における多評価者間κ係数と評価時間の比較

		改変評価票	改変樹枝状アルゴリズム
多評価者間κ係数	A群	0.21	0.53
	B群	0.54	0.50
	両群	0.35	0.51
評価時間（mean ± SD）(min)		97 ± 63	79 ± 27

康食品の摂取に伴う有害事象の因果関係評価に関与した経験のない学生であるという点からもその影響が顕著に表れたと推察される．したがって，改変評価票，改変樹枝状アルゴリズムのいずれも同程度の信頼性を有するものの，専門性の異なる集団から収集されるという有害事象報告の特性を考慮した場合，改変樹枝状アルゴリズムを活用することが適すると考えられた．しかしながら，評価者の資質による影響を十分に評価することは，類似の背景を持つ少数の評価者を対象とした本研究の限界を示しており，議論の余地が残された．

評価事例200例のカテゴリー分類は，これまでに改変評価票，樹枝状アルゴリズムで示した結果と同様，「可能性がある（Possible）」に集中した．これは，健康食品の摂取の中止や減量による情報が不足しているという情報の曖昧さが一因であると考えられた．また，「情報不足・評価不能（Lack of information）」に分類される事例も存在した．これは，消費者・患者から自発的に発信される情報のみでは，因果関係の判定に必要となる情報を十分に把握することが困難であり，お客様相談センターの担当者，販売店など情報の受け手の情報収集方式にも必ずしも一貫性がないことを示唆している．すなわち，一定の評価アルゴリズムを利用し，必要な情報を消費者・患者から聞き取り，詳細を把握することは因果関係評価を容易にし，結果として健康被害の拡大を阻止することに繋がると考えられる．また，漠然と有害事象の状況を問われる現状と比較して，順序立てて状況を問うことは消費者・患者にとっても情報提供のしやすさという点でメリットとなる．しかしながら，アルゴリズムに含まれる質問の回答様式の多くは「はい」，「いいえ」の２択ではなく，因果関係評価に対する一定の理解を要する．運用にあたっては，評価者に対する事例演習や説明が必要であると考えられた．

健康食品の利用目的として「病気の治療」を挙げる消費者も存在すること[13]から原疾患の有無や服用中の医薬品との相互作用も重要な問題となるが，改変評価票，改変樹枝状アルゴリズムのいずれにおいても疾患に関する情報は十分に得られず，因果関係評価における限界と改良の余地が残された．

また，有害事象には摂取によらず一定頻度で生じうるものも含まれる[14,15]．評価アルゴリズムを利用した大規模な情報の収集は，同一の症状を示す事象の集積にも繋がり，個別症例からは判定が困難な例を検出するうえでも活用が可能である．現在は，自発的に提供された情報に対してアルゴリズムを適用し，改変を重ねることでその質を高めることに留まっているが，消費者・患者からの聞き取り段階でアルゴリズムを活用することは，情報そのものの有用性を高め迅速に伝達することに繋がるだけでなく，シグナルマネジメントを基礎としたリスクマネジメントへの展開にも寄与するものと期待される．

結　論

今回最適化を図った改変樹枝状アルゴリズムは，改変評価票と比較して評価経験の少ない評価者においても高い信頼性を有し，評価時間の側面からも臨床現場における健康食品摂取に伴う有害事象の因果関係判定法として使用が可能であると考えられた．今後，臨床現場で使用する職種間の信頼性やアルゴリズムの適用による情報収集の効率化について有用性を検討する必要がある．

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Conflict of Interest

本研究論文の発表に関連して，開示すべきCOI関係にある企業等はない．

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文　献

1) 山田浩．日常診療からみた食の安全性．*ILSI*. 2005；(81)：18-22.
2) Timbo BB, Ross MP, McCarthy PV, Lin CT. Dietary supplements in a national survey: Prevalence of use and reports of adverse events. *J Am Diet Assoc*. 2006; **106**(12): 1966-74.
3) 山田浩．健康食品の有害事象の事例と解説　ダイエット関連事例．*Functional Food*. 2008；2(1)：96-9.
4) Frankos VH, Street DA, O'Neill RK. FDA regulation of dietary supplements and requirements regarding adverse event

reporting. *Clin Pharmacol Ther*. 2010; 87(2): 239-44.
5) Cellini M, Attipoe S, Seales P, Gray R, Ward A, Stephens M, et al. Dietary supplements: physician knowledge and adverse event reporting. *Med Sci Sports Exerc*. 2013; 45(1): 23-8.
6) 梅垣敬三. 健康食品の有害事象の事例と解説 有害事例の特徴. *Functional Food*. 2007; 1(1): 90-5.
7) Naranjo CA, Busto U, Sellers EM, Sandor P, Ruiz I, Roberts EA, et al. A method for estimating the probability of adverse drug reactions. *Clin Pharmacol Ther*. 1981; 30(2): 239-45.
8) Jones JK. Adverse drug reactions in the community health setting: approaches to recognizing, counseling, and reporting. *Fam Community Health*. 1982; 5(2): 58-67.
9) Agbabiaka TB, Savovic J, Ernst E. Methods for causality assessment of adverse drug reactions: a systematic review. *Drug Saf*. 2008; 31(1): 21-37.
10) 山田浩, 松本圭司, 清水雅之, 熊谷翼, 渡邉崇之, 近都正幸ほか. 健康食品摂取と健康上の有害事象の因果関係を評価するためのアルゴリズムの提案. *臨床薬理*. 2009; 40(4): 163-8.
11) 松本圭司, 高橋光明, 梅垣敬三, 山田浩. 健康食品摂取に伴う健康被害報告の因果関係評価法の構築:改訂評価票による評価者間信頼性評価. *臨床薬理*. 2011; 42(4): 211-4.
12) 山田浩, 一丸佳代, 小野彩奈, 高橋光明, 松本圭司, 小菅和仁ほか. 健康食品の摂取に伴う有害事象の因果関係評価のための樹枝状アルゴリズムの構築. *臨床薬理*. 2012; 43(6): 399-402.
13) 梅垣敬三. 健康食品の実態とその安全性・有効性. *食品衛生学雑誌*. 2010; 51(6): 396-401.
14) Rosenzweig P, Brohier S, Zipfel A. The placebo effect in healthy volunteers: influence of experimental conditions on the adverse events profile during phase I studies. *Clin Pharmacol Ther*. 1993; 54(5): 578-83.
15) Rief W, Avorn J, Barsky AJ. Medication-attributed adverse effects in placebo groups: implications for assessment of adverse effects. *Arch Intern Med*. 2006; 166(2): 155-60.

Research Article

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Dietary *Coleus forskohlii* extract generates dose-related hepatotoxicity in mice

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ABSTRACT: *Coleus forskohlii* root extract (CFE) represented by its bioactive constituent 'forskolin' is popularly used as a natural weight-lowering product, but the association of its use with liver-related risks is very limited. In the present study, the effect of standardized CFE with 10% forskolin on liver function of mice was examined. Mice were given 0–5% CFE in an AIN93G-based diet for 3–5 weeks. Food intake, body weights, relative organ weights and liver marker enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP)] combined with histophatological analysis were assessed. CFE (0–0.5%) only had minimal effects on food intake and body weight whereas a significant difference was observed in mice receiving the highest dose (5% CFE). The extract 0.05–5% dose-dependently decreased visceral fat weight by between 16% and 63%, and a dose-dependent several folds increase was observed in liver weights and plasma AST, ALT and ALP activities with quick onset apparent after only 1 week of 0.5% CFE intake. The hepatic effect persisted throughout the 3-weeks course but was restored towards normalization within 1 week after withdrawal of treatment. Liver histology of mice fed 0.5% CFE for 3 weeks showed hepatocyte hypertrophy and fat deposition. In contrast, none of the hepatic responses measured were altered when mice were given a diet containing pure forskolin alone at the dose corresponding to its content in 0.5% CFE. The present study clearly indicated that forskolin was not involved in the CFE-induced hepatotoxicity and was caused by other unidentified constituents in CFE which warrants further studies. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: forskolin; *Coleus forskohlii*; hepatotoxicity; liver marker enzymes; fatty liver; visceral fat

Introduction

Obesity is a chronic metabolic disorder and is associated with the genesis or development of various diseases, such as type 2 diabetes, cardiovascular disease, hypertension, fatty liver disease and certain forms of cancer (Grundy, 2004). The rise in obesity has caused an increasing demand for effective and safe anti-obesity agents, including herbal products (Egras *et al.*, 2011). Over the years, a variety of medicinal plants and their extracts have been reported to have beneficial effects in reducing the risk of obesity (Kamisoyama *et al.*, 2008, Stewart *et al.*, 2008). These natural compounds ameliorate obesity either by increasing energy expenditure or by inhibiting adipocyte differentiation.

The rhizome part of the perennial plant *Coleus forskohlii*, native to India, has been traditionally used in Ayurvedic medicine as a remedy for heart disease, respiratory, gastrointestinal and central nervous systems disorders (Ammon and Muller, 1985). Many of the beneficial effects of *C. forskohlii* consumption have been attributed to the pharmacological actions of forskolin, a major diterpene isolated from the root of *C. forskohlii*. Forskolin increases cyclic adenosine monophosphate (cAMP) via activation of the enzyme adenylate cyclase by binding to the glucose transporter owing to the right ring of the Decalin portion of forskolin having structural similarity with alpha-D-galactose (Abbadi and Morin, 1999; Laurenza *et al.*, 1989). Enhanced lipolysis as a result of elevation of cAMP by forskolin resulted in the breakdown of stored fats in animal cells (Okuda *et al.*, 1992) and human fat cells (Allen *et al.*, 1986). Anti-obesity effects have also been attributed to *C. forskohlii* extract (CFE) by reducing fat accumulation in ovariectomized rats (Han *et al.*, 2005), overweight females (Henderson *et al.*, 2005) and males (Godard *et al.*, 2005). Based on these findings, there are an increasing number of commercial dietary supplement products in which CFE is used as an herbal ingredient to promote weight loss.

There are examples of hepatotoxicity induced by many types of herbal remedies used as weight loss agents (Egras *et al.*, 2011). Considering the widespread use of CFE in herbal weight loss products there are only a limited number of studies on the involvement of CFE or its constituents regarding toxicity and the detrimental effects of this extract. Earlier, an *in vitro* study demonstrated that forskolin and its analog induced cytochrome P450 (CYP) family, CYP3A gene expression in primary hepatocytes (Ding and Staudinger, 2005). We recently reported that both CFE and pure forskolin induced CYP3A and glutathione *S*-transferase (GST) activities. However, only CFE showed a significant increase in liver weight with dose-related responses in mice (Virgona *et al.*, 2012). Therefore, whether the dose or duration of use may be correlated with the risk of liver damage remains unknown. It is also unclear if the safety profile of CFE is similar to other agents used in the management of body weight without hepatotoxicity effect. Thus, in the present study, we aimed to investigate the extent to which hepatic function of mice is affected by consumption of CFE. Four dose levels of a standardized CFE extract, and its main constituent forskolin were used to assess possible hepatotoxicity. Plasma marker enzymes

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for liver damage were measured to monitor treatment-related adverse effects. The extent of CFE treatment-related changes in liver tissues were assessed with histopathology.

Materials and Methods

Materials

Powdered CFE standardized with 10% forskolin used in the present study was extracted and prepared by Tokiwa Phytochemical Co., Ltd (Chiba, Japan). In brief, dried roots of *C. forskohlii* were crushed and applied to supercritical extraction with CO_2 gas. The obtained forskolin rich extract (20–30%) was incorporated into powdered dextrin to yield dry CFE powder with a forskolin concentration of 10%. The components of the CFE material were: water, 5.6%; protein, 0.3%; lipids, 22.7%; ash, 2.2%; and carbohydrates, 69.2%. The quality of the standardized CFE was determined by a validated HPLC method with an evaporative light scattering detector (ELSD) (Virgona *et al.*, 2010) using forskolin (purity > 99%; Biomol, Plymouth Meeting, PA, USA) as a standard. All other reagents were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Animals

In all the experiments, male ICR mice, 5 weeks old (CLEA Japan, Inc., Tokyo, Japan), were kept at a constant temperature (23 ± 1 °C) with a 12-h light–dark cycle with free access to water and the assigned diets for the length of each experiment. The mice were housed in individual polypropylene cages after a 7-day acclimatization period, maintained on an AIN93G semi-purified diet (Oriental Yeast Co., Ltd., Japan). On the seventh day of the acclimatization, mice were divided into groups of six and were given the experimental diets described later in this text. For all mice clinical observations, body weights and food consumptions were monitored and recorded every 2 days throughout the entire study. Mice were fasted overnight and were sacrificed under pentobarbital anesthesia during the next day. Blood was taken from the inferior vena cava with heparin as an anticoagulant. The plasma obtained from each mouse was frozen at −20 °C until measurement (within 24 h). After blood collection, the liver, kidney and visceral fat tissues were quickly removed from each mouse and weighed. The excised livers samples were fixed in 10% neutral-buffered formalin for histopathological examination. All procedures were in accordance with the National Institute of Health and Nutrition guidelines for the Care and Use of Laboratory Animals, and approved by an ethical committee. Efforts were also made to minimize the number of animals as well as their suffering.

Diets

The experimental diets were formulated on the basis of the abundance of a marker compound in CFE which is composed of 10% forskolin. The experimental diets consisting of: (a) control diet: AIN93G diet, composition of the diet was as in Reeves *et al.* (1993); (b) CFE diets: consisting of a control diet supplemented with 0.005–5% CFE, composition of the CFE diets were the same as the control diet except that a portion of dextrin (0.005–5%) was replaced with the corresponding amount of CFE; and (c) forskolin diet: the control diet supplemented with 0.05% pure forskolin (Biomol) replacing an equal amount of dextrin. The dose formulations were stored at approximately 4 °C in a refrigerator and were stable throughout the period of study.

Experimental Design

Three separate dietary CFE experiments were designed to examine the dose, duration and role of its main constituent on the hepatic function of mice.

Experiment 1: dose response effect of dietary CFE

Mice were randomly divided into five groups ($n = 6$ in each group) and were given either the control diet (AIN 93 G) or a control diet supplemented with 0.005%, 0.05%, 0.5% and 5% CFE (nominally containing 5, 50, 500 or 5000 mg forskolin per kg diet) for 3 weeks.

Experiment 2: time response and post treatment effect of CFE

Mice were given the control diet for up to 5 weeks, or the 0.5% CFE diet for 3 weeks (CFE groups) then switched to the control diet for up to 2 weeks (post-CFE fed groups). Time response to diet measurements ($n = 6$ mice in each group) of liver marker enzymes were performed at weeks 3, 4 and 5 for the control groups, weeks 1, 2 and 3 for the CFE treatment groups, and at weeks 4 and 5 for the post-CFE fed groups (post-CFE fed weeks 1 and 2, respectively).

Experiment 3: differential hepatic effects between intake of CFE and its principal constituent

Mice were randomly divided into three groups ($n = 6$) and were given free access to either the control diet, 0.5%CFE diet or 0.05% forskolin diet for 3 weeks.

HPLC Analysis

Forskolin content in the CFE and experimental diets was quantified by validated HPLC–ELSD. Briefly, samples of CFE and diets were accurately weighed into centrifuge tubes and sonicated in 3 ml of acetonitrile for 15 min. After centrifugation the supernatant was then transferred to a 25-ml volumetric flask. The procedure was repeated two more times and the respective supernatants combined. The sample supernatant (10 μl) was injected into a L column ODS 4.6 × 150 mm, 5-μm particle size (Chemical Inspection & Testing Institute, Tokyo, Japan) with a linear gradient elution system of water/acetonitrile. The temperature of the column was adjusted to 40 °C and a flow rate of 1.0 ml min^{-1}. Column effluent was monitored by UV absorption at 210 nm. The evaporator tube temperature of ELSD was set at 35 °C and a nebulizing gas flow-rate of 3.0 bars [Shimadzu HPLC-VP system, (Kyoto, Japan) coupled with double detectors of UV (SPD-10A) and ELSD (ELSD-LT)].

Biochemical Analysis

Plasma biochemistry measurements of the following parameters were determined according to the standard procedure (SRL Inc., Tokyo, Japan); aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), calcium (Ca), phosphorus (P), sodium (Na), potassium (K) and chlorine (Cl).

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Histopathological Examination of Liver Sections

The fixed liver samples were embedded in paraffin and sections of 3 μm were subjected to haematoxylin and eosin (H&E) staining or sectioned at 10 μm for staining with oil red O according to standard procedures (Biosafety Research Center, Foods, Drugs and Pesticides, Shizuoka, Japan). Each liver section stained with H&E was microscopically examined for distribution of lesions and fatty change, and oil red O for the evaluation of fatty droplets. The lesion and fatty change (macro/microvesicular) were graded semi-quantitatively 0–3, based on percent of hepatocytes in sections which were affected as, 0 = none, 1 = slight (up to 33%), 2 = moderate (up to 66%) and 3 = marked (> 66%). The histological evaluation of the liver sections was performed blindly.

Statistical Analysis

The data were subjected to one-way analysis of variance (ANOVA), followed by Dunnett's or Tukey's multiple comparison tests where appropriate. Results are presented as the mean ± standard error (SE) for the individual groups. Differences with $P < 0.05$ were considered to be significant. Statistical analyses were performed with Prism 5.04 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

HPLC Analysis of Forskolin

Concentration of forskolin in both CFE and experimental diets were calculated on the basis of the standard curve. The standardized CFE contained 10.88% forskolin w/w. The forskolin levels in CFE diets reflected the differences in dietary CFE supplementation (Table 1) ranging from 0.47 to 556 mg per 100-g diet. The pure forskolin diet was found to contain 55.2 ± 0.3 mg of forskolin per 100-g diet.

Dose Response Effect of Dietary CFE

Both food consumption values and estimated daily intakes of CFE in each group of mice were presented in Table 1, and their growth indexes are depicted in Fig. 1A. A slight but non-significant decrease in daily food intake was observed in treated mice (0.05–0.5% CFE groups); however, at the highest dose (5% CFE group) significantly suppressed food intake and body weight were noted. There were no statistically significant differences in the relative kidney weight and serum electrolytes such as calcium, potassium, sodium, chloride or phosphorus considered attributable to CFE treatment (data not shown). However, there were significant dose-related increases of 92% and 210% in the relative liver weight of the 0.5% and 5% CFE groups, respectively, compared with the control (Fig. 1B). A significant reduction of body fat was noted in the 0.05–5% CFE groups; the visceral fat weight was reduced by over 60% at the highest treatment dose of CFE. Similar to the results of substantial high liver weight, there were multiple-fold increases in plasma AST, ALT and ALP enzyme activities (Fig. 1C–E); these increases were statistically significant in the mice receiving 0.5% CFE or greater.

Time Response and Post-Treatment Effect of CFE

As there were apparent treatment-related hepatotoxicity effects of the 0.5% CFE dose as demonstrated by the significant elevation of liver marker enzymes, the same dose was then selected for a 5-week time-course study. After 1 week of CFE treatment, relative liver weight and visceral fat were approximately 75% higher and 30% lower, respectively, compared with the baseline (3 weeks) control group (Fig. 2A, B). These values remained at similar levels for weeks 2 and 3; however, they were largely corrected to the level of control by only 1 week of post-CFE treatment. At 2 weeks post-CFE, the relative liver and visceral fat weights had recovered to be almost identical to the corresponding control 5-weeks group. Elevation in the level of liver marker enzymes were immediately evident after 1 week of CFE treatment and remained higher than in controls for the entire 3 weeks of CFE feeding. The effect of CFE on plasma AST and ALT were maximal at week 2 (Fig. 2C, D) which resulted in increases of approximately 130% and 375%, respectively, compared with the week 3 control. Plasma ALP (Fig. 2E) was moderately (but non-significantly) increased by approximately 65% compared with the control group throughout the 3-week CFE diet period. After 1 week of post-CFE treatment, plasma AST, ALT and ALP levels were substantially restored towards normal.

Differential Hepatic Effects Between Intakes of CFE and its Principal Constituent (Forskolin)

Although all the constituents of the tested CFE involved in hepatotoxicity are not known, forskolin is the quantitatively important active component of CFE. The chromatographic

Table 1. Body weight and food intake of mice fed a diet containing *Coleus forskolli* extract (CFE) for 21 days

CFE % (w/w)	Analysed forskolin content (mg per 100-g diet)	Body weight (g)		Food intake (g mouse^{-1} day^{-1})	CFE intake (mg kg^{-1} BW day^{-1})
		Initial	Final		
0.0	0.0	33.9 ± 0.8	38.7 ± 1.6	4.97 ± 0.18	0
0.005	0.49 ± 0.05	33.3 ± 0.4	39.5 ± 1.0	4.57 ± 0.18	6.09
0.05	5.13 ± 0.12	33.3 ± 0.5	37.5 ± 1.2	4.47 ± 0.16	61.2
0.5	54.6 ± 0.8	33.6 ± 0.6	37.2 ± 1.5	4.50 ± 0.16	612
5.0	556 ± 1	33.3 ± 0.3	33.8 ± 0.6*	4.03 ± 0.19**	5915

Values are expressed as means ± standard error (SE).
*,
** Denote significant differences from the control (0% CFE), $P < 0.05$ and $P < 0.01$, respectively.

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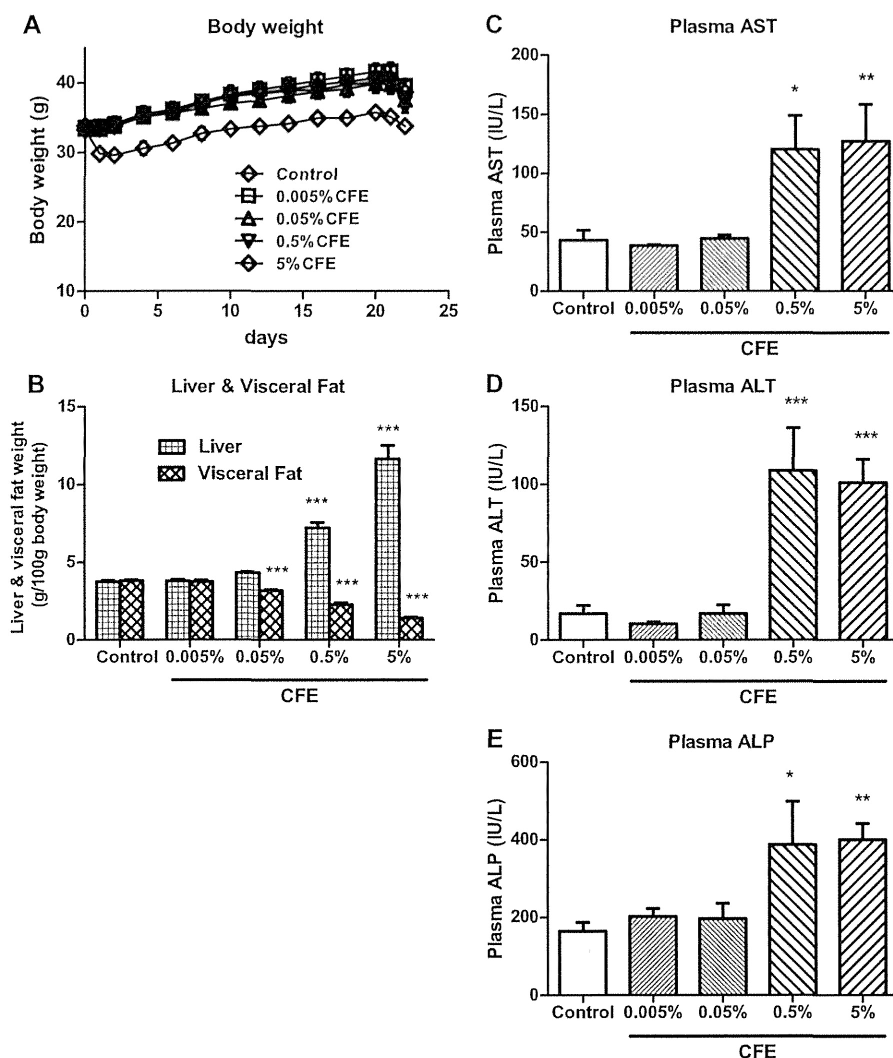

Figure 1. Dose-dependent effects of *Coleus forskohlii* extract (CFE) on: body weight in mice fed the experimental diets (A); relative liver and visceral fat weight (B); plasma markers of liver damage, aspartate aminotransferase (AST) (C), alanine aminotransferase (ALT) (D), alkaline phospatase (ALP) (E). Values are given as the mean ± standard error (SE). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 significantly different from the control group.

fingerprint of pure forskolin (Fig. 3A) compared with that of the CFE used in the present study (Fig. 3B) indicated that forskolin was the major constituent amongst other components as detected by HPLC-ELSD. Thus, the effect of treatment with forskolin alone was then studied using 0.05% of the pure compound compared with the 0.5% CFE dose equivalent. Forskolin or CFE had a minimal effect on weight gain; bodyweights were similar amongst the control, forskolin and CFE groups with no statistical difference (Fig. 3C), although the CFE group was marginally lower over the whole duration of the experiment. There was no significant difference in average food intake between the control (5.14 ± 0.26 g day^{-1} mouse^{-1}), forskolin (4.89 ± 0.19 g day^{-1} mouse^{-1}) and CFE (4.94 ± 0.25 g day^{-1} mouse^{-1}) treated groups. Consistent with the dose and time response findings, CFE showed significant multiple-fold elevation of liver marker enzymes AST, ALT and ALP (increases of 130%, 415% and 258%, respectively; Fig. 3D). Also there was a significant increase (99%) in relative liver weight and a significant reduction (29%) in visceral fat in the CFE mice group (Fig. 3E, F). However, the forskolin diet elicited either little or no effect compared with the control; there were no treatment-related effects of forskolin alone on liver marker enzymes or the relative liver weight. In fact the relative visceral fat weight of forskolin-treated mice was moderately (but non-significantly) 20% lower compared with the control. Histological features of the representative views (H&E and oil red O) and grading of liver sections are shown in Figs 4, 5 and Table 2, respectively. The liver tissue of mice belonging to the control and forskolin groups showed a normal histological architecture (Fig. 4A, B). However, CFE-fed mice exhibited profound histological changes, predominantly microvesicular fatty change in hepatocytes (Fig. 4C) and midlobular hypertrophy including individual cell necrosis and cellular infiltration (Fig. 4D). The livers of CFE mice exhibited a significant fatty change, single cell necrosis of hepatocytes moderate with cellular infiltration and hepatocellular hypertrophy (Table 2). In addition, oil red O staining (Fig. 5C) indicated marked hepatocyte fat deposition (stained red) in the liver tissue from CFE-treated mice but was normal in control- and forskolin-treated mice (Fig. 5A, B).

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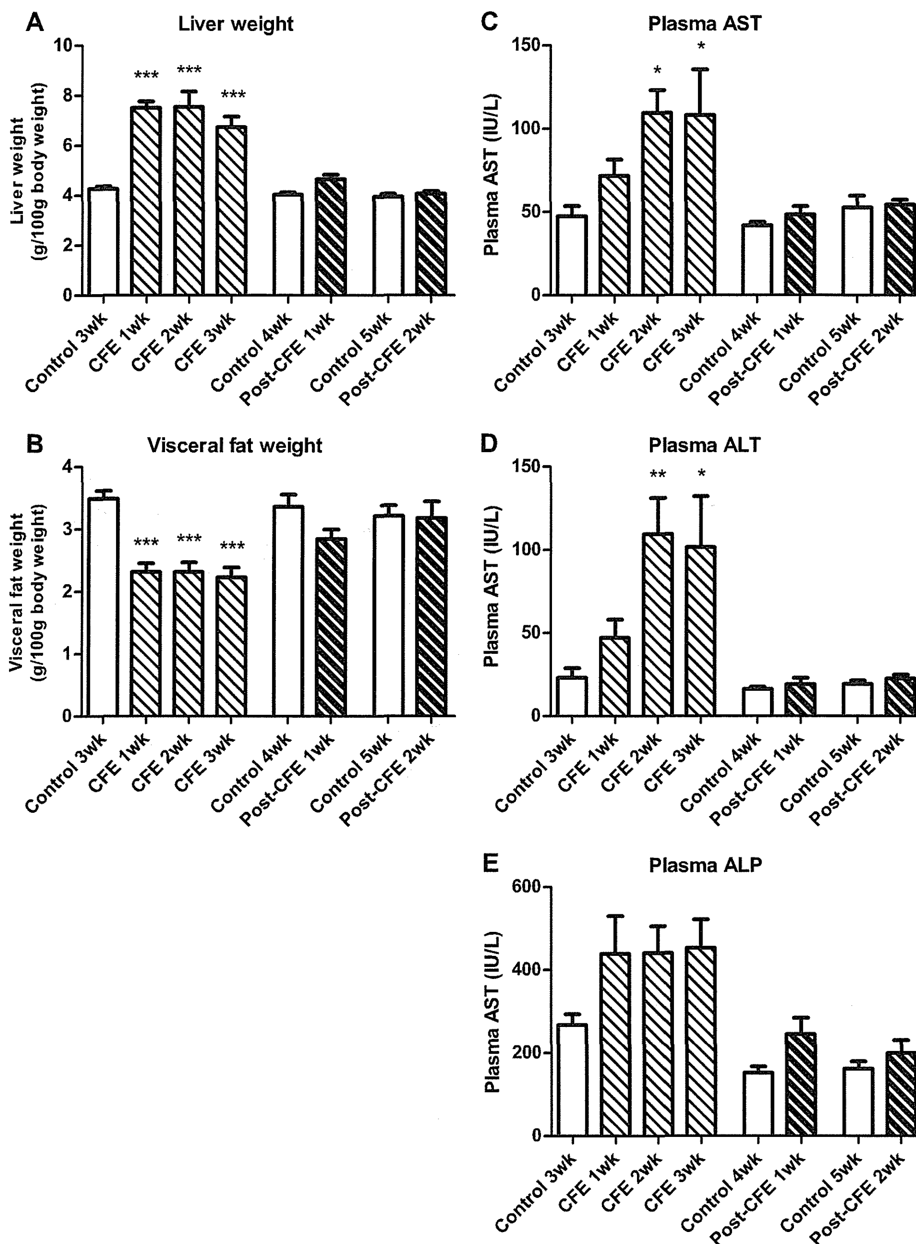

Figure 2. Time-dependent effects of *Coleus forskohlii* extract (CFE) on: relative liver weight (A); visceral fat weight (B); plasma markers of liver damage, aspartate aminotransferase (AST) (C), alanine aminotransferase (ALT) (D), alkaline phospatase (ALP) (E). Values are given as the mean ± standard error (SE). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ significantly different from the respective control group.

Discussion

Forskolin is one of the most extensively studied constituents of the *C. forskohlii* plant (Alasbahi and Melzig, 2012). Numerous positive bioactivity *in vitro* results have been reported with little *in vivo* results. Owing to the fact that the extract from the root of *C. forskohlii* showed CYP induction behaviour in mice (Virgona *et al.*, 2012), comparatively in the present study, CFE was evaluated for its hepatic effects of dose, duration of use and elucidation of the role of its major constituent 'forskolin' associated with these effects. We clearly demonstrated that CFE induced dose dependent hepatotoxicity; a significant effect was observed at a dietary CFE concentration greater than or equal to 0.5% and administration for longer than 1 week. Even although CFE produced decreased visceral fat tissue, it augmented not only liver mass but also hepatic lipid accumulation. Strong supporting evidence from the parameters measured indicated that forskolin, the main active compound in CFE, was not responsible for these events.

Anti-obesity effects by both decreased body weight gain and fat accumulation after CFE consumption has previously been demonstrated in ovariectomized rats (Han *et al.*, 2005). In the present study, a reduction of body weight gain was only observed in the highest dose (5%) although the degree of actual change was slight. This was considered at least partly to be related to the initial decreased food consumption perhaps

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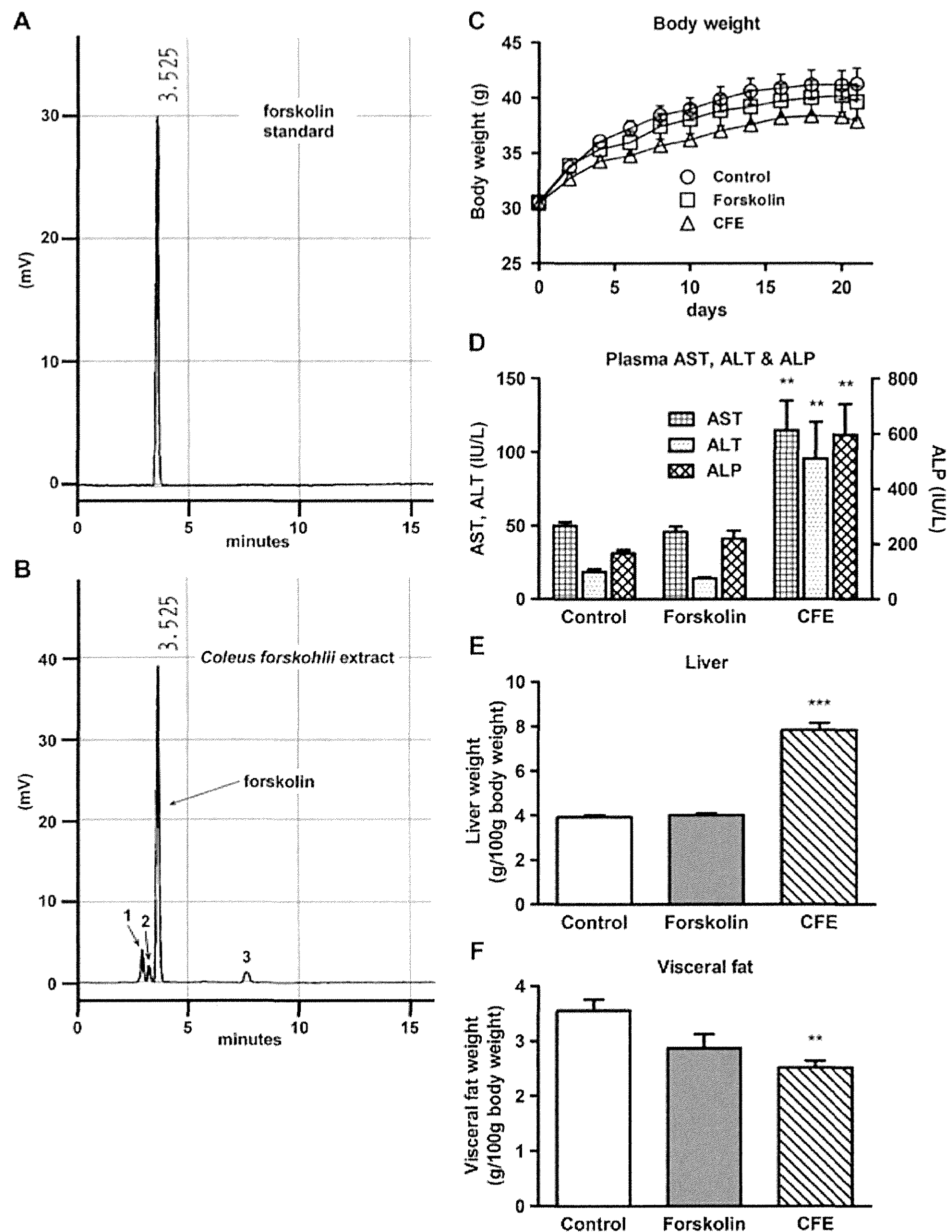

Figure 3. HPLC-ELSD analysis (A, B): chromatogram of pure forskolin at 3.525 min (A); chromatogram from the extract of *Coleus forskohlii* showing main forskolin peak at 3.525 min accompanied by 1, 2 and 3 unidentified components (B). Comparison of 0.05% pure forskolin diet with 0.5% *Coleus forskolli* extract (CFE) diet (C ~ F): body weight (C); plasma markers of liver damage, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phospatase (ALP) (D); relative visceral fat weight (E); relative liver weight (F). Values are given as the mean ± standard error (SE). $^{**}P < 0.01$, $^{***}P < 0.001$ significantly different from both the control and forskolin groups.

because of a repellent smell/taste of CFE at this concentration. Interestingly, extensive reduction in the visceral fat accumulation at the expense of an almost doubling of relative liver weight was seen in mice receiving 0.5% CFE but these phenomena did not occur in the pure forskolin-fed mice. In fact, a slight tendency towards decreased visceral fat mass, without any effect on liver weight, of mice receiving forskolin alone was noted. Thus, the effects of CFE on stored fat could be partially attributed to the lipolysis action of its forskolin content. Enhanced lipolysis leading to fat loss by forskolin has been reported both *in vitro* (Allen *et al.*, 1986; Okuda *et al.*, 1992) and *in vivo* (Han *et al.*, 2005). Obviously, impaired visceral adipose tissue development resulted in increased liver weight and thus the inability of CFE to suppress body weight gain cannot be ruled out. It appears that feeding CFE, especially at the highest dose, may lead to the induction of a whole or partial lack of adipose tissue, as in lipodystrophy or lipoatrophy. This quick collapse of visceral adipose tissue development caused by CFE appears to be reversible after only 1 week of post-CFE treatment.

The measurement of the activities of plasma biochemical parameters such as AST, ALT, and ALP in CFE mice dramatically showed several folds increase above the control. The transaminase enzymes such as AST and ALT and other hepatic markers including ALP are the most sensitive markers that play a major role in liver

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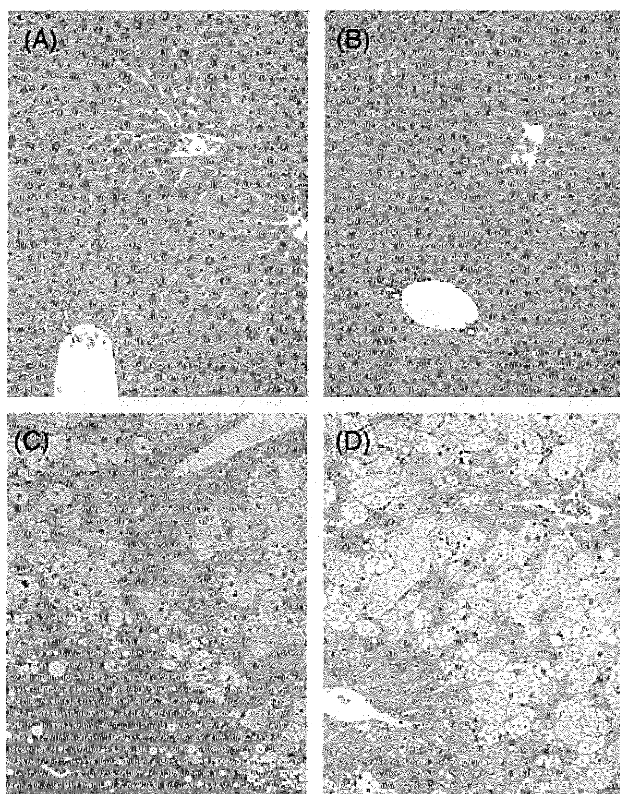

Figure 4. Representative histopathological changes of haematoxylin and eosin (H&E) stained liver sections of mice (original magnification 40×). Normal histological appearance of liver tissue of control mice (A); 0.05% forskolin diet group, liver section also show no abnormalities (B); liver sections of the 0.5% *Coleus forskolli* extract (CFE) diet group (C and D) – showed microvesicular fatty change in hepatocytes (c), apparent midlobular hypertrophy showing individual cell necrosis of hepatocytes, hepatocytes with cellular infiltration and clusters of foamy cells (D).

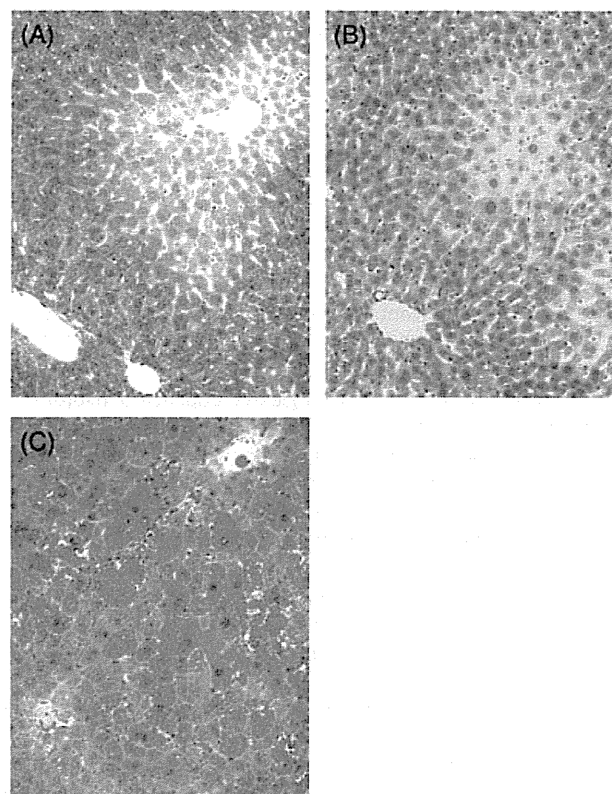

Figure 5. Representative histopathological changes of Oil red O-stained liver sections of mice (original magnification 40×). Control fed (a); 0.05% forskolin-fed mice (b); 0.5% *Coleus forskolli* extract (CFE) fed mice (c). Fat accumulation appears red in colour.

injury diagnosis (Sallie *et al.*, 1991). Elevation of AST, ALT and ALP activities in the plasma is the result of leakage from damaged cells and therefore reflects hepatocyte damage which is strongly associated with liver steatosis (Loria *et al.*, 2005).

In parallel with the alteration of liver function markers, these events were also confirmed by histological observation. In CFE mice, significant hepatic toxicity, including necrosis, hypertrophy and fatty change was observed. Hepatocyte fat accumulation was qualitatively characterized by the intensity of oil red O staining, which allows detection of lipid deposition. We found that CFE-treated mice had strong oil red O staining intensity indicating CFE caused predominantly microvesicular steatosis. Floettmann *et al.* (2010) found that once livers had exceeded a threshold weight of about 5.5% of total body weight that there is a correlation between increased liver weight and the presence of lipids analysed by oil red O staining intensities. In the present study, the relative liver weight of CFE mice at 7% is well above the threshold which further supports the fatty liver feature found from the oil red O staining result. The relative liver weight of forskolin-treated mice is about 4% and H&E and oil red O sections revealed no evidence of any lesion or fatty livers.

Several previous studies have established the association between impaired hepatic fat metabolism and the visceral fat depot (Unger *et al.*, 2010). Adipose tissue plays important roles in metabolic homeostasis which include an inert storage site for fat, and a major endocrine organ producing and releasing a variety of important bioactive substances into the bloodstream (Lara-Castro *et al.*, 2007). Deficiency of visceral adipose tissue has been associated with altered lipid metabolism most notably lipid accumulation in tissues such as the liver (Unger *et al.*, 2010). Adverse side effects as a result of a rapid and marked decrease in fat stored in adipose tissue, namely, severe liver steatosis have been reported in mice, and that the hepatic lipid accumulation is a result of uptake of mobilized fatty acids (FA) from adipose tissue and the liver's inability to sufficiently increase FA oxidation and export of synthesized triglycerides (Clement *et al.*, 2002; Wendel *et al.*, 2008). In the present studies, we demonstrated that a profoundly abnormal decrease in body adiposity observed in our CFE-fed mice drives fatty liver development clearly visible by the oil red O staining of lipid droplets. However, how the collapse of visceral fat mass by CFE triggers hepatocyte lipid deposition resulting in fatty liver remains to be defined.

An increased uptake of fatty acid in hepatocytes is associated with oxidative stress by overloaded mitochondrial beta-oxidation and is often found together with significant generation of reactive oxygen species, impaired exit of fatty acids and increased progression of steatosis (Gaemers and Groen, 2006). In addition, there is also a close relationship between steatosis and oxidative stress with reduced hepatic levels of glutathione (GSH) (Ibdah *et al.*, 2005). GST are a superfamily of multifunctional detoxification enzymes, which catalyze the conjugation of GSH to a wide variety of electrophilic compounds. GST isozymes also exhibit

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Table 2. Grading levels of dietary treatment related histopathological liver changes of mice in the control and treatment groups

Histopathological feature	Control	+ 0.05% Forskolin	+ 0.5% CFE
Haemorrhage	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.58
Hepatocyte single cell necrosis	0.0 ± 0.0	0.0 ± 0.0	1.25 ± 0.25***
Hepatocyte hypertrophy	0.0 ± 0.0	0.0 ± 0.0	1.75 ± 0.48**
Fatty change	1.0 ± 0.0	1.0 ± 0.0	2.75 ± 0.25***

Values are expressed as means ± standard error (SE).
**,
*** Denote significant differences from the control, $P < 0.01$ and $P < 0.001$, respectively.

GSH peroxidase activity and catalyse the reduction of hydroperoxides of fatty acids, and phospholipids (Frova, 2006). Previous studies have demonstrated that induction of the GSTs protected against CCl4-induced hepatotoxicity in mice by catalysing the decomposition of lipid hydroperoxides generated from oxidative damage of cellular lipid molecules (Ohnuma *et al.*, 2011; Yang *et al.*, 2001). Also there is a clear manifestation of excessive formation of hepatic lipid peroxidation associated with the decline in the levels of antioxidant enzymes including GST (Fukao *et al.*, 2004). In our previous study (Virgona *et al.*, 2012), we found that both CFE and forskolin induced GST and CYP3A activities in the liver of mice. Thus it is therefore conceivable that the forskolin moiety of CFE enhanced GST to help mitigate the adverse effects that CFE had on lipid storage in the liver. Su *et al.* (1999) reported that CYP3A activity is highly responsive to relatively small changes in hepatic lipids produced by dietary manipulation in a rat model of microvesicular steatosis.

The importance of visceral adipose tissue should be considered as an integral component of the disorder, as evidenced in the 0.5% CFE-fed mice; a reduction in visceral adipose tissue probably influenced the regulation of hepatic lipid homeostasis in the liver and led to hepatotoxicity with fatty liver. In the present study, CFE extract promotes lipid accumulation in liver tissue at a dose 6 to 29 times higher than those generally reported for humans (Godard *et al.*, 2005; Henderson *et al.*, 2005) based on the body surface area normalization method (Reagan-Shaw *et al.*, 2008). Even if it is difficult to transfer the human doses to laboratory rodents, we can reasonably state that the doses used in the present study are high. In some human studies (Hori *et al.*, 2009) the risk of the development of non-alcoholic fatty liver disease (NAFLD) increased with the number of high-risk GSTs genotypes. Therefore, the increased possibility of NAFLD by the intake of CFE products in humans should not be overlooked.

As mentioned above, the aetiology of the hepatotoxicity is still unknown. However, one fact is certain that forskolin, the principle component of CFE, is not responsible for this hepatotoxicity. HPLC-ELSD analysis of the constituents of the CFE extract showed that additional peaks (marked as 1, 2 and 3, Fig. 3B), albeit small, were present at the retention time of approximately 3 and 8 min. Therefore, further studies on the isolation of these and possible other unknown constituents which may have a role in CFE-induced fatty liver are needed.

In summary, the present results indicate that unknown component(s) in CFE but not forskolin causes hepatotoxicity. The hepatotoxicity is dose dependent, and more importantly has quick onset which can be reversed within 1 week by withdrawal of CFE. The feature of hepatotoxicity consisting of hepatocellular damage and enzyme leakage resulted in elevated liver marker enzymes. Histologically, CFE induces fatty liver. The present study also demonstrates an important effect of dietary CFE on adiposity. Marked reductions in adipose mass probably contribute to the abrupt and massive increase in liver size, hepatocellular injury and liver steatosis. Further study on these observations should provide mechanistic insight into the metabolic and molecular mediators of adipose restriction and the occurrence of lipid accumulation in the liver.

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REFERENCES

Abbadi M, Morin C. 1999. On the analogy between forskolin and D-glucose. *Bioorg. Med. Chem. Lett.* **9**: 1779–1782.

Alasbahi RH, Melzig MF. 2012. Forskolin and derivatives as tools for studying the role of cAMP. *Pharmazie* **67**: 5–13.

Allen DO, Ahmed B, Naseer K. 1986. Relationships between cyclic AMP levels and lipolysis in fat cells after isoproterenol and forskolin stimulation. *J. Pharmacol. Exp. Ther.* **238**: 659–664.

Ammon HP, Muller AB. 1985. Forskolin: from an ayurvedic remedy to a modern agent. *Planta Med.* **51**: 473–477.

Clement L, Poirier H, Niot I, Bocher V, Guerre-Millo M, Krief S, Staels B, Besnard P. 2002. Dietary *trans*-10,*cis*-12 conjugated linoleic acid induces hyperinsulinemia and fatty liver in the mouse. *J. Lipid Res.* **43**: 1400–1409.

Ding X, Staudinger JL. 2005. Induction of drug metabolism by forskolin: the role of the pregnane X receptor and the protein kinase a signal transduction pathway. *J. Pharmacol. Exp. Ther.* **312**: 849–856.

Egras AM, Hamillton WR, Lenz TL, Monaghan MS. 2011. An evidence-based review of fat modifying supplemental weight loss products. *J. Obes.* **2011**: 297–315.

Floettmann E, Gregory L, Teague J, Myatt J, Hammond C, Poucher SM, Jones HB. 2010. Prolonged inhibition of glycogen phosphorylase in livers of Zucker diabetic fatty rats models human glycogen storage diseases. *Toxicol. Pathol.* **38**: 393–401.

Frova C. 2006. Glutathione transferases in the genomics era: new insights and perspectives. *Biomol. Eng.* **23**: 149–169.

Fukao T, Hosono T, Misawa S, Seki T, Ariga T. 2004. Chemoprotective effect of diallyl trisulfide from garlic against carbon tetrachloride-induced acute liver injury of rats. *Biofactors* **21**: 171–174.

Gaemers IC, Groen AK. 2006. New insights in the pathogenesis of non-alcoholic fatty liver disease. *Curr. Opin. Lipidol.* **17**: 268–273.

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