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# Polyphenolic compounds in the fruits of Egyptian medicinal plants (*Terminalia bellerica*, *Terminalia chebula* and *Terminalia horrida*): Characterization, quantitation and determination of antioxidant capacities

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ABSTRACT

Thirty-four polyphenolic substances in methanol extracts of the fruits of *Terminalia bellerica*, *Terminalia chebula* and *Terminalia horrida*, three plants used in Egyptian folk medicine, were initially identified by HPLC–ESI-MS and quantitated by analytical HPLC after column chromatography on Sephadex LH-20. After purification by semi-preparative HPLC the compounds were identified by their mass and fragmentation patterns using ESI-MS–MS. For several compounds detailed $^{1}H/^{13}C$ NMR analysis at 600 MHz was performed. Two polyphenolics, namely 4-*O*-(4″-*O*-galloyl-α-L-rhamnopyranosyl)ellagic acid and 4-*O*-(3″,4″-di-*O*-galloyl-α-L-rhamnopyranosyl)ellagic acid were identified by NMR. Antioxidant capacities of the raw fruit extracts and the major isolated substances were determined using the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), oxygen radical absorbance capacity (ORAC) and ferric reducing ability of plasma (FRAP) *in vitro* assays and indicated that chebulic ellagitannins have high activity which may correlate with high potential as cancer chemopreventive agents. Therefore, further studies (metabolism, bioavailability and toxicity) of the polyphenolics in *Terminalia* species using preclinical models and *in vivo* human intervention trials are warranted.



## 1. Introduction

The three Terminalia trees (*Terminalia chebula* Retz, *Terminalia bellerica* and *Terminalia horrida*) belong to the family Combretaceae and are widespread in Egypt and other subtropical and tropical regions. The fruits are called black or chebulic myrobalans and are used in the leather tanning industry and in local traditional folk medicine in Egypt, India and Pakistan. Their trivial names in Egypt are Kebuli (*T. chebula*), Hind (*T. horrida*) and Bellileg (*T. bellerica*). Extracts of Terminalia fruits have been frequently applied in so-called folk medicines due to their laxative, astringent, purgative and diuretic properties. In Ayurvedic medicine a herbal formulation comprising equal parts of *T. chebula*, *T. bellerica* and *Embilica officinalis*, called "Triphala", is used to promote health, immunity and longevity (Kumar et al., 2008; Srikumar et al., 2005). Methanolic and aqueous extracts of *T. chebula* have been reported to exhibit a variety of biologic effects, e.g., antioxidant (Cheng et al., 2003; Lee et al., 2005, 2007; Saleem et al., 2001), antimicrobial (Malekzadeh et al., 2001), antianaphylactic (Shin et al., 2001), antidiabetic (Gao et al., 2008; Sabu and Kuttan, 2002; Saleem et al., 2001), antimutagenic (Kaur et al., 1998) and anticancer (Saleem et al., 2002) activities. *T. bellerica* extracts show antimutagenic (Padam et al., 1996), antimicrobial (Elizabeth, 2006), antiviral, antimalarial and antifungal activities (Valsaraj et al., 1997). Inhibitory effects have also been described for extracts of *T. bellerica*, *T. horrida* and *T. chebula* against human immunodeficiency virus reverse transcriptase (el Mekkawy et al., 1995).

Previous data on the characterization and quantitation of polyphenolic compounds present in Terminalia species is rather limited. Important studies in this area were presented by Tanaka

*Abbreviations:* AAPH, 2,2′-azobis (2-amidino-propane)-dihydrochloride; COLOC, C–H correlation via long-range coupling; COSY-45, 2D proton correlation spectroscopy with 45° readout pulse; cROESY, compensated rotating-frame Overhauser spectroscopy; DEPT, distortionless enhanced polarization transfer; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical; ESI, electrospray ionization; FRAP, ferric reducing ability of plasma; HHDP, hexahydroxydiphenoyl moiety (6,6′-dicarbonyl-2,2′,3,3′,4,4′-hexahydroxybiphenyl); HMBC, heteronuclear multiple-bond correlation 2D NMR; HSQC, heteronuclear single-quantum correlation 2D NMR; NOE, nuclear Overhauser effect; ORAC, oxygen radical absorbance capacity; ROESY, rotating-frame Overhauser effect; SERM, selective estrogen receptor modulator; SPS, secondary plant substance; TMS, tetramethylsilane; TPTZ, 2,4,6,-tri(2-pyridyl)-1,3,5-triazine, CAS: 3682-35-7; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid, CAS: 56305-04-5.

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et al. (1986) who characterized 12 different polyphenols in *Terminalia catappa* L. and by Juang et al. (2004) who identified and quantitated ten diverse polyphenols in *T. chebula*. However, only corilagin, punicalagin and chebulagic acid were found in both studies. Later, Juang and Sheu (2005) compared the concentrations of ten polyphenolic compounds in 28 commercial samples of *T. chebula* from Taiwan and found a high degree of sample variance (range: 126.5–360.3 g/kg, mean: 230 ± 81 g/kg).

The aim of this study was the comprehensive characterization of polyphenolic compounds in the fruits of three Terminalia species commonly used in traditional Egyptian folk medicine. The majority of the diverse polyphenolic compounds isolated and purified were studied in three common bioassay systems to establish antioxidant capacity and possible structure–activity relationships.

## 2. Results and discussion

### 2.1. Characterization of the polyphenols

In this study a total of 34 polyphenols were identified in methanol extracts of Terminalia fruits (Table 1, Figs. 1 and 2). These compounds are mainly hydrolyzable tannins, including simple gallate esters, ellagic acid derivatives and glycosides, and various ellagitannins. All substances were characterized by HPLC and ESI-MS, and the assumed molecular formulas, calculated exact masses and ESI-MS data are summarized in Table 1. Where possible, identification was carried out by comparison with authentic reference compounds or with previously isolated substances which had been unambiguously identified by NMR. For several compounds sufficient purified material was available for detailed NMR analysis in $CD_3OD$ solution which led to complete and unambiguous assignments for all $^1H$ and $^{13}C$ resonances (Tables 2–6) and complete determination of structural linkages and stereochemistry (Fig. 2). The remaining substances for which NMR data were not obtained in this study are summarized in Fig. 1. For those compounds which may have several isomeric structures with the same exact mass value (e.g., different positions of galloyl substituents), unambiguous structures could not be determined in every case. This situation is appropriately noted in Table 1 and Fig. 1.

#### 2.1.1. Gallic acid and simple gallate esters

In this subgroup the following known secondary plant substances (SPS) were identified: gallic acid (**1**), methyl gallate (**2**), and the glucose derivatives with di- (**3**), tri- (**4**), tetra- (**5**) and penta-*O*-galloyl (**6**) substituents. The likely substitution patterns based on previous literature data are 1,6-di- (Owen et al., 2003), 1,3,6-tri- or 3,4,6-tri- (Juang et al., 2004), and 1,3,4,6-tetra-*O*-galloyl for β-D-glucopyranose (Barreto et al., 2008). In addition, the compound 3,4,5-tri-*O*-galloyl-(3*R*,4*S*,5*R*)-shikimic acid (**7**) was confirmed conclusively by NMR (Table 2) and has not been previously identified in Terminalia extracts, although it has been isolated from other plant species (Nonaka et al., 1990).

#### 2.1.2. Chebulic acid and chebulic ellagitannins

The known substances from this subgroup, which were confirmed by NMR (Fig. 2, Tables 3 and 4), are the mono-, and tri-*O*-galloyl derivatives of 2,4-*O*-chebuloyl-β-D-glucose, namely, chebulanin (**10**) (also known as terminalic acid) (Liu et al., 1998) and chebulinic acid (**13**), as well as chebulagic acid (**12**) which features a bridging 3,6-*O*-(*R*)-HHDP unit instead of two galloyl residues (Yoshida et al., 1982).

In addition, the previously unreported chebulinic acid analog (**16**) with only two galloyl residues was confirmed by the MS fragmentation pattern with peaks at *m*/*z* 803 $[M-H]^-$, 651 $[M-galloyl]^-$, 633 $[M-H-gallic]^-$, and 481 $[M-galloyl-gallic]^-$, as well as the base peak at *m*/*z* 337 for $[chebulic-H-H_2O]^-$ and further peaks at 319, 293, 275 corresponding to neutral loss of $H_2O$ or $CO_2$. NMR data have not yet been obtained for **16** so that the exact digalloyl configuration (more likely 1,6- or 1,3-) was not determined.

Related compounds in this class which were confirmed by NMR (Fig. 2, Tables 3 and 4) are methyl neochebulagate (**14**) and methyl neochebulinate (**15**). Mechanistically (Haslam and Uddin, 1967), the diacyl chebuloyl moiety can be reversibly converted to the monoacyl neochebuloyl form by hydrolysis at glucose C2, hydrolytic opening of the lactone linkage C1′–O–C5″ (see Fig. 2), 180° rotation of the galloyl residue around the C3′–C6″ bond, followed by closure of a new lactone ring between C7′ and 2′-OH. This interconversion scheme can be visualized on the basis of the atom numbering scheme used in Fig. 2 (e.g., compare **12** and **14**). The presence of the methyl ester at C1′ in **14** and **15** was confirmed by NMR. Hydrolysis of the methyl ester and the acyl linkage at glucose C4 in these compounds would result in the release of the neochebuloyl group as free chebulic acid (Yoshida et al., 1982), which was isolated as compound **9**.

At this point we wish to point out that there are some confusing and inconsistent aspects concerning the nomenclature of chebulic derivatives in the literature. Using the numbering scheme in Fig. 1, the absolute stereochemical configuration is (2*S*,3*S*,4*S*) for chebulic acid (**9**) from *T. chebula*, as determined by Yoshida et al. (1982) and as shown for the analagous 2′,3′,4′ positions in the chebuloyl and neochebuloyl moieties in Fig. 2. Note that the configuration of the lactone ring in chebulic acid corresponds exactly to that shown for the methyl esters of neochebulagate (**14**) and neochebulinate (**15**) in Fig. 2. In contrast, the chebuloyl moieties in chebulagic and chebulinic acids have a rearranged lactone, i.e. not the form that occurs in chebulic acid. To make matters worse, Lee et al. (2007) have described neochebulic acid as the diastereomer with the configuration (2*S*,3*S*,4*R*) and the same lactone ring as in chebulic acid.

The MS fragmentation patterns for **14** and **15** show neutral loss of gallic acid and methanol from the $[M-H]^-$ molecular ion as well as loss of a $[methyl\ neochebuloyl]^+$ moiety (*m*/*z* 353) from M to give the negative ions at *m*/*z* 463 and 465, respectively. Both compounds also show fragment ions characteristic for $[methyl\ neochebulate-H-H_2O]^-$ at *m*/*z* 351, in contrast to the peak at 337 observed for the chebulate fragment of **9**, **12**, and **13**. Finally, the previously unidentified compound **11** was tentatively determined by MS to be methyl neochebulanin (Fig. 1) with only one galloyl residue (presumed to be at glucose C1, as in chebulanin). The fragmentation pattern showed neutral loss of gallic acid, water, and methanol as well as the peak at *m*/*z* 351 corresponding to the methyl neochebulate moiety.

#### 2.1.3. Non-chebulic ellagitannins

Known substances of this type, which were isolated from Terminalia extracts in this study, are tellimagrandin (**17**, Okuda et al., 1983) and corilagin (**18**, Hatano et al., 1988) (Table 1, Fig. 1). More complex diacyl substituents derived from three or four galloyls, i.e. (*S*)-flavogallonyl or gallagyl, can also be found as bridging substituents, as in the isolated known compounds punicalin (**20**), punicalagin (**21**), and terflavin B (**22**) (Table 1, Fig. 1, Tanaka et al., 1986a,b).

#### 2.1.4. Ellagic acid and derivatives

Ellagic acid (**27**), the lactonized form of HHDP when released by hydrolysis, and a number of related compounds were isolated from the Terminalia extracts. Mono- and dimethyl ellagic acids (**33**) and (**32**) (Khallouki et al., 2007) respectively, and gallagic acid (**19**, Cerda et al., 2003) were determined by MS analysis. Gallagic acid is the fully lactonized form of the gallagyl moiety in punicalin (**20**) and