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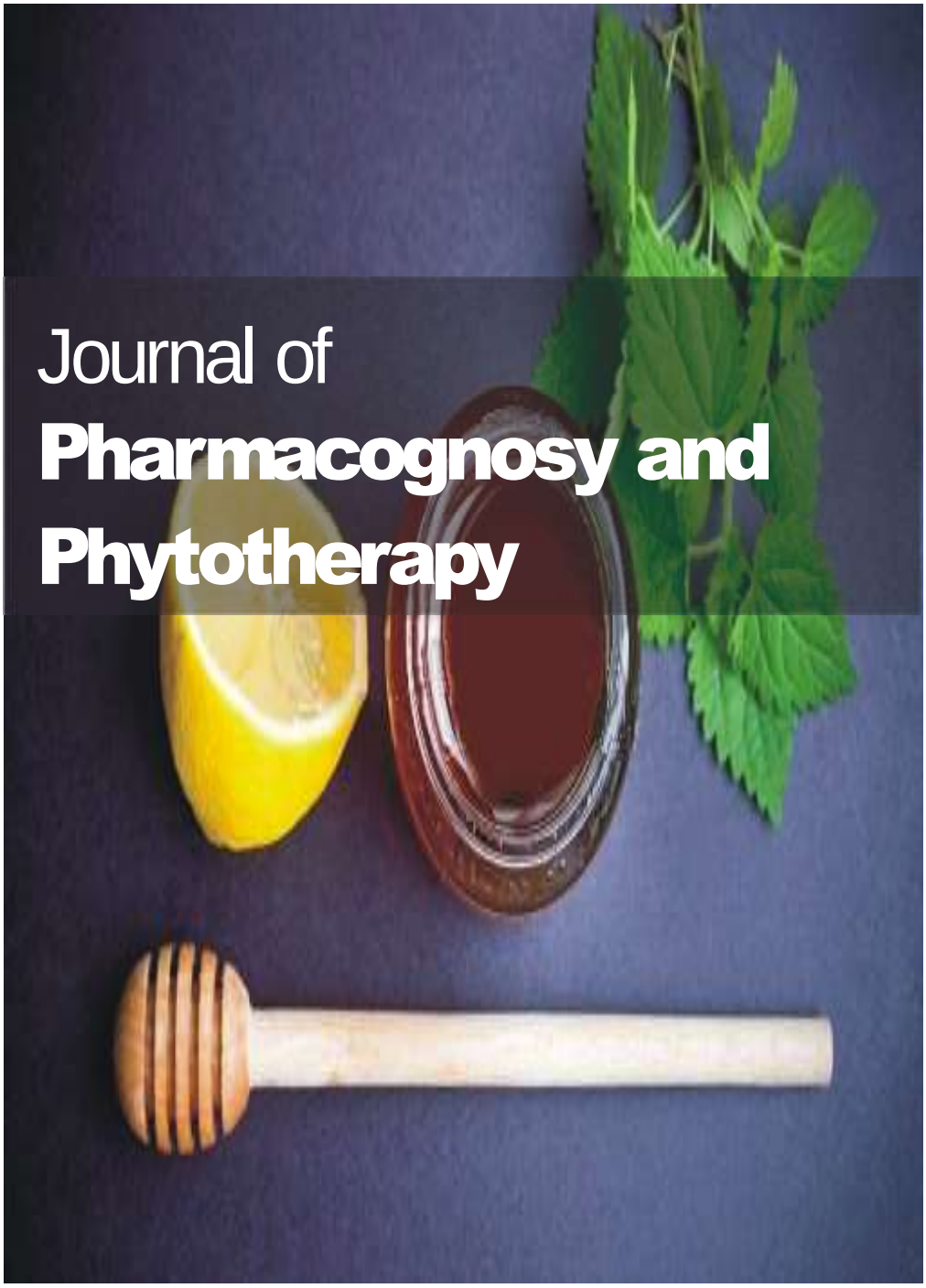

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Table of Content

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Full Length Research Paper

Pharmacognostic, physicochemical and phytochemical evaluation of the leaves of *Fadogia cienkowski* Schweinf (Rubiaceae)

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Fadogia cienkowskii **is a shrub used in folklore medicine. Evaluations of the leaves were carried out to determine the macroscopic, microscopic, chemomicroscopic, physicochemical and phytochemical profiles using standard methods. The macroscopic examination revealed fresh leaves are green, odourless with a bitter taste. The leaf is oblong-elliptic in shape and sub-acute at apex; rounded at the base with entire margin. Microscopic examination indicated the presence of calcium oxalate crystals, starch grains, xylem, phloem, trichomes, epidermal cells, collenchyma cells, paracytic stomata and reticulate vessels. Chemomicroscopic characters present are lignin, starch, cellulose, mucilage and calcium oxalate crystals. The physicochemical evaluation indicated 4.6% moisture content, 1.4% total ash value, 0.8% acid insoluble ash value, 0.4% water soluble ash value, 7.8% water soluble extractive value and 9.0% alcohol soluble extractive value. The phytochemical evaluation revealed the presence of tannins (17.6%), saponins (1%), glycosides (2.5%), alkaloids (3.3%), steroids (1.1%), terpenoids (6.6%), phenols (8.8%), flavonoids (17.7%) and the absence of hydrogen cyanide. This study is useful in pharmacognostic standardization of this plant. The parameters laid down will be useful and suitable for compilation of a monograph and help in identifying this plant in its crude form and prevent it from adulteration and ensure its therapeutic efficacy.**

Key words: *Fadogia cienkowskii*, pharmacognostic, phytochemical, physicochemical, macroscopic, microscopic, chemomicroscopic.

INTRODUCTION

Medicinal plants are important in healthcare system throughout the world for their proven and effective therapeutic properties (Helmstädter and Staiger, 2014). An estimated 80% of the world's population is relying on

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medicines that contain compounds of herbal origin (Ekor, 2013). The International Union for Conservation of Nature has suggested that approximately 50,000 to 80,000 flowering plants are used for medicinal purposes (Chen et al., 2016). Although medicinal plants have been used globally, their wider usage is limited to a few countries like Japan, India, China, Pakistan, Thailand, Iran, and some African countries (Bahmani et al., 2014; Iwu, 2014; Li, 2016; Sivasankari et al., 2014). Other countries are also encouraging the use of plant-based medicinal products in their healthcare systems. For example, Natural Health Product Regulations of Canada for the plant-based product in healthcare encourages usage of modern technology and evidence- based scientific support towards promoting medicinal plants and the associated products (Tomlinson and Akerele, 2015).

However, a key obstacle, which has hindered the acceptance of the alternative medicines in the developed countries, is the lack of documentation and stringent quality control. There is the need for documentation of research work carried out on traditional medicines (Dahanukar et al., 2000). With this backdrop, it becomes extremely important to make an effort towards standardization of the plant material to be used as medicine. The process of standardization can be achieved by stepwise pharmacognostic studies (Ozarkar, 2005). These studies help in identification and authentication of the plant material. Correct identification and quality assurance of the starting materials is an essential prerequisite to ensure reproducible quality of herbal medicine which will contribute to its safety and efficacy. Simple pharmacognostic techniques used in standardization of plant material include its morphological, anatomical and biochemical characteristics (Anonymous, 1998).

Pharmacognostic studies ensure plant identity, lays down standardization parameters which will help and prevents adulterations. Such studies will help in authentication of the plants and ensure reproducible quality of herbal products which will lead to safety and efficacy of natural products (Sumitra, 2014).

Pharmacognostic standardization of crude drugs is a series of laboratory experiment which reveals and assembles a set of inherent peculiar characteristics such as constant parameter, definite, qualitative and quantitative values or specific and unique features on the basis of which similar herbal medicine claim to be the same, can be compared for the purpose of authenticity, efficacy, genuiness, purity, reproducibility and overall quality assurance. The broad use of herbal drugs in conventional medicines, standardization becomes an important measure for ensuring quality, purity and authenticity of the crude drugs. First step in this context is authentification of plant species which can be done by morphological and anatomical analysis or pharmacognostic analysis. It is one of the simplest and cheapest methods for establishing the correct identification of the source materials (Nirmal et al., 2012; Kumar et al., 2012a).

Fadogia cienkowskii belongs to the family Rubiaceae. It is locally called ‘Ogwu-agu’ in Igbo and ‘Ufu-ewureje’ in Igede tribe of Benue State within the middle belt of Nigeria. The leaves were highly acknowledged for their wide therapeutic efficacy in the relief of headache, general body debility, inflammation, diarrhoea and other ailments especially in infants. The plant is a shrub of less than 1 m high usually found in the savannah region and found to be widely dispersed into the drier parts of tropical Africa (Emeline et al., 2012).

The central and peripherally mediated nervous effects, acute toxicity studies, effect on phenobarbitone-induced sleeping time, local anaesthetic effects, analgesic activity and muscle relaxant effects were reported by Ode et al. (2015). As there are incomplete pharmacognostic work recorded on the leaves of *F. cienkowskii* by Chukwube et al. (2018). The present study reports the detailed pharmacognostic, physicochemical and phytochemical evaluation of the leaves of *F. cienkowskii* Schweinf (Rubiaceae). These parameters will be useful in complete authentification and standardization of the crude extract, which can guarantee the quality and purity of the drug and maintain its therapeutic efficacy (Figure 1).

MATERIALS AND METHODS

Plant materials

F. cienkowskii leaves were collected in July 2018 from Enugu state, Nigeria. The plant was *identified* and authenticated by a taxonomist in the Pharmacognosy and Traditional Medicine Department of Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University Awka, Nigeria. Herbarium number PCG474/A/005.

Equipments

Microscope (Finlab, Nig), Hot air oven (Genlab, UK), Electronic weighing balance (Ohaus Corp, USA), water bath (Serological, England), beakers (Pyrex;10, 50, 100 and 1000 ml), measuring cylinders, hand grinding machine (Ohaus Corp, USA), syringes and needles (1, 2 and 10 ml capacity), refrigerator (Thermocool, England), cotton wool (Pyrex).

Reagents and chemicals

Concentrated sulphuric acid (Versha Chemicals, Belgaum), naphthol solution in ethanol (Molisch reagents) (Nalco Chemicals, USA), Ammonium solution (Shakti Chemicals, India), Aluminum chloride (Neel Chemicals, India), Fehling solution A and B (Alpha Chemika, India), Hager’s reagent (saturated solution of picric acid) (Alpha Chemika, India), Wagner’s reagent (iodine and potassium iodide) (Alpha Chemika, India).

Preparation of plant material

The leaves were dipped in water to remove dust and unwanted particle. They were air dried at room temperature for two weeks.

The dried leaves were pulverized with an analytical milling

Figure 1. Habit photograph of *F. cienkowskii*.

machine and sieved to control the particle size. Then it was stored in an airtight container for further analysis (Bruce et al., 2016).

Extraction

A quantity (600 g) of the powdered leaves was extracted using ethanol (2500 ml) with occasional stirring for 72 h by cold maceration. The mixture was sieved using porcelain cloth and filtered with a filter paper. The filtrate was dried *in vacuo* at 40°C. The extract was stored in a refrigerator for use (Onyegbule et al., 2019).

Pharmacognostic studies

Macroscopic examination

Macroscopic studies were carried out by using organoleptic evaluation method. The shape, size, colour, odour, taste, base, texture, margin, apex of the leaf of plant were observed (Evans, 2002).

Microscopic examination

Microscopic studies were carried out by preparing thin sections of leaf. The thin sections were further washed with water, staining was done by clearing in chloral hydrate solution then heat fixed and allowed to cool, then mounted using glycerine. The specimen was gently covered with a cover slip and placed on the stage of the microscope for observation (10x, 40x) (Khandelwal, 2008).

Quantitative investigation

Quantitative leaf microscopy to determine palisade ratio, stomata number, stomata index, and vein – islet number and vein let termination number were carried out on epidermal strips (Evans, 2002).

Chemomicroscopic examination

Examination of the powder for lignin, starch, mucilage, calcium oxalate crystals, cellulose, fatty oil and protein were carried out using standard techniques (Evans, 2002).

Physicochemical analysis

The parameters which were studied are moisture content, ash values and extractive values (Eleazu and Eleazu, 2012; AOAC, 2005; Tatiya et al., 2012).

Phytochemical analysis

Qualitative phytochemical analysis

The plant crude extracts were tested for the presence Reducing sugar, Hydrogen cyanide, Soluble carbohydrate, Tannins, Alkaloids, Steroids, Terpenoids, Phenol, Flavonoids, Saponins and Glycosides using standard methods (Evans, 2002).

Quantitative phytochemical analysis

The coarse powder of the plant material were tested to determine the quantity of Reducing sugar, Hydrogen cyanide, Soluble carbohydrate, Tannins, Alkaloids, Steroids, Terpenoids, Phenol, Flavonoids, Saponins and Glycosides present (Edeoga and Gomina, 2000).

RESULTS

Pharmacognostic evaluation

Macroscopic characteristics of F. cienkowskii

Macroscopic characteristics of *F. cienkowskii* leaf are given in Table 1. The fresh leaves are green in colour, odourless with a bitter taste. The leaf is oblong-elliptic in shape and sub-acute at apex; rounded at the base with entire margin. The leaves are arranged in whorls of 3 at each node or rarely opposite. There surface is pubescent. They measure up to 8 cm in length and 2.5 cm in breath (Table 1).

Microscopic examination

The result of microscopic examination is presented in Figure 2. Microscopic examination indicate the presence

Table 1. Macroscopic characteristics of *F. cienkowskii.*

Feature	Observation
Shape	Oblong-elliptic
Size	8 cm in length and 2.5 cm in breath
Colour	Green
Taste	Bitter
Texture	Rough
Apex	Sub- acute
Base	Round
Margin	Entire
Surface	Pubescent

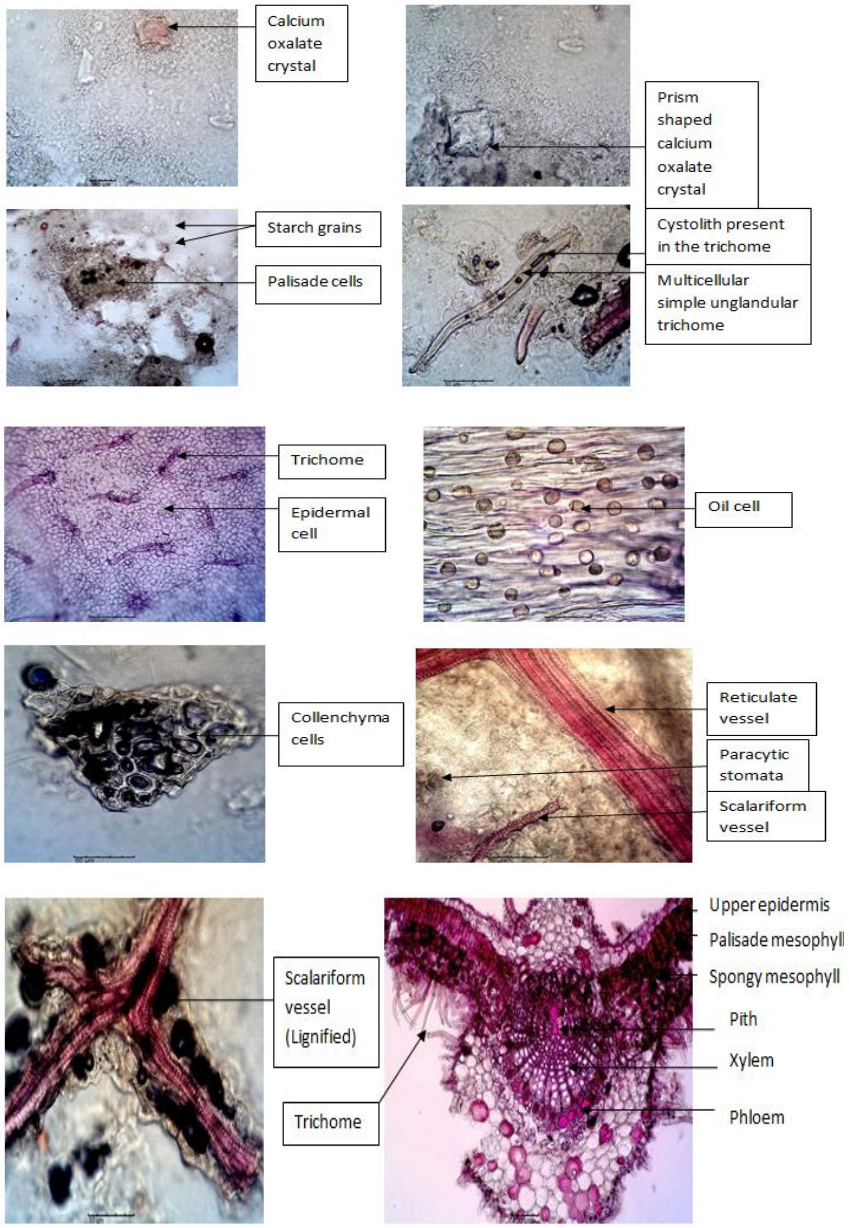

Figure 2. Transverse section of the leaf of *F. cienkowskii* X100.

of the following features: calcium oxalate crystals, starch grains, lignified tissues, cystolith, xylem phloem, scalariform vessels, pith, trichomes, spongy and palisade mesophyll, oil cells, epidermal cells, collenchyma cells, paracytic stomata and reticulate vessels (Figure 2). The diagnostic characteristics are:

(i) The upper epidermis is composed of polygonal cells with very slightly wavy walls which are irregularly thickened and beaded, in the areas over the veins the cells are more elongated; covering trichomes or cicatrices where the trichomes have been attached and some of these give a faint reaction for lignin.
(ii) Oil cells which are usually fragmented, large and spherical surrounded by moderately thick walled parenchymatous cells.
(iii) The cluster crystals of calcium oxalate which occur in a layer of cells in the spongy mesophyll immediately below the palisade.
(iv) The prisms of calcium oxalate, which are found scattered and frequently associated with the groups of fibres; they vary considerably in size and are occasionally quite large and irregularly shaped.
(v) The numerous vessels from the stem, which usually occur in small groups; they are fairly large, lignified and reticulately thickened or bordered pitted; they are frequently associated with thin-walled, lignified fibres and lignified parenchymatous cells; paracytic stomata are fairly numerous but rather faint and distinct.
(vi) The occasional fragments of collenchyma from the midrib composed of fairly large cells.
(vii) Long warty, cystolithic covering trichomes are present with cellular simple unglandulartrichomes.
(viii) Starch grains which are simple and frequently found massed together in groups, a small number of compound grains occur in two or three components; the underlying palisade cells are fairly large, thin walled and loosely packed.
(ix) The numerous vessels from the stem, which usually occur in small groups; they are fairly large, lignified and reticulately thickened or bordered pitted.
(x) The transverse section of the leaf showed the presence of the outermost covering tissues- the upper and the lower epidermises, which are multiseriate and lack chloroplasts. There was presence of closely packed palisade mesophyll cells with numerous chloroplasts (the main photosynthetic organ) and scattered spongy mesophyll cells that are loosely fitted to leave air spaces. The midrib bears the vascular bundle which comprises the phloem (exteriorly located) and the xylem (interiorly located)- the main conducting organs. Some mass of parenchymatous cells formed the pith at the centre (Jackson and Snowdon, 1974).

Quantitative leaf microscopy of F. cienkowskii

The result of quantitative leaf microscopy is presented on Table 2. The quantitative leaf microscopy is to determine palisade ratio, stomata number, stomata index, vein-islet number and vein let termination number were carried out on epidermal strips (Table 2).

Chemomicroscopic examination of the leaves of F. cienkowskii

The result of chemomicroscopic examination is presented on Table 3. The chemomicroscopic examination of the leaves revealed the presence of lignin, starch, mucilage, calcium oxalate crystals, cellulose, fatty oil and protein (Table 3).

Physicochemical analysis

The result of phytochemical analysis is presented on Table 4. The physicochemical analysis of *F. cienkowskii* powdered leaves reveals the parameters such as moisture content, total ash values, acid insoluble ash values, water soluble ash values, alcohol soluble extractive value and water soluble extractive values (Table 4).

Phytochemical analysis

Qualitative phytochemical analysis

The result of qualitative phytochemical analysis is presented on Table 5. The qualitative phytochemical analysis of *Fadogia cienkowskii* leaf extract reveals the presence of tannins, saponins, glycosides, reducing sugars, alkaloid, steroids, terpenoids, phenols, flavonoids and the absence of hydrogen cyanide (Table 5).

Quantitative phytochemical test

The result of quantitative phytochemical test is presented on Table 6. The quantitative phytochemical test in *F. cienkowskii* leaf extract revealed that flavonoids (17.7%) and tannins (17.6%) as the highest phytoconstituents; while steroids and saponins are the lowest phytochemical constituents (Table 6).

DISCUSSION

Macroscopic characteristics reveal that the leaves are green in colour, odourless with a bitter taste. The leaf is oblong-elliptic in shape and sub-acute at apex; rounded at the base with entire margin. The leaves are arranged in whorls of 3 at each node or rarely opposite. There surface is pubescent. They measure up to 8 cm in length and 2.5 cm in breath. This will aid in the physical or phenotypic identification of the plant.

Microscopic examination indicate the presence of calcium

Table 2. Quantitative leaf microscopy of *F. cienkowskii.*

Parameter	Range (mm^{-2})
Palisade ratio	8.50-9.50
Stomata number	17.50-21.45
Stomata index	7.60-10.66
Vein islet number	4.50-5.23
Vein let termination number	3.40-4.34

Table 3. Chemomicroscopic characteristics of *F. cienkowskii.*

Test reagent	Observation	Inference
Sample+Phloroglucinol + Conc. HCl	Red colour observed	Lignin present
Sample+Iodine	Blue colour observed	Starch present
Sample+ Ruthenium red	Red or dark pink colour observed	Mucilage present
Sample+Hydrochloric acid	Bright crystals dissolved	Calcium oxalates
Crystal present		
Sample+ Chlor-Zinc Iodine or **N/50 iodine + 66% H_2SO_4**	Blue colour observed	Cellulose present
Sample+ Sudan IV reagent	Pink colour observed	Fatty oils Present
Sample+ 1% Picric acid and Million's reagent	Red colour observed	Protein present

Table 4. Physicochemical analysis of *F. cienkowskii* leaves.

Parameter	% composition
Moisture content value	4.6
Total ash value	1.4
Acid insoluble ash value	0.8
Water soluble ash value	0.4
Alcohol soluble extractive value	9.0
Water soluble extractive value	7.8

vessels, when compared with Chukwube et al. (2018), which reported the presence of calcium oxalate crystals of various configurations, starches of various oxalate crystals, starch grains, lignified tissues, cystolith, xylem phloem, scalariform vessels, pith, trichomes, spongy and palisade mesophyll, oil cells, epidermal cells, collenchyma cells, paracytic stomata and reticulate shapes, trichomes, stomata of various types and their quantitative values and of course the vessels and fibers that confer rigidity to the plant tissues.

The quantitative leaf microscopy contains palisade ratio (8.50-9.50 mm^{-2}), stomata number (17.50-21.45 mm^{-2}), stomata index (7.60-10.66 mm^{-2}), vein - islet number (4.50-5.23 mm^{-2}) and vein let termination number (3.40-4.34 mm^{-2}) on epidermal strips. The chemomicroscopic examination of the leaves revealed the presence of lignin, starch, mucilage, calcium oxalate crystals, cellulose, fatty oil and protein. Abere et al. (2007) reported the presence of lignin, starch, mucilage, calcium oxalate crystals and cellulose on the chemomicroscopic examination of the leaves of *Mitracarpus scaber* Zucc (Rubiaceae).

Pharmacognostic and physicochemical studies of whole plant act as a reliable tool for plant identification and detecting adulteration (Desai and Chanda, 2014; Zhao et al., 2011; Raj and Radhamany, 2012). Studies of macroscopic and microscopic study can be valuable source of information which is usually and helpful in evaluation of purity and quality of a crude drugs. The pharmacognostic evaluation indicates that *F. cienkowskii* leaves contains the moisture content value (4.6%) as compared with Chukwube et al. (2018), which reported the moisture content value (2.33%). Therefore the moisture content of the plant is not too high (falls within the limit of the general requirement of 8-14%), indicating less probability of microbial degradation. Excess moisture in crude drug may lead to the breakdown of important constituent and the growth of microorganisms especially during storage of drug (Adesina et al., 2008).

Table 5. Qualitative Phytochemical Analysis of *F. cienkowskii* leaf extract.

Phytochemical	Crude extract
Saponins	+
Tannins	++++
Flavonoids	++++
Steroids	+
Terpenoids	+++
Alkaloids	++
Phenol	+++
Hydrogen cyanide	-
Reducing sugars	++
Glycosides	++

(+) = Present in small concentration, (++) = Present in moderately high concentration, (+++) = Present in high concentration, (++++) = Abundantly Present, (-) = Not Present.

Table 6. Quantitative phytochemical test of *F. cienkowskii* leaf extract.

Phytochemical	Crude extract
Saponins	1.0
Tannins	17.6
Flavonoids	17.7
Steroids	1.1
Terpenoids	6.6
Alkaloids	3.3
Phenol	8.8
Reducing sugars	4.6
Glycosides	2.5

Total ash value is (1.4%) as compared with Chukwube et al. (2018), which reveals the total ash value (3.85%), which can also be used to detect foreign organic matter and adulteration of sand or earth (Kunle et al., 2002). Acid insoluble ash value is (0.8%) as compared with Chukwube et al. (2018), which reported the acid insoluble value of (1.0%), and also compared to that of *Atropa belladonna* L. leaves which is not more than 4% (British Pharmacopoeia, 2011), water soluble ash value is (0.4%), as compared with Chukwube et al. (2018), reveals the water soluble ash value of (0.50%). The water soluble ash is used to estimate the amount of inorganic compound present in drugs (Tatiya et al., 2012).

The extractive values are useful to evaluate the chemical constituents present in the crude drug and also help in estimation of specific constituents soluble in a particular solvent (Ozarkar, 2005). Estimation of extractive values determines the amount of the active constituents in a given amount of plant material when extracted with a particular solvent. The extractions of any crude drug with a particular solvent yield a solution containing different phytoconstituents. The compositions of these phytoconstituents depend upon the nature of the drug and the solvent used. It also gives an indication whether the crude drug is exhausted or not (Tatiya et al. 2012).

Water soluble extractive value of *F. cienkowskii* leaves is 7.8%, as compared with Chukwube et al. (2018), which reported the water soluble extractive value of (3.40%), and also compared to that of *Azadirachta indica* A. Juss. leaves which is less than 20% (British Pharmacopoeia, 2011). The alcohol soluble extractive value of *F. cienkowskii* leaves is 9.0%, compared with Chukwube et al. (2018), which reported the alcohol soluble extractive value of 4.40%. This suggests that the use of alcohol as an extractive solvent is a better choice for the polar metabolites present in the plant. The qualitative phytochemical analysis of *F. cienkowskii* leaf extract reveals the presence of tannins, saponins, glycosides, reducing sugars, alkaloid, steroids, terpenoids, phenols, flavonoids and the absence of hydrogen cyanide, when compared with Chukwube et al. (2018), reported that the phytochemical analysis contains high amounts of alkaloids, tannins, saponins, flavonoids and moderate

amounts of carbohydrates, glycosides, saponins, resins and terpenoids with low concentrations of proteins and steroids. Alkaloids are known to be the largest group of secondary metabolites found in plants. They are claimed to have powerful effects on humans and animals and hence can be used as analgesics (Kam and Lie, 2002). Alkaloids are found to have antimicrobial activity by inhibiting DNA topoisomerase (Bonjean and De Paw-Gillet, 1998).

The quantitative phytochemical test in *F. cienkowskii* leaf extract revealed that flavonoids (17.7%) and tannins (17.6%) are the highest phytochemical constituents. Tannins reduce the risk of coronary heart disease (Ranjith, 2010). Saponins, present in plants have been suggested as possible ant carcinogens. Flavonoids and phenols are excellent sources of natural antioxidants (*Ali* et al., 2008). Steroids have been reported in clinical studies as anti-inflammatory and analgesic agents and also used in the treatment of congestive heart failure (Said*u* et al., 2012). Tannins are also suggested to have anticancer activities (Liq et al., 2006) and hence could be used for cancer prevention.

Conclusion

The current investigation reveals the pharmacognostic features, physicochemical and phytochemical properties of *F. cienkowskii*. These parameters could be useful in the preparation of the herbal section of proposed Nigerian Pharmacopoeia. Any crude drug which is claimed to be *F. cienkowskii* but whose characters significantly deviate from the accepted standard above would then be rejected as contaminated, adulterated or fake. The high content of poly-phenolic secondary metabolites (alkaloids and flavonoids) in *F. cienkowskii*, and its used in complementary medicine are indications that the plant is of great potential for wide range of applications in ethnomedicine.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Tea tree oil a new natural adjuvant for inhibiting glioblastoma growth

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Tea Tree oil (TTO), the essential oil from the Australian native Melaleuca alternifolia has demonstrated a variety of beneficial efficacies including antimicrobial, antifungal, antiviral and anti-inflammatory. This report discusses data obtained on the in vitro activity of TTO on human glioblastoma cells U87MG. Cell viability was examined by 2-(4,5-dimethyl-2-thiazolyl)-3,5-diphenyl-2H-tetrazolium bromide MTT assay. Growth was investigated by incubating cells with various concentrations of TTO (0.025 and 0.05 %) for 24, 48 or 72 h and daily cell count. Cell cycle and apoptosis assay were assessed by flow citometry. TTO decreased cell viability in a dose and time-dependent manner. . The cell cycle distribution showed that TTO enhanced the accumulation of the cells in G0/G1 phase. The analysis by Western blot of protein related to cell cycle (CDK2 and p27), cell apoptosis (caspase 6 and 8), necrosis (TNFR1 and RIP1) demonstrated that TTO induces U87MG growth inhibition by more synergic mechanisms: necrosis, low level apoptosis and cell cycle arrest. TTO induces also in vivo glioblastoma tumor growth inhibition in a murine subcutaneous model.

Key words: Brain cancer, tea tree oil, terpinol, glioblastoma, natural drug, adjuvant chemotherapy, temozolomide, apoptosis, cell cycle.

INTRODUCTION

Glioblastoma (GBM) is the most common primary malignant form of brain cancer, with a median survival of 7-15 months from the time of diagnosis. Hallmarks of the aggressive cancer include extensive infiltration and strong vascular proliferation into the surrounding brain parenchyma (Wei et al., 2014; Kim et al., 2015; Van-Tellingen et al., 2015). GBM is characterized by a pronounced mitotic activity, high neoangiogenesis, cellular heterogeneity, necrosis and high proliferative rates (Cicero et al., 2013; De-Almeida et al., 2012). Furthermore, the presence of cancer stem cells, was able to proliferate and generate glial neoplastic cell, contribute to the unfavourable prognosis of GBM patients (Surawicz et al., 1998). Conventional therapy for glioblastoma, tumor resection followed by radiotherapy and chemotherapy typically temozolomide (TMZ), is limited in

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efficacy due to high rates of recurrence, overall resistance to therapy, and devastating neurological deterioration (Kim et al., 2015; Lin et al., 2015). Even though numerous efforts have been made to identify the molecular pathways and potential “druggable” targets involved in gliomagenesis (Shergalis et al., 2018), gross-total resection of tumor tissue, followed by adjuvant chemo- and radiotherapy remain the standard of care (Stupp and Weber, 2005). Current therapeutic approaches to glioblastoma are often associated with the development of multidrug resistance, important side effects, and high cost, underscoring the unmet need for more efficacious and less toxic interventions. Numerous experimental data and other clinical-pathological studies have shown that overexpression of the xenobiotic transporter P-glycoprotein and MGMT not methylated lead to drug-resistant phenotype, often associated with more aggressive behavior glioma (Diao et al., 2019; Sarkaria et al., 2008). Resistance to cancer agents through drug metabolism, drug target mutation (Gottesman, 2002), compartmentalization (Kitazono et al., 1999) and through primary resistance; such as over-2 expression of MDR1 (the gene for P-glycoprotein) and especially apoptotic gene mutations of cancer cells, which render them resistant to apoptosis, means the search for novel antitumour agents that may overcome this is warranted. Tea tree oil (TTO), the essential oil from the Australian native *Melaleuca alternifolia* has demonstrated a variety of beneficial efficacies including antimicrobial (Carson et al., 2006; Akthar et al., 2014), antifungal (Hammer et al., 2003), antiviral (Schnitzler et al., 2001) and anti-inflammatory (Hart et al., 2000). TTO consists of over 100 components; the most represented are: terpinen-4-ol, γ-terpinene, α-terpinene and 1,8-cineole. It is the most abundant component, terpinen-4-ol that is the likely mediator of the *in vitro* and *in vivo* efficacy TTO (Mondello et al., 2006). Amongst the activities listed above, anti-cancerous efficacy has also been identified (Gautam et al., 2014). A single study demonstrated terpinen-4-ol and TTO *in vitro* anticancer activity. Human melanoma and Adriamycin resistant melanoma cells treated with TTO and terpinen-4-ol underwent caspase dependent apoptosis, a process thought to involve plasma membrane interaction via lipid reorganisation (Calcabrini et al., 2004;Tawona et al., 2014). Interestingly, both TTO and terpinen-4-ol were more effective against the resistant cell line suggesting that neither are substrates for P-glycoprotein (P-gp), a very useful property in the treatment of multidrug resistant (MDR) tumours. Only one study has demonstrated *in vitro* anticancer efficacy of 1,8-cineole against two human leukemia cells lines through apoptosis (Moteki et al., 2002). Accordingly, further study of the potential anticancer activity of TTO and its major components is warranted. Currently, no investigation of TTO or terpinen-4-ol has been conducted *in vivo* and before *in vitro* data can be translated clinically, *in vivo* studies must be completed. In this study we investigate the effect of TTO and components *in vitro* coupled with an investigation of the effects of TTO on tumour development in immuno-competent murine tumour models. Studies on human melanoma cells have shown that both oil and its main active component, terpinen-4-ol, are able to induce caspase-dependent apoptosis (Calcabrini et al., 2004). Other research has shown that tea tree oil has *in vitro* antitumor activity on breast cancer cells, causing a cell cycle arrest in the S phase (Assmann et al., 2018). The vegetal kingdom has always represented an attractive source for therapeutics and several examples do exist for natural products being included in current protocols to tackle the limits of chemotherapy. One of the main objectives of brain cancer research is therefore to identify new effective therapeutic strategies against drug-resistant tumors, in fact the increase in resistance and the side effects of current therapeutic approaches are leading scientists to explore alternative medicines to the traditional one as an option for finding new chemical entities for cancer treatment (Gautam et al., 2014). Among phytochemicals, essential oils have been considered attractive for their wide variety of bioactivities. Anticancer potential of essential oils has been explored and several studies are now available in the literature. Traditionally, essential oils have been used for their biological activities including antiseptic, analgesic, sedative, anti-inflammatory, spasmolytic, and locally anesthetic properties (Sharifi et al., 2017). Furthermore, they are used in aromatherapy for health improvement due to their sedative or stimulant properties (Hadfield et al., 2001). Essential oils (also called volatile or ethereal oils) are aromatic, highly volatile, hydrophobic liquids produced by aromatic plants as secondary metabolites. Essential oils (EO) have been the subject of research for alternative medicine research; EOs-mediated therapy cannot replace standard chemotherapy and radiotherapy but can be used in combination with cancer therapy to reduce drug side effects (Gautam et al., 2014). On the basis of their chemical structures the constituents of essential oils are classified as terpene hydrocarbons, divided into monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20); terpenes containing oxygen (terpenoids), such as alcohols, ketones, aldehydes, esters, lactones, coumarins and phenylpropanoids. Among the phytochemicals, essential oils were considered interesting for their wide variety of bioactivity (Bozin et al., 2006). The anti-tumor potential of oils has been explored and numerous studies are now available in the literature. Traditionally, EOs are used for their biological activities including antiseptic, analgesic, sedative, anti-inflammatory, spasmolytic and local anesthetic properties (Javad et al., ,2017). Furthermore, they are used in aromatherapy for health improvement and especially to reduce the agony of patients with brain cancer (Hadfield et al., 2001). It has been suggested that

Table 1. Cell cycle FACS analysis

Sample	% cells G0/G1	% cells S	% cells G2/M
CT 24h	66.53 ± 0.08	16.47 ± 0.06	16.97 ± 0.5
TTO 0.025% 24 h	65.97 ± 0.8	16.44 ± 0.09	17.26 ± 0,7
CT 48h	68.3 ± 1.28	16.66 ± 0.67	15.1 ± 0.64
TTO 0.025% 48 h (first day induction)	65.24 ± 0.7	17.15 ± 0.4	17.51 ± 0.33
TTO 0.025% 48 h (daily induction)	65.07 ± 1.6	16.63 ± 1.35	17.92 ± 0.35
CT 72h	68.57 ± 0.9	15.14 ± 0.07	16.29 ± 0.9
TTO 0.025% 72 h (first day induction)	62.39 ± 2.3	19.15 ± 1.7	18.46 ±0.6
TTO 0.025% 72 h (daily induction)	77.21 ± 0.15 *	9.27 ± 1.36 *	12.7 ± 0.84 *

topical application of aromatic oils may exert antibacterial, anti-inflammatory and analgesic effects. For cancer patients, the benefits obtained include reduced levels of anxiety and relief from emotional stress, pain, muscle tension and fatigue (Fellowes et al., 2004). There are a lot of evidence that essential oils and their constituents act according to multiple pathways and mechanisms that involve apoptosis, cell cycle arrest, antimetastatic and antiangiogenic effect (Gautam et al., 2014). Tea Tree Oil is an essential oil, derived from the *M. alternifolia* plant, used largely for its antimicrobial properties and incorporated as an active ingredient in many topical formulations used for the treatment of skin diseases (Carson et al., 2006). It is difficult to identify the single component responsible for each activity of Tea Tree Oil as the oil, as shown in Table 1, consists of a mix of substances. The antitumor activity is carried out above all by the terpene fraction which is the one present in greatest concentration. Studies on human melanoma cells have shown that both oil and its main active component, terpinen-4-ol, are able to induce caspase-dependent apoptosis (Calcabrini et al., 2004). Other research has shown that tea tree oil has *in vitro* antitumor activity on breast cancer cells, causing a cell cycle arrest in the S phase (Assmann et al., 2018). The ability to inhibit the growth of cancer cells has been shown in a dose-dependent manner in cancer of the colon, pancreas, prostate and stomach (Shapira et al., 2016). The main purpose of the present study was to investigate the effects of TTO on glioma cells both *in vitro* and *in vivo* and to explore the possible anticancer action and mechanisms that lead TTO-induced apoptosis , cell cycle arrest and necrosis.

MATERIALS AND METHODS

Cell culture

For in vitro and in vivo experiments we used a human glioblastoma continuous cell line U87MG, from Sigma Aldrich Collection (LGC Promochem, Teddington, UK). Glioblastoma cells were grown in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10 % fetal bovine serum, 2 mmol/L-glutamine, 50 U/mLl Penicillin and 50 µg/mLl Streptomycin, at Streptomycin, at 37°C, 5 % CO2, and 95 % of humidity. For treatment, we used Tea Tree Oil 38 % (ESI and Temozolomide (Sigma Aldrich).

Growth inhibition of U87MG by TTO treatment

To evaluate, in vitro, the response to treatment with TTO, human glioblastoma cells (U87MG) were plated in 48-well plates, 1×104 cells per well, in Dulbecco 's Modified Eagle' s Medium (DMEM, Invitrogen) supplemented with 10 % fetal calf serum (Invitrogen) and incubated at 37 °C in an atmosphere containing 5 % CO2. The day after the cells were treated with TTO 0.025 and 0.05 % TTO. The substance used is pure TTO 38 %, produced by ESI srl Registered office Corso Ferrari 74/6 17011 Albisola Superiore (SV) Italy Tax Code: 01099380105 VAT number: 11264680155 REA number: SV-102611.

For the experiment, the TTO was reconstituted in 0.5% dimethylsulfoxide (DMSO) and Tween 80 (0.002%) (Bag and Chattopadhyay, 2015). At 24, 48 and 72 hrs after treatment we made a cell count by Burker chamber, in the presence of trypan blue dye (Sigma) to discriminate live cells from dead ones. The same experiment was repeated in presence of canonical chemotherapic TMZ. Cells were seeded in 48-well plates (1×104 cells/well) and were treated for 24, 48, and 72 hrs with 0.025 and 0.05 % TTO alone or in combination with 10 µM TMZ. At the end of the treatment, cell count was performed.

Cell viability assays

U87MG cells were seeded in 96-well plates (5x103 cells/well) in DMEM 10 % FBS and allowed to adhere for 12 hrs. Cells were exposed to different concentrations of TTO (0.025 and 0.05 %) for 24, 48 and 72 hou. After treatment, 5 mg/mLl MTT (Methylthiazolyldiphenyl-tetrazolium bromide; Sigma–Aldrich) in 100 µl of DMEM without phenol red was added to the cells for 2 hrs. We washed cells with PBS (phosphate-buffered saline) and added 100 µl of DMSO. MTT metabolized concentrations was determined by spectrophotometer measurement of absorbance at 570 nm.

Analysis of cell cycle, apoptosis and necroptosis proteins after treatment with TTO

We analyzed cell cycle, apoptosis and necroptosis proteins in U87Mg control and U87Mg after short and long treatment with TTO 0.025 %. In short-term treatments we plated U87Mg (800,000 per plate) in DMEM without serum for 48 hrs. After re-addition of FBS we carried out inductions with TTO 0.025 % for 2, 4, 8, 12 and 24 h. For long-term treatments, we plated U87Mg in DMEM 10 % FBS and we induced with TTO 0.025 % only on the first day or daily for

24 rs, 48 rs and 72 hrs. Proteins were extracted from U87MG in Triton X-100 lysis buffer (Tris-HCL 10 mM, EDTA 1 mM, NaCl 150 mM, Triton X-100 1 %, NaF 1 mM, 1 mM Na4P2O7, 1 mM Na3VO4, protease inhibitors 1X). Proteins (40 µg) were separated by SDS-PAGE on a polyacrylamide gel and transferred to nitrocellulose membranes (Amersham) by electroblotting. The membranes were incubated for 2 h at room temperature in 5 % milk or BSA diluted in T-TBS 1X, and then incubated over night at 4°C with primary antibodies specific and 1 h with secondary antibody. Each membrane was incubated with mouse monoclonal anti-βactin (1:50000; Santa Cruz Biotechnology). Protein were detected by chemiluminescence using ECL Western blotting (Amersham). The signals were detected by a digital scanner and quantified by densitometric analysis (Scion Image software). For cell cycle proteins analysis we used anti- CDK2, anti-P21 and anti-P27 primary antibodies (1:1000), all from Cell Cycle Regulation Sampler Kit (Cell Signaling Technology); we also analysed P53 protein with mouse monoclonal antibody anti-P53 (Roche Diagnostic). For the analysis of the proteins involved in apoptosis we used anti-Caspase 6, anti-Caspase 8 and anti-Parp primary antibodies (1:1000) from the Procaspase Antibody Sampler Kit (Cell Signaling technology), while for necroptotic proteins we used anti-TNFR1, anti-RIP and anti-TRADD primary antibodies (1:1000), all from Death Receptor antibody sampler Kit (Cell Signaling technology); as secondary antibodies we used an anti-rabbit or anti-mouse (1: 2000; Cell Signaling technology).

FACS analysis by flow cytometry

The U87MG human GBM cells were plated (8 × 105) in DMEM with 10 % FBS and treated with TTO (0.025 %) for 24, 48 and 72 hrs. After treatment, cells were trypsinized, washed in sample buffer (glucose 0.1 % in HBSS), fixed in 70 % ethanol, and stored at 4 °C over night until the day of analysis. Before analysis, Propidium iodide (50 µg/mLl) was added for 30 min at room temperature. Flow Cytometry (FC) analysis of the cell cycle was performed with Gallios instrument (Beckman Coulter).

Apoptosis assay

U87MG were treated with TTO 0.025 % for 24, 48 and 72 h. Cells were collected for apoptosis assay with Annexin V-FITC apoptosis detection kit (Cell Signaling). After centrifugation at 1200 rpm for 5 min, the pellet (5 x 105 cells) was resuspended in 1ml of 1 x binding buffer with 5 µl of Annexin V-FITC conjugated and 10 µl of Propidium iodide solution. The reaction complex was incubated for 10 minutes on ice in the dark. Samples were subjected to apoptosis analysis assay by flow cytometry. The expression of Annexin V was tested also by immunofluorescence on U87MG treated with TTO 0.025 % for 24 hrs.

U87MG cell implantation

Male nude mice CD1 (Charles River, Calco, CO, Italy; 20-22 g body weight) were housed in a controlled conditions (temperature 22 °C; humidity, 40 %) on a 12 hrs light/dark cycle and observed daily. The experiments were carried out according to the regulations for the care and use of animals promulgated by National Institutes of Health. After anesthesia with ketamine 100 mg/Kg and xyilazine 10 mg/Kg i.p., mice were subcutaneously implanted with 5 × 106 U87MG cells/0.2 mLl (matrigel and DMEM). At 7 days after inoculation treatments are carried out with TTO 3.5 % intratumoral, twice a week, for 3 weeks.The dose administered in vivo was calculated according to evidence in the literature (Shapira et al., 2016).Tumor size was measured by caliper. Subsequently, the mice were sacrificed by cervical dislocation after anesthesia, and tumors removed, were fixed in 4 % formalin, dehydrated in ethanol at increasing concentrations and embedded in paraffin. From each tumors were cut sections of 4 µm thickness. The sections were stained with Mayer's hemalum (Diapath) and Eosyn (Diapath) and subjected to microscopic evaluation.

Immunofluorescence on tumor tissue

Tissue sections of 4 µm thickness were deparaffinized in xylene, rehydrated in alcohol with decreasing concentration and permeabilized in Triton 0.1% for 30 min. We performed an incubation with 10 % goat serum for 1 h and then with primary antibody anti-TNFR1 (1: 100, Cell Signaling) over night at 4°C. Secondary antibody anti rabbit -Cy3 was added (1: 200 Vector) for 1 h and DAPI mounting medium (Vector). The slides have been analysed by a fluorescence microscope at 40x magnification.

Apoptosis detection of tumor tissue by TUNEL staining

Tissue sections of 4 µm thickness were deparaffinized in xylene, rehydrated in alcohol with decreasing concentration and fixed in 4% methanol. TUNEL tests were performed by a commercial kit, Deadend fluorometric Tunel System (Promega) following the manufacturer's instructions. The slides were counterstained with DAPI and analysed by a fluorescence microscope at 40x magnification.

Statistical analysis

Statistical analysis was performed using statistical software (GraphPad Prism Software, version 5.0). Values are presented as the Mean±S.E.M. Student's t-test was used to compare two groups. Analysis of variance (one-way ANOVA) was used to compare three and more conditions and Dunnett post-test was used to compare mean values for all groups. Differences between groups were considered statistically significant when the null hypothesis pH0 was than P< 0.05.

RESULTS

Effect of TTO on the of human GBM cells growth

The effect of TTO on GBM cell (U87MG) proliferation was analyzed long term, with cell count performed at 24, 48, and 72 hrs after TTO treatment. As shown in Figure 1 Hyperlink reference not valid.A, the continuous cell line U87MG had a significant reduction in proliferation with 0.025 and 0.05 % TTO; a more significant reduction appears at 72 hrs of treatment. The TTO concentration that caused the greatest growth inhibition in cell line U87MG was 0.05 %. The growth inhibition is dose and time dependent.

TTO inhibits the viability of U87MG glioblastoma cells

To determine the effect of TTO on the cell viability of U87MG glioblastoma cells, U87MG were treated at various concentrations of TTO 0.025, 0.05% for different

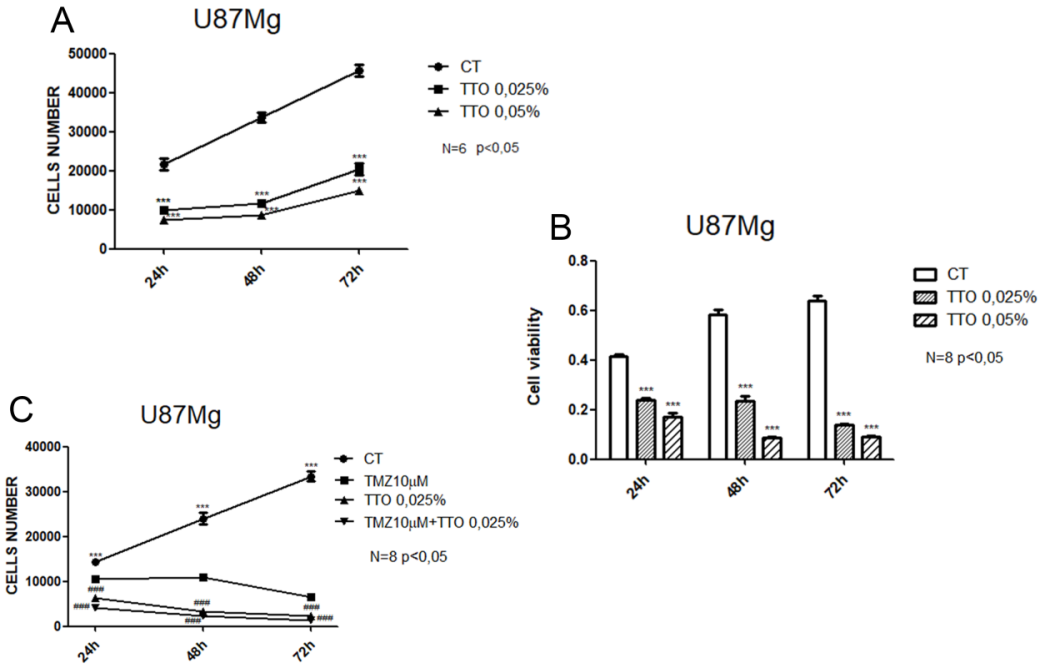

Figure 1. TTO induces U87MG growth inhibition. (A). Dose-response and time course Cell cultures were exposed to tea tree oil (0.025 and 0.05 %); the drug was applied daily and cell number per was counted at 24, 48, and 72 hrs. Counts for each time interval represents the mean ± S.E.M. of six wells. One-way ANOVA test ***p<0.05 compared to control cells. (B). Tea tree oil reduces cell viability. Tea tree oil leads a time and dose -dependent growth inhibition as measured by MTT assay. (C). Synergistic effect of Tea tree oil and classic chemotherapy (TMZ) in U87MG.
Cell count at 24, 48, and 72 hrs after treatment with tea tree oil (0.025 and 0.05 %) and TMZ (10 µM), alone or in combination. Values are the means ± SEM of eight individual determinations. One-way ANOVA test ***p<0.05 compared to control cells.

time (24, 48 and 72 hrs). Cells with 0.1 % DMSO were used as a control. The MTT assay showed that TTO inhibited the viability of U87MG cells in a time and dose-dependent manner (Figure 1B). TTO concentrations 0.05 % markedly suppressed the cell viability of U87MG cells (PP < 0.005).

TTO treatment amplified the effects of TMZ on the proliferation of human glioma cells in vitro

The continuous cell line U87MG were treated with TMZ (10 µM) alone or in combination with TTO 0.025%, and after 24, 48, and 72 h, the activity of the drugs on cell count was evaluated. The TMZ treatment appeared to be effective, but a statistically significant reduction in growth was observed in cells treated with a combination of the 2 drugs (10 µM TMZ plus 0.025% TTO) already after 24 h of treatment (Figure 1C).

Western blot analysis

To assess the molecular mechanism of TT0 -induced cell-cycle arrest, we investigated the expression levels of cell cycle related proteins in U87MG control and TTO -treated. TTO treatment (0.025%) causes a reduction of cyclin D2 in U87MG cells at 2, 4, 8 and 12 h (Supplementary Figure 1) of treatment and at long term exposure (24 and 48 h) (Figure 2A) while p21 levels was induced significatively only at 48 h by TTO treatment (0.025%) (Figure 2B). Cyclin-dependent kinase inhibitor p27 was also investigated, western blot analysis shows an increase of p27 at 48 h from TTO induction (Figure 2C). Finally, we analyzed the expression of the p53 oncogene that undergoes a significant increase at 48 h of treatment with TTO, p53 would participate in TTO-induced glioblastoma growth inhibition (Figure 2D).

TTO leads apoptotic effect in U87MG

To investigate the pro-apoptotic effect of TTO in U87MG glioblastoma cells PARP expression was analyzed by western blot on U87MG TTO treated with 0.025% for 24, 48 and 72 h. The expression of PARP (Figure 4C) reveals in the cells treated with TTO an accumulation of PARP (116Kd) and cannot be seen of cleavage form.

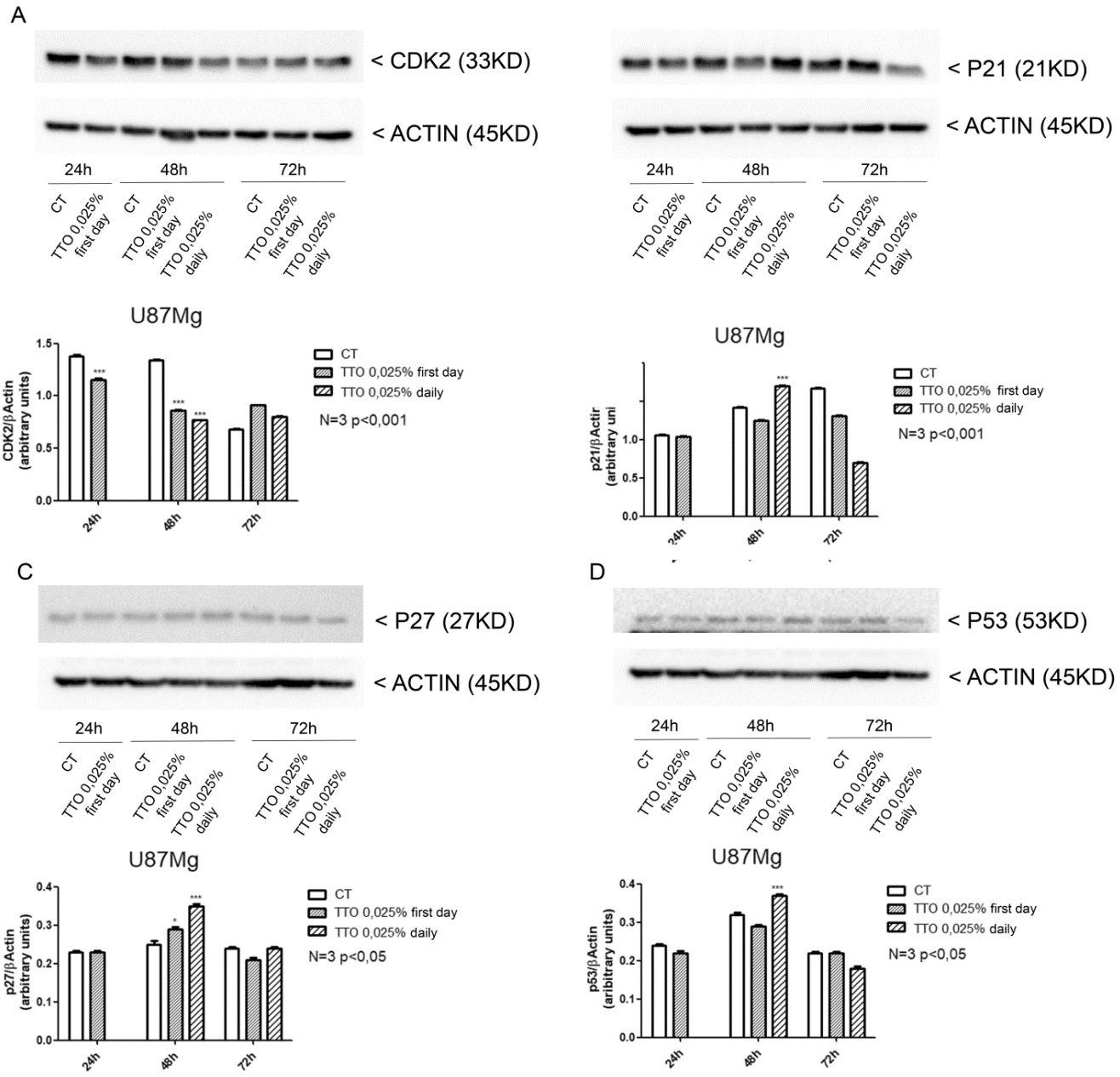

Figure 2. Down regulation of cyclin D2 and induction of p21, p27 and p53 in U87MG cell line TTO treated.
Western blotting and densitometric analysis of A) cyclin D2, B) p21, C) p27, D) p53 in continuous GBM cell line U87MG after treatment with TTO (0.025%) applied to the cultures only on first day or daily at 24, 48, and 72h. Thirty micrograms of protein were loaded for each lane. The blot was repeated 3 times with similar results. Values are the means ± SEM of 3 individual determinationns. One-way ANOVA and Dunnett test; ***p < 0.05.

Since the cleavage of PARP protein occurs only in case of apoptosis, increased expression of normal form in cells treated with TTO suggest the switch to other mechanism than control growth inhibition and Necrosis. The expression of caspase 6 and caspase 8 was also analyzed; Figure 4A and B shows that there is an increase of cleavage caspase 6 and caspase 8 after 72 h TTO treatment, witnessing the presence of a real apoptotic process also.

TTO induces necrosis in U87MG

We study the protein that normally induces inflammation TNFR1 by western blot of cell lysate of U87MG treated with 0.025% TTO for 24, 48 and 72 h. The results

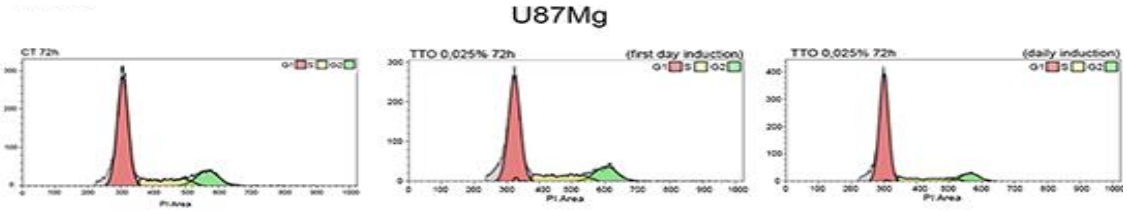

Figure 3. Tea tree oil induces cell cycle block.
Representative flow cytometric analysis of propidium iodide staining in U87Mg vehicle and TTO (0.025%) treated cells only on first day or at 24,48,72 h. TTO reduces cell growth by increase the cell percentage in G0/G1 phase with maximum effect after 72 h of treatment.

showed an increase of TNFR1 at 24 h while RIP at long term exposure of TTO (48 h) (Figures 4D and E) and increase of TNFR1 was found just at short treatment times (2, 4 and 8 h) whereas TRADD increase only at 4 rshrs of treatment (Supplementary Figure 1). The increase in these proteins suggests the coexistence of a phenomenon of necrosis.

TTO induces G0/G1 cell cycle arrest in U87MG cells

To explore how TTO inhibits the proliferation of U87MG cells, FACS was performed to determine cell cycle distribution. After 24 h plating U87MG were treated with 0.025% of TTO for 24, 48, and 72 h. After 72 h of treatment is visible the maxim effect on cell cycle arrest, the percentage of U87MG cells staying at G0/G1 phase were 68.57± 0.9 in the control while in treated cell is 77± 0.15 and 37.9 ± 1.1 (Figure 3). The increase of G0/G1 phase cell population was accompanied by a concomitant decrease in % cells in S and G2 phase (Figure 3). Our results indicated that TTO triggers cell cycle arrest in the G0 / G1 phase in a time and dose dependent manner showing a significant effect after 72 h. To demonstrate the presence of apoptosis in TTO-treated cells a FACS experiment with Annexin V was performed. The results of the analysis show that with increasing treatment time: (24, 48 and 72 h) the peak relative to cells is increased in late apoptosis phase. (Table 2). Annexin V immunofluorescence reveals an increase of phosphatidylserine externalisation in TTO treated cells (supplementary Figure 2).

In vivo experiment

We implanted U87MG cells subcutaneously onto right side of nude mice (N8), after one week from implantation, mice were divided randomly in two groups: control and TTO treated one. Mice were treated with TTO 3.5% by intratumoral injectionintratumoral injection twice for week for 3 weeks. At the end of the treatments animals were sacrificed and tumors taken from subcutaneous were weighed and undergoing to histological analysis. Tumor size was evaluated weekly by caliper. The evaluation of tumor size, by caliper measurements analysis confirm that, in mice treated with TTO, tumor size significantlysize significantly reduced about 80% (Figure 5C). In Figure 5D there is a representative graph of tumor growth that is visibly inhibited massively by treatment with TTO.

Cytologically, tumors were composed of large pleomorphic cells with abundant eosinophilic cytoplasm (Figure 5E). Hematoxylin -Eosin staining shows that tumors are composed by dysmorphic cells, typical of glioblastoma, with high cell density; the tumors treated with TTO show the same cell morphology but a marked reduction in cell density with large areas of necrosis. Finally, to show that even in the tissues there is an evidence of pro-apoptotic effect of TTO we exposed the same slide to TUNEL assay.

TUNEL assay and immunofluorescence of pro-inflammatory receptor TNRF1

To consider the possibility that TTO induced apoptosis in vivo, paraffin-embedded sections of tumors were assessment of apoptosis by TUNEL staining. In the control group, the structure of cells was intact and the nucleus was completely stained blue. However, in the TTO -treated group, the percentage of TUNEL-positive cells (green) markedly increased (Figure 5F), confirming the increase in apoptotic tumor cells of DNA fragmentation after TTO treatment. On the same sections we analyzed by Immunofluorescence the pro-inflammatory receptor TNFR1 as shown in (Figure 5G) TNFR1 expression increase visible in tumor slide from TTO treated.

DISCUSSION

Despite standard treatments for glioblastoma (GMB), relapse and drug resistance remain the main causes of treatment failure in many patients.

Glioblastoma multiforme (GBM), which is a Grade IV brain tumor according to the World Health Organization (WHO) classification, is the most common form of

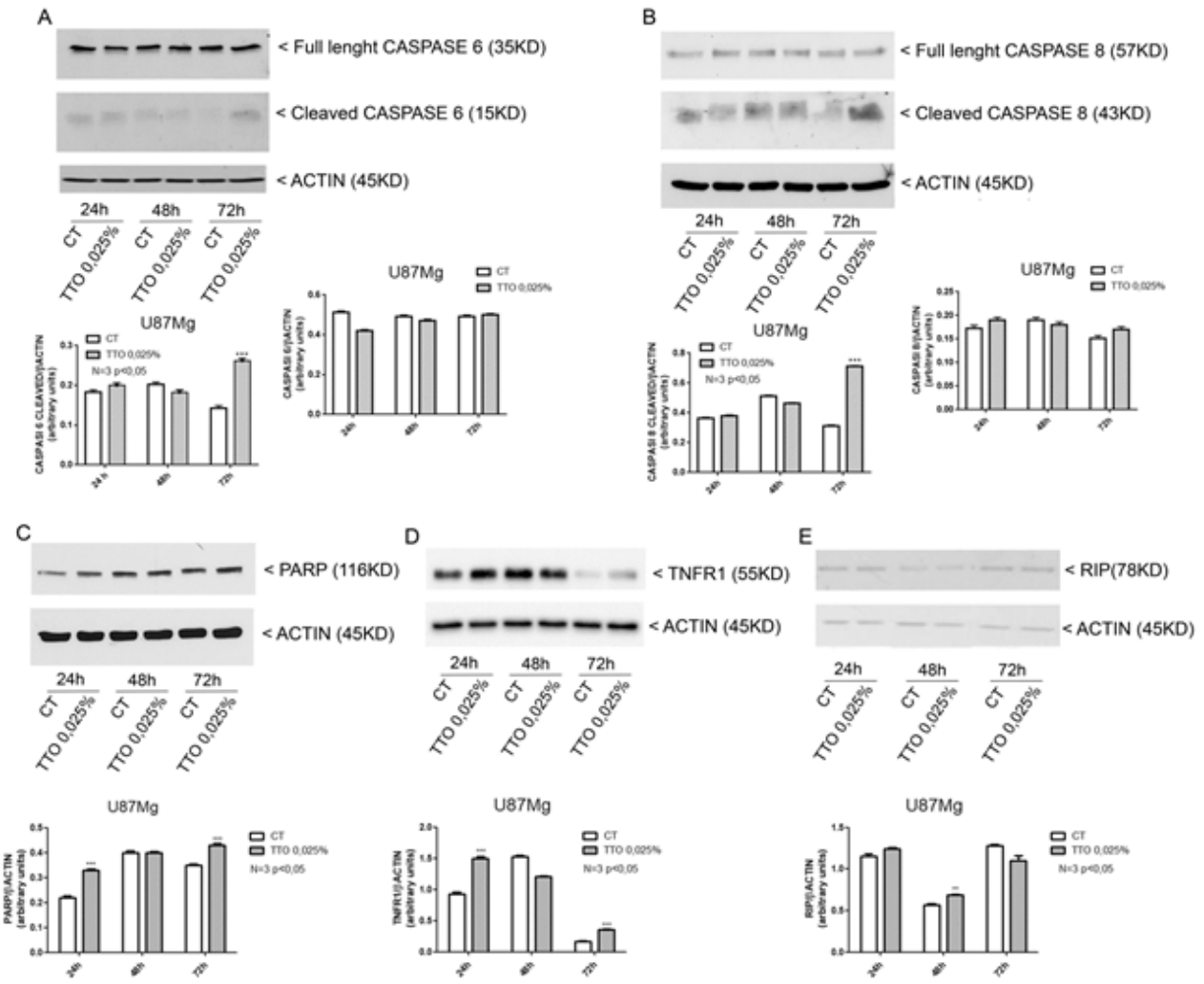

Figure 4. TTO regulation of apoptosis and necrosis.
Effects of TTO on the apoptosis pathways. U87MG glioblastoma cells were treated with TTO (0.025%) for 24, 48 and 72 h. The total cell lysates were then subjected to Western blotting to analyze PARP(C) and caspase 6(A) and 8(B). In figure is shown the activation of both caspase (6, 8) by cleavage only at 72 h. The same cell lysates were then subjected to Western blotting to analyze TNFR1 and RIP necrosis protein. TTO increases TNRF1 after 24h and RIP too at 48 h. Values are means +/-SEM of 3 individual determinations. One-way ANOVA and Dunnett test; ***p < 0.05.

primary brain tumor in the central nervous system (CNS), and its aggressive nature and evasiveness to treatments make it one of the most lethal cancers. Less than 3% of glioblastoma patients are still alive at 5 years after diagnosis. (Ohgaki et al., 2005).

Current treatments for GBM range from common chemotherapeutic agents such as temozolomide (TMZ) in conjunction with radiotherapy (Stupp protocol 23) (Stupp and Weber, 2005). Over the past 10 years, however, therapeutic agents have not significantly increased the median survival of patients with glioblastoma. The 5- year survival rate for patients with the same disease, after treatment including surgical resection, radiotherapy and chemotherapy is less than 9.8 %. In order to improve the results of brain tumor therapy, in our laboratory we investigated the use of natural and less toxic substances that can be used as adjuvant drugs in the treatment of glioblastoma. Complementary medicines with natural compounds (Vengoji et al., 2018; Arcella et al., 2015 Kamarudin et al, 2019; Correa et al, 2019) have become increasingly popular in recent decades. In this study we investigated about the effect Tea Tree oil, an essential oil that comes from an Australian plant, M. alternifolia, made up of different substances including terpinen4-ol, considered the active component, which, as shown by other studies has anticancer effect, is able to induce a significant inhibition of pancreatic, prostatic, gastric and colorectal cancer tumor cells (Nakayama et al., 2017; He et al, 2000). In this study, for the first time, the effect of TTO on human GMB cell line was studied, both in vitro and in vivo. An in vitro proliferation test was performed on U87MGg cells to evaluate their proliferation in the

Table 2. Apoptotic rate of U87MG cells control (C) and treated with TTO for 24, 48 and 72 h detected through annexin V-FITC/PI staining and analyzed by FACS.

Sample	Annexin V positive cells (%)	Annexin V+ PI positive cells (%)	PI positive cells (%)
CT 24 h	3.06 ± 0.12	4.6 ± 0.06	0.16 ± 0.006
TTO 0.025% 24 h (daily induction)	3.67 ± 0.36	6.52 ± 0.37 ***	0.6 ± 0.057
CT 48h	2.92 ± 0.59	4.86 ± 0.7	2.3 ± 0.67
TTO 0.025% 48 h (daily induction)	3.6 ± 0.11	8.26 ± 0.84 *	0.9 ± 0.18
CT 72h	4 ± 0.24	8.14 ± 0.54	0.69 ± 0.1
TTO 0.025% 72 h (daily induction)	4.5 ± 0.48	10.76 ± 1.38	0.84 ± 0.2

presence of different concentrations of TTO (0.025 and 0.05 %). Both doses tested caused a growth inhibition of about 50 %, a block confirmed also by the MTT toxicity test. The inhibition of TTO on glioblastoma cell growth was tested also in combination of TTO with canonical chemoterapic for brain tumors temozolomide, the results of cell count and MTT assay shown an interestingly synergic effect. At this point we tried to investigate the expression of proteins involved in apoptosis, necrosis and control of the cell cycle to establish with which mechanism the growth block occurs. Western blot analysis showed that, in cells treated with TTO for 2, 4, 8, 12 and 24 hrs CDK2 protein decrease significatively while the tumoroncosuppressor p27 increase only at 8 h from TTO treatment. We found also an increase of caspase. 8 and caspase 6 cleavage, and p53 increases. These results suggest that the cause of the growth inhibition of U87MG glioblastoma cells by TTO is a results of more synergic mechanism: may be an increase of apoptosis and a block of the cell cycle in G0/G1 phase. This hypothesis was confirmed by FACS analysis: longterm treatment 72 h with TTO in U87MG caused an increase of cells in the G0/G1 phase.

Apoptosis and cell blocking therefore would not represent the unique and preponderant cause of TTO cell growth inhibition, as it is not massive apoptosis so it is conceivable that other mechanisms can act synergistic cooperating in blocking the cell growth. It could occur a phenomenon of necroptosis, for which we analyzed the expression of TNFR1 and TRADD which represents the most widely studied model of programmed necrosis (Justyna et al., 2014; Bing et al. 2018). The results showed an increase of TNFR1 and RIP at long term exposition of TTO (24, 48 and 72 hrs) and an increase of TNFR1 and TRADD at short treatment times (2, 4, 8 and and 12 hrs) which reveals that treatment with Tea Tree Oil could also lead to necroptosis. Necroptosis is a pro-inflammatory cell death in which swelling of the organelles, rupture of the cell membrane and uncontrolled release of intracellular contents into the surrounding tissue occurs. Necroptosis is a regulated necrosis mediated by death receptors (Berghe et al., 2014). It is well known that involvement of receptor like Fas, TNF, and TRAIL can lead to cell death through the recruitment of caspase-8 leading to initiation of extrinsic apoptotic pathway (Taylor et al., 2008). Considering the evidence of our results (increase of cleavage caspase 8, TNFR1, RIP, after 3 days TTO treatment) we have assumed necroptosis as the probably molecular mechanisms of necroptosis to death U87 Mg by TTO treatments. In contrast to apoptosis, necroptosis is caspase independent. These data together suggest that the growth block of U87MG, after TTO treatment, is determined by several control mechanisms: apoptosis, necrosis and cell blockade in G0/G1 phase. The cell line, U87MG, which has an amazing growth rate, replicates every 24 h, logarithmic growth, would explain the typical virulence of the brain tumor. The fact that a natural substance like TTO can block the growth of glioblastoma has comforted us to start an in vivo study in a more physiological context. In the in vivo studies, mice were subcutaneously injected with human glioblastoma U87MG, after 1 week were divided in two groups: control and treated one with TTO 3.5% (intratumoral administration two days a week for three weeks). Mice treated with TTO showed a 80% reduction in the tumor mass compared with tumor in control mice, as evidenced by volume. The histological analysis of the slide of removed tumors reveals typical hyperchromic pleomorphic elements; in the section from mice treated with TTO it can be evident in a big area of necrosis confirming that necrosis is one of the mechanisms underlying the growth block of glioblastoma by TTO. The presence of necrosis mechanism was confirmed by TUNEL assay too; in the section by tumor TTO treated for 3 weeks, as shown in the images, it is visible increase of DNA fragmentation.

Conclusion

A growing body of scientific reports has recently focused on the potential of essential oils as anticancer treatment in the attempt to overcome the development of multidrug resistance and important side effects associated with the antitumor drugs currently used (Russo et al, 2015). Our

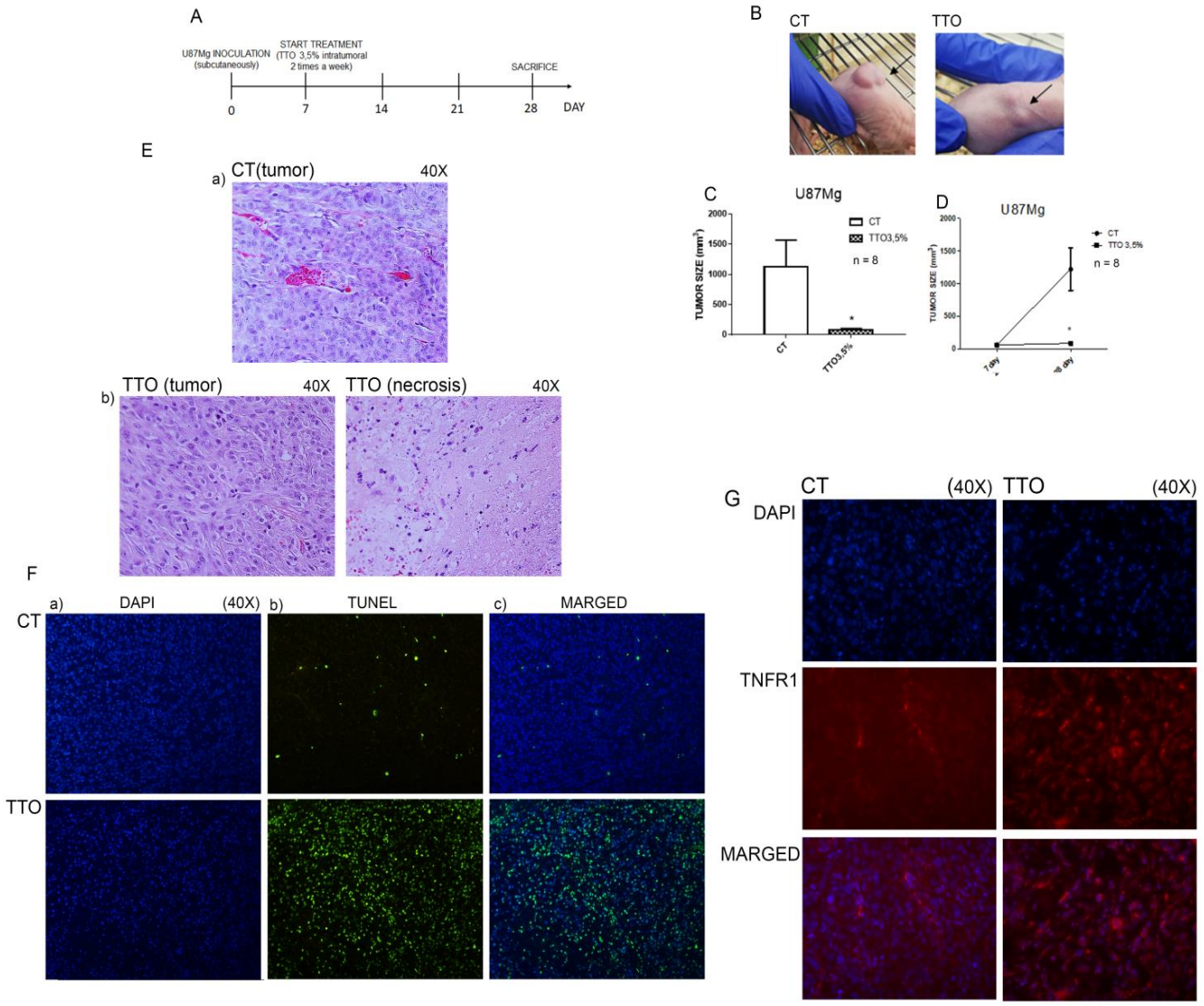

Figure 5. Long-term TTO treatment suppresses glioblastoma growth *in vivo*.
UM87MG cells were subcutaneously injected into right of nude mice in order to produce an orthotopic xenograft model of glioblastoma.
(A). Time course of U87MG subcutaneously growth in nude mice CT and TTO treated (3.5%) twice week for 3 week by intratumoral injection.
(B). Representative image of subcutaneously tumor in control mice and TTO treated at the end of treatment (after 21 days).
(C). Final tumor size from mice CT and TTO treated; it is evident almost total inhibition of tumor growth (80%) in mice TTO treated. Values are means ± SEM of eight individual determinations. T-test *p < 0.05, **p <0 .001, ***p < 0.0001.
(D). Tumor growth in mice CT and TTO treated.
(E). Hematoxylin- Eosin staining of subcutaneously tumor. In slide from control mice treated with vehicle (a) clear eosinophilic high density tumoral cell were apparent; (b) TTO treated slide shows a reduction of density cell and a clear necrosis area.
(F). TUNEL assay on sections of subcutaneously tumors Control (CT) and TTO treated a) DAPI stained, b) TUNEL staining of apoptotic cells c) Merged; in TTO mice treated there is an high staining showing the increase of DNA fragmentation.
(G). Immunohistochemical distribution of TNF receptor-1 (TNFR1) proteins on transverse sections of subcutaneously tumors Control (CT left) and TTO treated (right). Picture shows in slide from TTO mice treated (right) TNFR1 expression increase.

results indicated that TTO, the essential oil isolated from Melaleuca Alternifolia, is a natural compound that possesses the activity of inhibiting glioblastoma cell growth in vitro and in vivo at a dose- and time-dependent manner, and the mechanisms were associated with cell cycle arrest, triggering DNA damage and inducing apoptosis and necrosis. It is tempting to speculate that TTO might be utilized as a potential therapeutic agent against brain tumor. This is only a preliminary study to prove the antitumor effect of TTO on human glioblastoma

cells.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Dedicated to my father. We thank Rosanna de Rosa (English teacher) for the assistance rendered to edit the manuscript. The authors are also grateful to Dr. Vittorio Maglione (IRCCS Neuromed) for processing the Annexin V samples at the FACS.

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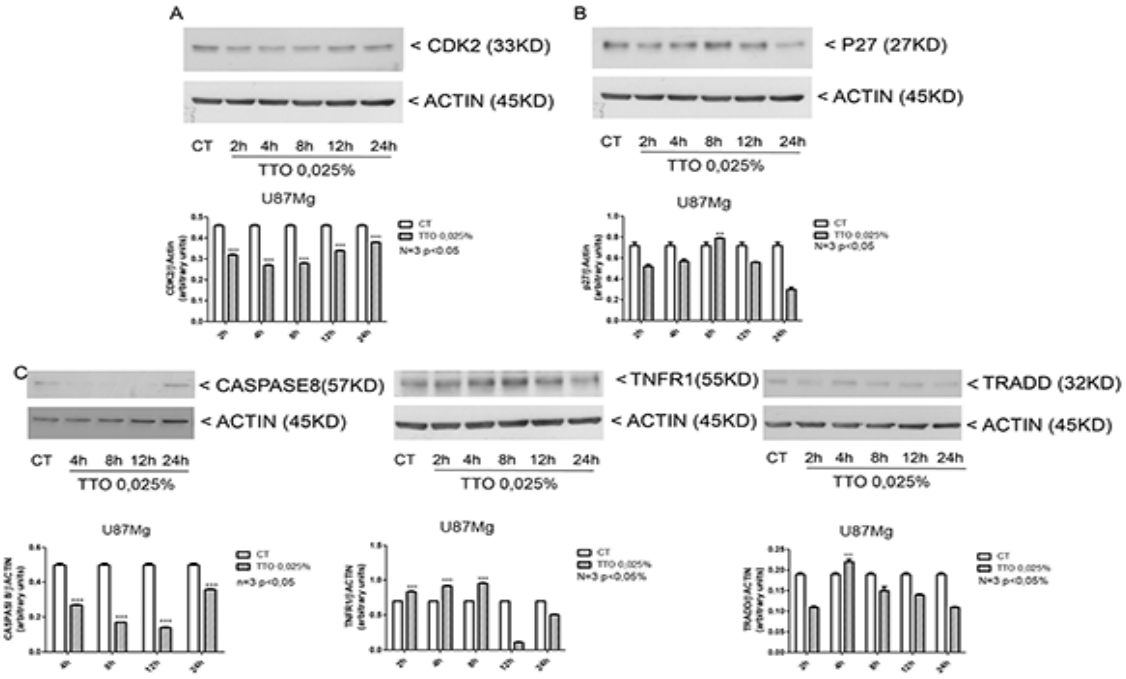
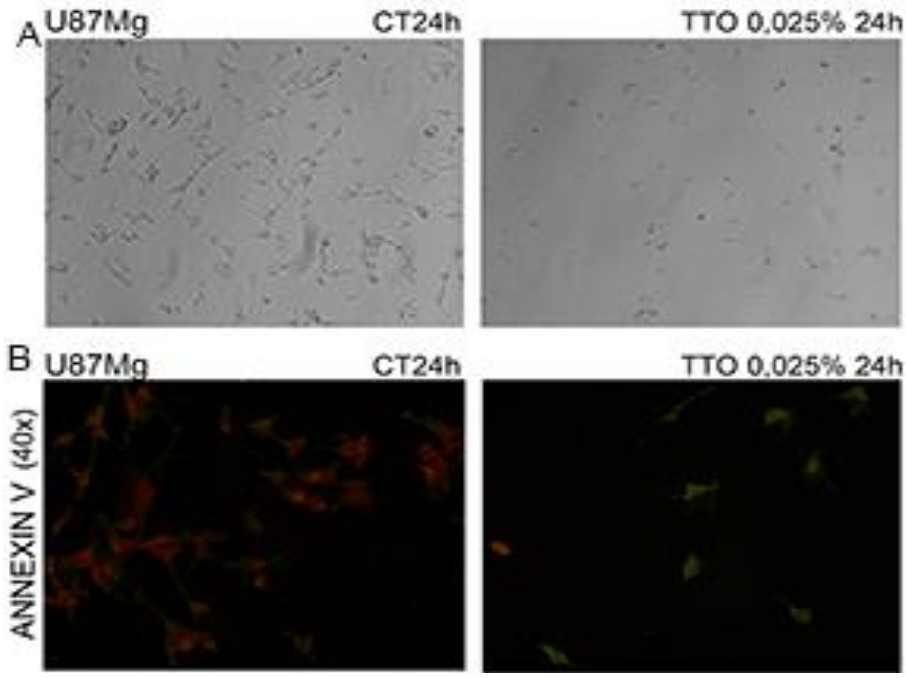


Supplementary Figure 1. Short-term TTO treatment in U87MG western blot analysis of cell cycle , apoptosis and necrosis regulation protein.
Western blotting and densitometric analysis of A) cyclin D2, B) p27, C) caspase 8 , D) TNFR1, E) TRADD Thirty micrograms of protein were loaded for each lane. The blot was repeated 3 times with similar results. Values are the means ± SEM of 3 individual determinations. One-way ANOVA and Dunnett test; ***p < 0.05.



Supplementary Figure 2. Representative picture display the results of the cells at 24 h, after TTO treatment.
(A) Cell morphology 20 x magnification, (B) Fluorescence with annexin V- FITC coniugated. The percentage of apoptotic cells (: Annexin V-FITC + / PI +) with phosphatidylserine externalisation increases significantly.

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Full Length Research Paper

A comparative study of phytochemical profile and antioxidant activity of Sahelian plants used in the treatment of infectious diseases in northern part of Burkina Faso: *Acacia seyal* Delile and *Acacia tortilis* (Forssk.) Hayne subsp. *raddiana* (Savi)

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***Acacia seyal* and *Acacia tortilis* are used in the treatment of infectious diseases in traditional medicine by population in Northern Burkina Faso. Phytochemical screening by tube test and on HPTLC plates showed the presence of important chemical compounds in these plants. Determination of total phenolic content using method of Folin-Ciocalteu Reagent (FCR) and antioxidant activity by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) test, showed that this biological activity is related to phenolic content. The trunk bark of *A. tortilis* had an important antioxidant activity with IC_{50} value of 0.01±0.01 µg/mL due to its highest content ($p<0.05$) of total phenolic compounds (383.19±0.07 mg GAE/g), of condensed tannins (18.21±0.04%) and flavonoids (66.09±0.06 mg QE/g). This antiradical activity was comparable to that of Trolox used as reference. Radical scavenging activity of leaves of *A. seyal* was also significant with IC_{50} value of 0.02±0.01 µg/mL. Its total phenolic compounds, condensed tannins and flavonoids were estimated at 371.43±0.09 mg GAE/g, 14.24±0.00% and 52.72±0.10 mg QE/g, respectively. This study shows that local plants adapted to drought could make an interesting source of molecules with antioxidant property in the prevention and the treatment of infectious diseases.**

Key words: Sahel plants, infectious diseases, phenolic compounds, radical scavenging.

INTRODUCTION

Climatic warming is the most serious environmental problems which has led to the development of highly transmissible and virulent infectious diseases such as Ebola, Dengue, Zika, or Severe Acute Respiratory Syndrome (Debil, 2013).

These emerging or re-emerging diseases have occurred in recent decades and constitute real threats to public health (Chidiac and Ferry, 2016). The tropical regions of

Africa affected by dryness are the most vulnerable. Increasing temperatures in these regions favor the proliferation of mosquitoes. Malaria and dengue are the most common vector infectious diseases in these areas (Githeko et al., 2000). Indeed, climate change is responsible for 6% of malaria cases in some low-income countries and the capacity of mosquitoes carrying the dengue virus to transmit this infection has increased by 9.4% since 1950 (Kraemer et al., 2015). According to World Health Organization, the incidence of these diseases will increase each year with climatic warming (WHO, 2014). Burkina Faso has experienced drought since 1970, which has allowed desertification to gain ground every year. Previous studies showed that infectious diseases are the most common in this country (Besancenot et al., 2004). The high cost of imported medicines and the cultural attachment to effectiveness of recipes based on plant makes that at least 80% of rural people living in developing countries depend on traditional medicine for their needs in healthcare (OMS, 2013). The renewed interest in herbal medicines in developed countries could be explained by the emergence and expansion of various pathologies of bacterial and/or viral origin, which often resist to conventional medicine treatments. It is also due to undesirable effects of some synthetic pharmaceutical drugs (Moore et al., 1985). In addition, thanks to World Health Organization's politics of promoting traditional medicine, herbal medicines occupy today a considerable place in international pharmaceutical trade (OMS, 2013). The *Acacia* genus of Fabaceae-Mimosoideae family is widely spread in arid zones, tropical forests and driest regions of the world including Sahelian countries of Africa. It contains more than 1350 species, present in bushes form and sometimes as large trees (Ibrahim and Aref, 2000). Acacia species are used in planting and sylvo-pastoral programs to mitigate the effects of desertification. They are the most available and play an important socio-economic and therapeutic role in the Sahel regions. *Acacias* are used daily by local populations as food, medicine, energy, building materials, fodder for cattle and as source of large quantities of gum arabic (*Acacia senegal* and *Acacia seyal*) (Guinko, 1997). Previous work showed that *Acacia* spp. have very efficient pharmacological properties. Indeed, the presence of some chemical groups in these species, such as tannins known for their astringent, antiparasitic and antibacterial properties as well as anthocyanins and flavonoids recognized for their anti-inflammatory, antioxidant and antiradical effects would justify the preferential choice of the genus Acacia in the treatment of some infections (MacRae et al., 1989; Okuda et al., 1991).

Other studies showed that one of the consequences of exposure of populations to high intensities of solar radiation is the increase of oxidative stress which causes an overproduction of free radicals in body and the alteration or suppression of cellular immunity. This reinforces the risks of infectious diseases (Favier, 2006). It is therefore important to promote alternative and local therapy based on plants potential antioxidant in order to reinforce the resilience of the population facing the effects of climate.

The objective of this study was to compare the chemical profile and the antioxidant activity of *A. seyal* and *Acacia tortilis* used in the treatment of infectious diseases in Northern Burkina Faso.

MATERIALS AND METHODS

Plant

A. seyal called Bulbi in Fulfulde language is a thorny bush, sometimes a tree up to 12 m tall. Leaves are twice composed and the bark often smooth breaks loose by irregular plates. *A. tortilis* or *Acacia* false gum called Tchiluki is also a thorny which can reach more than 15 m high. The peak is displayed in parasol, leaves are twice pinnate and the bark is often red-brown. *A. seyal* and *A. tortilis* are used in some regions of Africa to treat different diseases in traditional medicine such as jaundice, bilious fevers, skin allergies, diabetes, hypertension or as diuretic (Jaouadi et al., 2015). These two species are used by populations in Northern Burkina Faso in traditional recipes for the treatment of infectious diseases such as yellow fever, dysentery, gonorrhea, schistosomiasis, ulcers, and pulmonary infections but especially against malaria and dengue.

The different parts (leaves and trunk bark) of *A. seyal* and *A. tortilis* were harvested in Mamassirou village (Soum province) in March 2019. After identification by the Botanical team of Ouaga 1 Pr Joseph Ki-ZERBO University, specimens were deposited in a herbarium under registration 4S/2019 and 5S/2019, respectively. Each plant material was washed and dried in an airy room, protected from sunlight for a week and finely ground by a mechanical grinder.

Chemical material

The chemicals products used were: 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma, St Louis, MO, USA), 6-hydroxy 2,5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) and Folin-Ciocalteu Reagent (Sigma Chemical Company, Steinheim, Germany), NEU Reagent (Natural Products - Poly Ethylene Glycol), NaOH, Na_2CO_3, Methanol (E. Merck, Darmstadt, Germany), Ethyl acetate (SSI, France), Sulfuric acid, Aluminium trichloride and Acetic acid (Labosi, France), n-Hexane (SDS, France), Gallic acid, Quercetin (Sigma-Aldrich, Germany) and $AlCl_3$ (Fluka Chemika, Switzerland)

Extraction and phytochemical screening

One hundred grams of dry matter was extracted by maceration at

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low temperature (4°C) for 24 h with methanol. The extraction was repeated twice with the marc obtained after each filtration. In total, 1 L of solvent was used. The different filtrates were collected and then concentrated to dryness (100 mL) on a rotary evaporator at a temperature below 40°C. Phytochemical screening of extracts was carried out by following the method described by Ciulei (1982) for tube tests and on HPTLC plates according to the analytical technique used by Kavit et al. (2013). Plates with aluminium support Silica Gel 60 F_{254} were used. The spots of extracts were deposited by using the system of Linomat 5 (Camag, Muttez; Switzerland) spray on automated instrument for HPTLC. Eluent system (Ethyl acetate: Formic acid: Acetic acid: Water, 100: 11:11: 26, v/v/v/v) was used for the migration of flavonoids, phenolic acids, sterols and triterpenes. Another system (Ethyl acetate: Water: Methanol: n-Hexane, 11,9:1,6:1,4:3,5, v/v/v/v) was used for the migration of tannins. Flavonoids and phenolic acids were revealed with Neu's reagent in the presence of UV light (366 nm), sterols and triterpenes were revealed by 3% H_2SO_4 in EtOH (96%) and tannins by $FeCl_3$ 2%.

Determination of total phenolic content

Total phenolic content was estimated using the Folin-Ciocalteu Reagent (FCR) as described by Singleton et al. (1999) and used by Ramde-Tiendrebeogo et al. (2012). The reaction mixture consists of 1 mL of extract, 1 mL of FCR 2N and 3 mL of 20% sodium carbonate solution. This mixture is left to stand at room temperature for 40 min and then the absorbance was measured (spectrophotometer UV, Shimadzu) at 760 nm. In the control tube, the extract volume was replaced by distilled water. Calculation was based on a calibration curve obtained with increasing concentrations of gallic acid (Y=0.0664X-0.0009; R^2=0.9991). The total phenolic content was expressed as milligrams of Gallic Acid Equivalent per gram of dry material (mg GAE/g).

Determination of tannins content

Condensed tannins

The condensed tannins were estimated according to method described by Price et al. (1978) and used by Ba et al. (2010). The reagent was vanillin 1% (1 g of vanillin dissolved in 100 mL of 70% sulfuric acid). 2 mL of this reagent was added to 1 mL of extract. The absorbance of the mixture was read at 500 nm (spectrophotometer UV, Shimadzu) after 15 min of incubation in a water bath at 20°C. The condensed tannins content T (%) was determined using the following formula:

$T\% = 5.2\ 10^{-2} \times A.V/P$

where $5.2\ 10^{-2}$ = equivalent constant of cyanidine, A= absorbance, V = extract volume and P = sample weight.

Hydrolyzable tannins

The hydrolyzable tannins were estimated according to method described by Mole and Waterman (1987) and used by Mboko et al. (2017). 1 mL of the extract and 3.5 mL of the reagent ($FeCl_3$ 10^{-2} M in HCl 10^{-3} M) were mixed. The absorbance of the mixture was read at 660 nm (spectrophotometer UV, Shimadzu) after 15 s. The hydrolysable tannins content T (%) was determined using the following formula:

$T\% = A.\ PM.\ V.\ FD/\mathcal{E}_{mole}.\ P$

where A = Absorbance, $\mathcal{E}_{mole}$ = 2169 (for gallic acid), PM = weight of gallic acid (170.12 g/mol), V = volume of extract, P = sample weight and FD = dilution factor.

Determination of total flavonoid content

Total flavonoid content was determined according to method of Alothman et al. (2009). The extract was prepared at a concentration of 1 mg/mL in methanol. 1 mL of this extract was mixed with 3 mL of double-distilled water followed by 0.3 mL of $NaNO_2$ at 5% (m/v). 5 min later, 0.3 mL of $AlCl_3$ 10% (m/v) was added. The whole was incubated at room temperature for 6 min. 1 mL of NaOH 1 N was added. The absorbance of the mixture was measured at 510 nm using a microplate reader (MP 96 spectrophotometer, SAFAS). Calculation was based on a calibration curve obtained with increasing concentration of quercetin solution (Y=0.0001X+0.0014; R^2=0.9891) following the same procedure. The flavonoid content of the sample, expressed as milligrams of Quercetin Equivalent per gram of plant material (mg QE/g) was obtained by relating the absorbance read on the calibration curve.

Antiradical activity by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) test

The antiradical activity by DPPH test was evaluated according to the method of Kim et al. (2003) used by Ramde-Tiendrebeogo et al. (2012). Ten numbered tubes (1-10) were primed. The DPPH radical was dissolved in methanol (2 mg/50 mL). 0.5 mL of the extract was put in tube 1 to which 2 mL of methanolic solution of DPPH radical (0.04 mg/mL) was added. Concentrations range of extracts or standard (quercetin) were prepared by cascade dilution. After 10 min incubation at 37°C protected from light, the absorbance of residual DPPH was measured at 517 nm (spectrophotometer SAFAS). Antiradical activity of a sample (calculated by the following formula) is given as percentage of reduced DPPH:

$I\% = (A_0\text{-}A_S)/A_0 \times 100$

where I = percentage of inhibition, A_0 = absorbance of control, and A_S = absorbance of sample. For each sample the concentration (µg/mL) required to reduce by 50% the activity of DPPH (IC_{50}) was determined.

Statistical analysis

The results are expressed as mean ± SEM (n = 3). The data were analyzed using One Way Analysis of Variance (ANOVA) followed by Dunnett's posttest for multiple comparisons (Graph Pad Prism version 5.0 for Windows, Graph Pad Software, San Diego California USA). Differences were considered statistically significant for a p value less than 0.05.

RESULTS

Chemical screening of extracts

Preliminary phytochemical screening using tube tests revealed the presence of chemical groups including alkaloids, carbohydrates, flavonoids, saponins, sterols, tannins, phenolic acids, and triterpenes (Table 1). The determination of constituents by HPTLC and the revelation of the spots, showed that certain compounds

Table 1. Chemical composition of leaves and trunk bark extracts of *Acacia seyal* and *Acacia tortilis*.

Chemical group	*Acacia seyal*		*Acacia tortilis*	
	Leaves	Trunk bark	Leaves	Trunk bark
Alkaloid bases	-	-	-	-
Alkaloid salts	+	+	+	+
Carbohydrates	+	+	+	+
Polyphenols	+	+	+	+
Flavonoids	+	+	+	+
Saponins	+	+	+	+
Sterols	+	+	+	+
Triterpenes	+	+	+	+
Tannins	+	+	+	+
Carotenoids	+	+	+	+
Anthracenosids aglycones	-	-	-	-
Phenolic acids	+	+	+	+

+ Present, - absent.

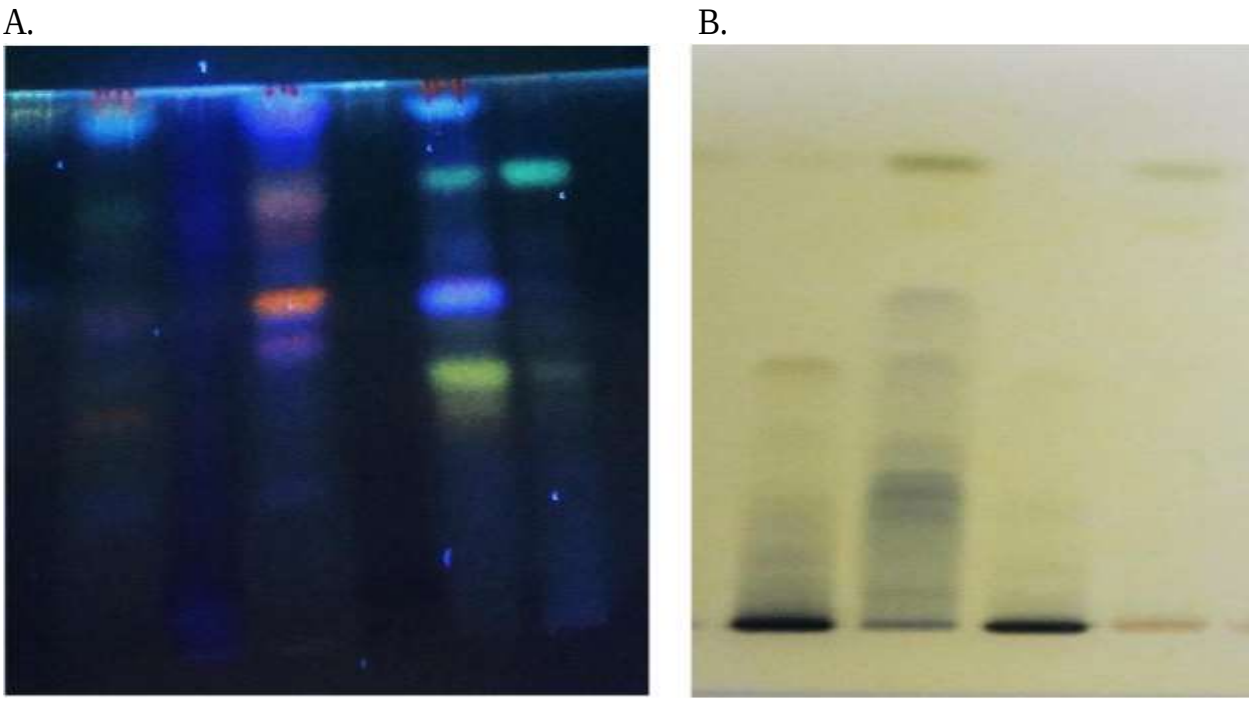

Figure 1. Determination of phenolic compounds of leaves and trunk bark extracts of *Acacia seyal* and *Acacia tortilis* on HPTLC plates.

react positively to Neu reagent, to 3% H_2SO_4 in EtOH (96%) and to $FeCl_3$ 2% (Figure 1A and B). Flavonoids were yellow-orange and phenolic acids blue. Terpenes were purple while sterols were brown. The presence of tannins was confirmed.

Antioxidant activity and phenolic contents

The contents of phenolic compounds and the concentration inhibiting 50% of DPPH are regrouped in Table 2. The trunk bark of *A. tortilis* showed the highest

Table 2. Phenolic contents of leaves and trunk bark and antiradical activity by the DPPH test.

Plant	*Acacia seyal*		*Acacia tortilis*		*Reference substance*
	Leaves	Trunk bark	Leaves	Trunk bark	Trolox
Total phenolic mg GAE/g	371.43 ± 0.09[a]	310.33 ± 0.06[ab]	307.89 ± 0.15[ab]	383.19 ± 0.07	-
Condensed tannins (%)	14.24 ± 0.00	8.85 ± 0.07	7.37 ± 0.00	18.21 ± 0.04	-
Hydrolyzable tannins (%)	7.11 ± 0.20	12.77 ± 0.07	11.59 ± 0.09	10.21 ± 0.08	-
Flavonoids mg QE/g	52.72 ± 0.10	41.15 ± 0.04	43.52 ± 0.14	66.09 ± 0.06	-
Antiradical activity IC_{50} (µg/mL)	0.02 ± 0.01[c]	0.03 ± 0.02[c]	0.03 ± 0.01[c]	0.01 ± 0.01	0.01 ± 0.00

Values are mean±SEM (n = 3); [a]p < 0.05 against *Acacia tortilis* trunk bark; [b]p<0.05 against leaves of *Acacia seyal*; [c]p < 0.05 against Trolox. GAE, Gallic acid equivalent; QE, quercetin equivalent.

content of total phenol compounds (383.19±0.07 mg GAE/g) (P < 0.05) than its leaves and the different parts of *A. seyal*. In addition, it had the highest percentage of condensed tannins (18.21±0.04%). *A. seyal* trunk bark had the highest percentage of hydrolysable tannins (12.77±0.07%). Flavonoid content ranged from 66.09±0.06 mg QE/g for the highest at the lowest 41.15±0.04 mg QE/g obtained, respectively with the trunk bark of *A. tortilis* and *A. seyal*. The best activity of DPPH scavenging (P < 0.05) was obtained with the extract of *A. tortilis* trunk bark (0.01±0.01 µg/mL, IC_{50}) which had higher level of total phenolic, condensed tannins and flavonoids. This antiradical activity was comparable to that of Trolox used as reference. The leaves of *Acacia seyal* which had phenolic content, condensed tannins and flavonoids estimated to 371.43±0.09 mg GAE/g, 14.24±0.00% and 52.72±0.10 mg QE/g, respectively showed significant antiradical activity with IC_{50} value equal to 0.02±0.01 µg/mL compared to its trunk bark and *A. tortilis* leaves.

DISCUSSION

Oxidative stress is an imbalance of the oxidant-antioxidant balance in favor of oxidants. It develops when free radicals or oxidative molecules are produced faster than they can be neutralized by the body (Houssaini et al., 1997). Oxidative stress is secondary to the establishment of pathology but contributes to its immune or vascular complications (Atamer et al., 2008). Previous studies showed that reducing oxidative stress can greatly improve the health status of populations (Rorive et al., 2005). It is therefore important to evaluate the antioxidant potential of natural resources that can act in the prevention or treatment of infectious diseases. Results showed that A. seyal and *A. tortilis* have an antioxidant property due to their ability to trap free radical DPPH. But, methanol extract of the trunk bark of *A. tortilis* which had the highest content of total phenolic compounds (383.19±0.07 mg GAE/g), of condensed tannins (18.21±0.04%) and flavonoids (66.09±0.06 mg QE/g) also had the best antioxidant activity (0.01±0.01 µg/mL, IC_{50}) comparable to that of Trolox (0.01±0.00 µg/mL, IC_{50}) used as reference. The present results are in agreement with previous studies which reported the antioxidant activity of some *Acacia* spp. and showed that this antioxidant activity is related to phenolic content (Abdel-Farid et al., 2014). The result obtained with a raw extract of A. tortilis trunk bark shows significant antiradical activity of the active principle contained in this plant. Tannins and flavonoids are recognized for their important antioxidant activity. Indeed the protective effects of flavonoids in biological systems are linked to their ability to transfer electrons to free radicals, to activate antioxidant enzymes or to inhibit oxidases (Bruneton, 2009; Santi et al., 2018). Previous work showed that Vicenin and Rutin which are two flavonoids isolated in A. tortilis have the ability to remove hydroxides and peroxides (Seigler, 2003; Jaouadi et al., 2015). The tannins have antiradical and antioxidant properties expressed by their inhibiting effect on lipid peroxidation and radical-scavenging ability on DPPH radical (Bouchet et al., 1998). The antiradical activity obtained with *A. seyal* leaves is quite remarkable (0.02±0.01 µg/mL, IC_{50}). The use of its leaves is to encouraged due to the fact that *A. seyal* being an important source of gum arabic marketing, using its leaves allows to avoid destruction of the plant and to preserve its durability. Other phytoconstituents exist in both plants as shown in Table 1, such as alkaloids, triterpenes, sterols, saponins, and phenolic acids. This could justify their multiple uses in traditional medicine.

A. seyal and A. tortilis are woody plants, perennial, widely distributed and adapted to drought of Sahelian countries. They contribute to soil protection against erosion by wind and runoff. Also, the richness of these plants in total phenolic compounds, tannins and flavonoids with antiradical properties as demonstrated by this study as well as by other authors makes them interesting source of bioactive molecules. It is therefore urgent to promote the therapeutic experience of Sahelian local populations in order to develop improved traditional medicines or to isolate new bioactive molecules for the

prevention and treatment of infectious diseases that continue making more victims each year.

Conclusion

The objective of this study was to compare the phytochemical profile and antioxidant activity of two Sahelian plants (*A. seyal* and *A. tortilis*) used in the treatment of infectious diseases by populations in Northern Burkina Faso. Results showed significant antiradical activity of *A. tortilis* trunk bark and the leaves of *A. seyal* related to the importance of their phenolic contents. This study shows that local plants adapted to drought could contribute to health security of populations living in the Sahel region. Further bioguided studies should make it possible to isolate and identify new bioactive molecules for the treatment of the most common infectious diseases.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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