Reprinted from: Perspectives on new crops and new uses. 1999.
J. Janick (ed.), ASHS Press, Alexandria, VA.

Antimicrobial and Cytotoxic Activity of the Extracts of Khat Callus Cultures

Hamid Elhag, Jaber S. Mossa, and Mahmoud M. El-Olemy*

Khat, (*Catha edulis* Forssk., Celastraceae) is an evergreen tree indigenous to East Africa and Yemen. The fresh young leaves are commonly chewed, known as the Khat habit, to alleviate hunger and to produce stimulating effects (CNS). Such effects were shown to be due to phenylalkylamine alkaloids, primarily cathinone (Kalix 1990; Crombie et al. 1990). Habitual use of khat is often associated with social and medical problems (Shadan and Shellard 1972).

Previous work on khat cultures, in our laboratories, has dealt with in vitro micropropagation (Elhag 1991) and the production of secondary metabolites by micropropagated plantlets and callus cultures (El-Domiaty et al. 1994). In the course of our work with khat tissue cultures, the production of dark colored pigments was observed as a typical characteristic of the callus culture (Elhag and Mossa 1996). The present investigation deals with the isolation, identification, and biological effects of such pigments.

METHODOLOGY

Callus Culture

Leaves from young twigs of micropropagated greenhouse grown plants (Elhag 1991) were used as explants for callus induction and as fresh material for extraction and chemical analysis. As described previously by El-Domiaty et al. (1994) callus induction from leaf sections was best achieved on MSB5 basal medium (Murashige and Skoog inorganic salts with Gamborg-B5 vitamins) supplemented with 3.0 mg/L of either IBA (indolebutyric acid) or NAA (naphtalene acetic acid). PVP (polyvinylpyrrolidone) at a concentration of 0.1% in the medium was found beneficial in enhancing callus growth. However, PVP was excluded from the culture medium after the establishment of proliferating cultures. Callus tissues collected form several subcultures on IBA or NAA-containing media were used for extraction and analysis of pigments.

Extraction and Isolation

The freeze-dried powdered callus of *C. edulis* (25 g) was extracted with MeOH at room temperature. The MeOH extract was evaporated and the residue (3.9 g) was partitioned between $CHCl_3$ and H_2O. The dark brown residue (0.89 g) left after evaporation of $CHCl_3$ was chromatographed on silica gel column (2 × 20 cm) and eluted with $CHCl_3$ (300 ml) and $CHCl_3$ containing a trace of acetic acid (10:0.01, 200 ml). Two fractions were collected, fraction A (230 mg) and fraction B (110 mg). Fraction A (containing the pigments) was further chromatographed on reversed phase silica gel (C-18, 25–40 μ) using medium pressure column (2 × 18 cm, 75% MeOH in 5% aqueous acetic acid) with a flow rate of 3 ml/min. Upon evaporation, fractions eluted between 25–60 ml gave 32 mg of compound 1 (0.13%), while fractions 120–175 ml gave 120 mg of compound 2 (0.48%).

Antimicrobial Assays

Evaluation of the antimicrobial activity of callus extracts (petroleum ether, $CHCl_3$, MeOH, and aqueous successive extracts) and the isolated compounds 1 and 2 was conducted according to the disk diffusion and agar dilution methods (Mitscher et al. 1972; Jayasuria 1988). Chloramphenicol and streptomycin were used as positive controls. The solvent DMSO was used as the negative control for all the experiments. The bacteria used were obtained from the National Collection of Type Culture (NCTC), Central Public Health Laboratory, London; and the Center for Disease Control, Atlanta, Georgia, US. The minimum inhibitory concentration (MIC) of the two compounds (22β-hydroxytingenone and tingenone) was determined by the two-fold serial dilution assay (Hufford et al. 1975). The MIC was taken as the lowest concentration that inhibited growth

*The authors thank King Abdelaziz City for Science and Technology (KACST), Riyadh, Saudi Arabia, for financial support (Project No. AT-15-39.

after 48h of incubation at 37°C for *B. subtilis*, *S. aureus*, and *S. durans* and after 72 hr for *Mycobacterium* strains.

Cytotoxicity and Anti-HIV

The assays were performed according to the standard procedures of the in vitro primary screen of NCI (Weislow et al. 1989; Grever et al. 1992).

EXPERIMENTAL RESULTS

Extract Identification

Khat callus produced on MSB5 medium containing IBA or NAA continued to proliferate as compact hard clumps with snow-white top surfaces and dark-pigmented lower parts. The dark pigments partly diffused into the medium at the point of contact with the agar medium. This dark pigmentation was thought to result from the high content of polyphenols (tannins) that is known for the intact plant (El-Sissi and Abdalla 1966). However, the initial TLC (Si gel, EtOAc-HCOOH-H_2O, 90:6:5) screening of the EtOH extract of the callus detected only a trace of the polyphenolic precursors of tannins gallocatechin and epigallocatechin. On the other hand, intact khat plants did not show the same pigments detected in the callus (TLC : Si gel, EtOAc-HCOOH-toluene, 9:1:10).

The MeOH extract of the freeze-dried callus was fractionated between H_2O and $CHCl_3$. The colored chloroformic fraction yielded two orange pigments using combined normal and reversed phase chromatography. Compound 1 gave, $[\alpha]_D$ -317.4°; UV spectrum [λ_{max} MeOH; 420, 286. and 246 nm], suggested the presence of a chromophore. IR spectrum showed bands at ν_{max} 3400 (OH), 1710 (carbonyl), and 1580 cm^{-1} (conjugated C = C). The UV spectrum and IR bands, in addition to the positive Liebermann-Burchard test suggested a quinone methide triterpene structure (Gonzalez et al. 1983; Fernando et al. 1988; Likhitwitayawuid et al. 1993). This suggestion was substantiated by the presence of proton signals at δ 6.55 (d, *J* = 2, Hz, H-1), 7.07 (dd, *J* = 7 & 2 Hz, H-6), and 6.36 (d, *J* = 7 Hz, H-7) in the ^{1}H-NMR spectrum ($CDC1_3$) (Likhitwitayawuid et al. 1993). The ^{1}H-^{1}H COSY showed a proton signal at δ 4.56 (d, *J* = 5 Hz, H-22) correlated to the signal at δ 3.66 (which is not directly correlated to any carbon signal in ^{1}H-^{13}C HETCOR), while the proton at δ 4.56 correlated to the signal at δ 76.4 in the ^{13}C NMR spectrum, (C-22). Thus the proton signal at δ 4.56 should be assigned to H-22 and the signal at δ 3.66 to OH proton. Compound 1 was thus identified as 22β-hydroxytingenone; this was confirmed by direct comparison with the reported spectral data (Kutney et al. 1981; Bavovada et al. 1990; Likhitwitayawuid et al. 1993).

Compound 2 gave $[\alpha]_D$ -307.4°; its UV spectrum [λ_{max}) MeOH : 420, 286 and 252 nm], was quite similar to that of compound 1, suggesting the presence of a similar chromophore, IR spectrum of compound 2 showed the same characterstic bands of 1. ^{1}H- and ^{13}C-NMR data of compound 2 were similar to that of 1 except that 2 showed the absence of hydroxyl group at C-22, as it appeared at δ 52.6 in ^{13}C-NMR and one of the H-22 protons appeared at δ 2.92 (H, d, J= 14 Hz) in ^{1}H-NMR. Compound 2 was thus identified as tingenone; this was confirmed by comparison with reported data (Gonzalez et al. 1975; Kutney et al. 1981; Ngassapa et al. 1994).

Antimicrobial Activity

Initial antimicrobial screening of the crude callus extracts was conducted using the disk diffusion method and was confirmed with bioautography (Jayasuria 1988). The highest growth inhibition was found in the petroleum ether and chloroformic successive extracts, the latter being more active. Further fractionation and purification of the chloroformic-soluble fraction of the methanolic extract (as described in Materials and Methods), resulted in the isolation of two active compounds (1) and (2). The isolated compounds were identified as 22β-hydroxytingenone (1) and tingenone (2) by various spectral methods. Both compounds exhibited significant activities against *B. subtilis*, *S. aureus*, and *S. durans* (MIC 0.6 μg/ml), being more potent than the positive control chloramphenicol (Table 1). Both compounds were also found to be more potent against *Mycobacterium* species (MIC was 5.0 μg/ml for both compounds) than the positive controls, streptomycin and

Table 1. MIC values for 22β-hydroxytingenone (compound 1) and tingenone (compound 2) isolated from khat callus cultures.

Microorganisms	MIC values (μg/ml) (1)	(2)	Streptomycin	Chloramphenicol	Isonicotinic acid hydrazide
B. subtilis[z]	0.6	0.6	NT[x]	4.0	NT
S. aureus[z]	0.6	0.6	NT	8.0	NT
St. durans[z]	0.6	0.6	NT	4.0	NT
M. chelonei[y]	5.0	5.0	10.0	NT	10.0
M. smegmatis[y]	5.0	5.0	10.0	NT	10.0
M. intracellulare[y]	5.0	5.0	10.0	NT	10.0
M. xenopi[y]	5.0	5.0	10.0	NT	10.0
E. coli	inactive	inactive	NT	NT	NT
C. albicans	inactive	inactive	NT	NT	NT

[z]MIC values after 48 hr of incubation at 37° C
[y]Incubated for 72 hr at 37° C
[x]NT = not tested.

isonicotinic acid hydrazide (Table 1). However, both compounds; were found to be inactive against the gram-negative bacteria *E Coli* and the fungus *C. albicans* (Table 1).

Table 2. Results of antitumor evaluation of 22β-hydroxy-tingenone in the NCI in vitro primary screen.

Panel	ED_{50} (μg/ml)
Leukemia	0.54
Non-small cell lung cancer	4.4
Colon cancer	1.31
CNS cancer	3.3
Melonama	1.71
Ovarian cancer	2.35
Renal cancer	1.61
Prostate cancer	0.85
Breast cancer	1.11

Cytotoxic Activity

Compounds 1 and 2 have been reported to have cytotoxic activity (Kutney et al. 1981, Bavovada et al. 1990; Ngassapa et al. 1994); however, compound 1 was tested only against a few cancer cell line systems (Bavovada et al. 1990). In the present study, compound 1 was therefore tested using NCI (USA) in vitro primary anticancer and anti-HIV screening. Table 2 shows that 22β-hydroxytingenone (1) exhibited significant cytotoxic activities against leukemia (ED_{50} 0.54 μg/ml) and prostate cancer (ED_{50} 0.85 mg/ml), while the least activity was observed against non-small cell lung cancer (ED_{50} 4.4 μg/ml). While compound 1 showed non-selective broad cytotoxicity against all tested panels (Boyd and Paul 1995), it was inactive when tested against HIV virus. Tingenone 2, was also shown to exhibit strong non-selective broad cytotoxicity against several cancer cell-line systems (Ngassapa et al. 1994).

CONCLUSIONS

The cultural conditions for khat callus induction and growth were established. Best callus induction and growth occurred on MSB5 medium supplemented with 3.0 mg/L of either NAA or IBA. The production of dark pigments was observed at the start of callus induction and continued with subcultures as a typical characteristic of khat callus.

The isolation of 22β-hydroxytingenone and tingenone from khat callus cultures is reported for the first time. They could not be detected in the mother plant grown in the greenhouse. The crude callus extracts and the isolated compounds, 22β-hydroxytingenone (compound 1) and tingenone (compound 2), showed high antibacterial activity against gram positive and myocbacteria and broad cytotoxic activity against several cell-line systems.

Large scale production of khat cultures for the commercial production of such biologically active components is a promising system worthy of further investigation. It would be ironic if khat, which is considered a plant of abuse, turned out to be a miracle plant with efficacious medical properties.

REFERENCES

Bavovada, R., G. Blasko, H.L. Sheih, J.M. Pezzuto, and G.A. Cordell. 1990. Spectral assignment and cytotoxicity of 22-hydroxytingenone from *Glyptopetalum sclerocarpum.* Planta Med. 56:380–382.

Boyd, M.R. and K.D. Paul. 1995. Some practical considerations and application of the national cancer institute in vitro anticancer drug discovery screen. Drug Develop. Res. 34:91–109.

Crombie, L., W.M.L. Crombie, and D.A. Whiting. 1990. Alkaloids of khat (*Catha edulis*). Alkaloids 39:139–164.

El-Domiaty, M.M., H.M. Elhag, F.S. El-Feraly, I.A. al-Meshal, and M.M. El-Olemy. 1994. Studies on (-)-cathinone formation in micropropagated plants and tissue cultures of *Catha edulis* (Khat). Int. J. Pharmacog. 32:135–141.

Elhag, H.M. 1991. In vitro propagation of *Catha edulis.* HortScience 26:212.

Elhag, H.M. and J.S. Mossa. 1996. *Catha edulis*: In vitro culture and the production of cathinone and other secondary metabolites. p. 76–86. In: Y.P.S. Bajaj (ed.), Biotechnology in agriculture and forestry. Springer Verlag, Berlin.

El-Sissi, H.I. and M.F. Abdalla. 1966. Polyphenolics of the leaves of *Catha edulis.* Planta Med. 14:76–79.

Fernando, H.C., A.A. Leise Gunatilaka, V. Kumar, and G. Weeratunga. 1988. Two new quinone-methide from *Cassine balae*: Revised structure of balaenolol. Tetrahedron Lett. 29:387–390.

Gonzalez, A.G., C.G. Francisco, R. Friere, R. Hernandez, J.A. Salazar, and E. Surez. 1975. Inguesterin, a new quinonoid triterpene from *Catha cassinoides.* Phytochemistry 14:1067–1077.

Gonzalez, A.G., B.M. Fraga, C.M. Gonzalez, A.G. Ravelo, and E. Ferro. 1983. X-ray analysis of netzhaulcoyone, a triterpene quinone methide from *Orthosphenia mexicana*. Tetrahendron Lett. 24:3033–3036.

Grever, M.R., A.A. Sheprtz, and B.A. Chabner. 1992. The National Cancer Institute cancer drug discovery and development program. Seminars Oncol. 19:622.

Hufford, C.D., M.J. Funderburk, J.M. Morgan, and L.W. Robertson. 1975. Two antimicrobial alkaloids from heartwood of *L. tulipifera*. J. Pharm. Sci. 4:789–792.

Jayasuria, H. 1988. Biological and chemical investigations of native Mississippi plants. *H. drummondi* (grev. and T and G). Ph.D. Thesis, Univ. of Missippi, Oxford.

Kalix, P. 1990. Pharmacological properties of the stimulant khat. Pharmacol. Ther. 48:397–416.

Kutney, J.P., M.H. Beale, P.J. Salisbury, K.L. Stuart, B.R. Worth, P.M. Townsley, W.T. Chalmers, K. Nilsson, and G.G. Jacoli. 1981. Isolation and characterization of natural products from plant tissue cultures of *Maytenus bachananii*. Phytochemistry 20:653–657.

Likhitwitayawuid, K., R. Bavovada, L-Z. Lin, and G.A. Cordell. 1993. Revised structure of 20-hydroxytingenone and ^{13}C-NMR assignments of 22 B-hydroxytingenone. Phytochemistry 34:759–763.

Mitscher, L.A., R.P. Leu, M.S. Bathala, W.N. Wu, and J.L. Beal. 1972. Antimicrobial agents from higher plants. 1. Introduction, rationale and methodology. Lloydia 35:157–166.

Ngassapa, O., D.D. Soejarto, J.M. Pezzuto, and N.R. Farnsworth. 1994. Quinone-methide triterpenes and salaspermic acid from *Kokoona ochracea.* J. Nat. Prod. 57:1–8.

Shadon, P. and E.J. Shellard. 1972. An anatomical study of Ethiopian khat (leaf of *Catha edulis* Forssk.) J. Pharm. Pharmacol. 14:110–118.

Weislow, O.W., R. Kiser, D. Fine, J. Bader, R.H. Shoemaker, and M.R. Boyd. 1989. New soluble-formazan assay for HIV-1 cytopathic effects: Application to high-flux screening of synthetic and natural products for AIDS-antiviral activity. J. Nat. Cancer Inst. 81:577–586.