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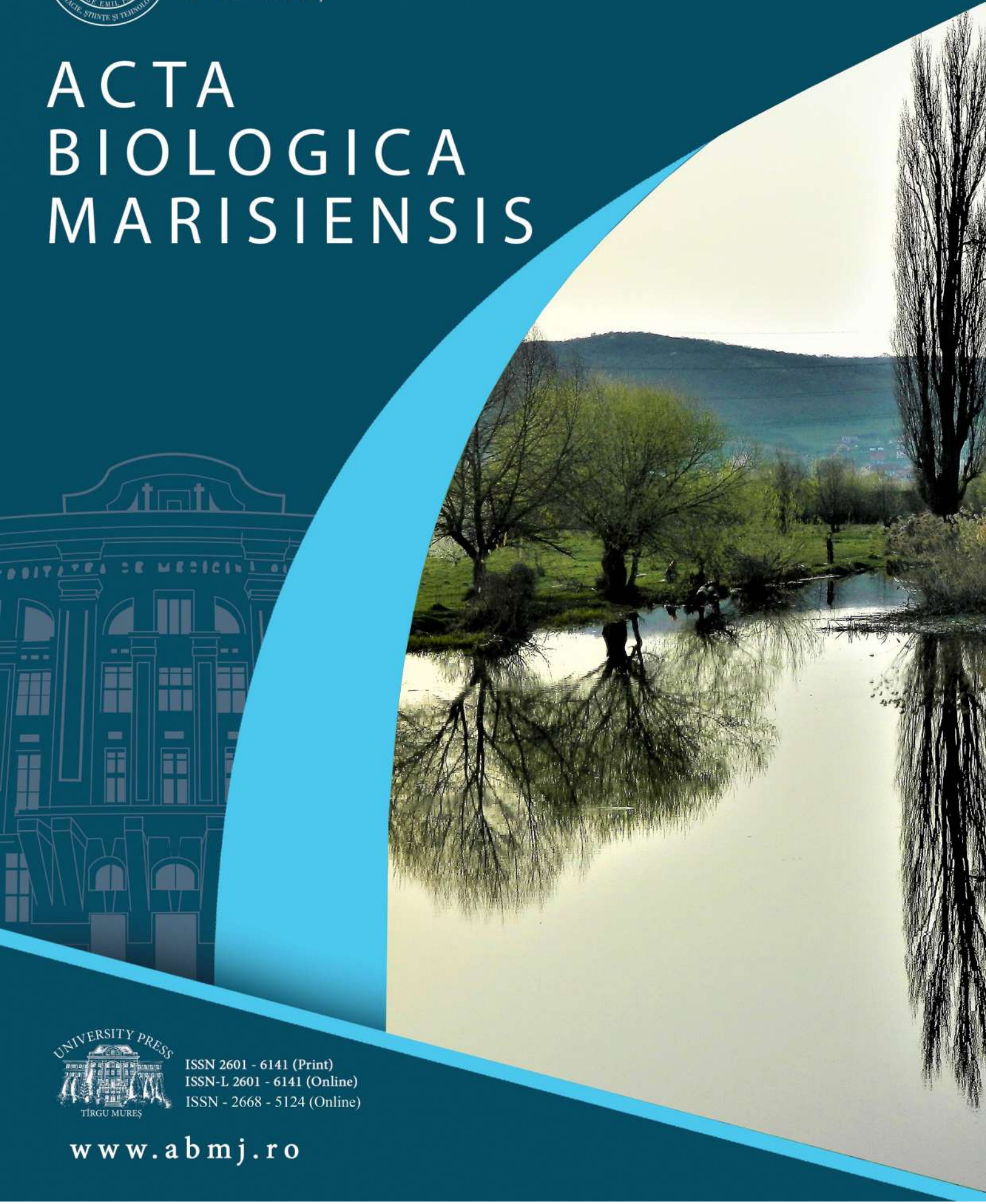

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IN VITRO EFFECTS OF VARIOUS PROBIOTIC PRODUCTS ON GROWTH AND BIOFILM FORMATION OF CLINICAL UPEC STRAINS

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Abstract: Uropathogenic *Escherichia coli* (UPEC) is the most prevalent pathogen causing urinary tract infections (UTIs). UPECs have various virulence factors such as adhesins, biofilm forming and toxin producing etc., to survive in urinary tract. Under certain circumstances probiotics are preferred for prevention and treatment of UTIs. In this study, we aimed to investigate the *in-vitro* effects of four different *Lactobacillus* spp. cell-free supernatants on growth and biofilm formation inhibition in clinically isolated UPEC strains. Growths of 50 UPEC strains were determined in 96-well microplate and measured in a spectrophotometer after four hours incubation at 37°C. Biofilm formation was detected by crystal violet staining method on three UPEC strains. Statistical analysis of growth and biofilm formation experiments were performed by Kruskal-Wallis and one-way ANOVA Tukey's multiple-comparison tests, respectively. All tested cell-free supernatans of lactobacilli inhibited growths (p<0. 0001) and biofilm formation (p<0.05) of UPECs. All results were found to be statistically significant. As a conclusion, our findings supported previous studies which reported the high efficiency of these four *Lactobacillus* spp. in the prevention of UTIs.

Keywords: *Lactobacillus*, growth, biofilm formation, UPEC, probiotics.

1. Introduction

Uropathogenic Escherichia coli (UPEC) have various virulence factors such as fimbrial adhesins, biofilm formation, siderophore, toxins, cytotoxic necrotizing factor-1, bacteriocins, endonuclease activity, and outer membrane protease. They are known as the leading pathogens causing urinary tract infections (UTI) (Mandal, 2001; Ruiz et al., 2002; Miyazaki et al., 2002; Bower et al., 2005; Sabaté et al., 2006; Yamamoto, 2007; Uzun et al., 2015). Biofilm formation capacity of UPEC strains is also an important advantage for the persistence and recurrence in infections caused by them. Besides, biofilm formation protects from host immunity and antimicrobial components (Freestone, 2013). Urinary tract infections (UTI) are seen about 40% in women, 12% in men for their life time and often recur within 6 to 12 months nearly in 25% of infected women (Kulkarni et al., 2009; Sivick

and Mobley, 2010; Li et al., 2010). Recurrent infections could be due to unsuccessful treatments which is related to antibiotic resistance and invasive infections (Hunstad and Justice, 2010; Andersen et al., 2012). Therefore some alternative strategies such as probiotics are useful and more cost effective for their treatment (Geerlings et al., 2014; Beerepoot et al., 2012; Delley et al., 2015; Lee et al., 2016).

Probiotics are known as live microorganisms which beneficially regulate host health (FAO/WHO, 2001). It is well known that the beneficial effects of lactobacilli depend on secreting several strong antimicrobial compounds such as organic acids, benzoic acid, acetic acid, formic acid different types of bacteriocins, bacteriocin-like inhibitory substances and hydrogen peroxide (Lash et al., 2005; Kim and Kim, 2009).

Lactobacillus spp. and *Bifidobacterium* spp. are the most administered bacteria, especially for prevention and control of oral, gastrointestinal and urogenital system diseases (Saavedra 2000; Tomas et al., 2003; Morelli et al., 2004; Servin, 2004; Morais, 2006; Segarra-Newnham, 2007; Guarino et al., 2009; Miyazaki et al., 2010; Guandalini, 2011; Wagner and Johnson, 2012; Behnsen et al., 2013; Turroni et al., 2014; Wu et al., 2015). For example, *Lactobacillus plantarum*, *Lactobacillus reuteri, Lactobacillus rhamnosus* and *Lactobacillus acidophilus* are known to inhibit biofilm formation of pathogens (Miyazaki et al., 2010; Wagner and Johnson, 2012; Wu et al., 2015). Cadieux et al., (2009) mentioned the antagonist effects of urogenital lactobacilli for UPECs and explained that their lethal effects do not occur only directly, but they can provide stress conditions for bacteria.

In the present study, we aimed to evaluate the in-vitro effects of four different lactobacilli cell-free supernatants on growth and biofilm formation in UPEC strains isolated from UTI patients.

2. Materials and Methods

Bacterial strains, media and culture conditions

In the present study cell-free supernatants of *Lactobacillus rhamnosus* ATCC 53103, *Lactobacillus fermentum* ATCC 9338, *Lactobacillus acidophilus* ATCC 314, and *Lactobacillus plantarum* ATCC 14917 were tested for their effects on growth and biofilm formation in UPECs. They were chosen according to their high usage in commercial probiotics (Karska-Wysocki, 2010; Nigam et al., 2012); therefore we prepared cell-free supernatants. All strains were stored in −80 °C prior to the experiments. De Man, Rogosa and Sharpe (MRS) broth and (MRS) agar (Conda, Spain) were used for isolation of all lactobacilli. Cultures were performed in anaerobic atmosphere (10% H_2, 5% CO_2 and 85% N_2) at 37°C for 48 hours. 50 UPEC strains from our culture collection were included in the present study; they were previously isolated from symptomatic, acute, uncomplicated UTI patients and they were proven for their pathogenic abilities (Uzun et al., 2015). Bacteria were kept in −80°C and revived after 10 years via inoculating into Tryptic Soy Broth (TSB) and Tryptic Soy Agar (TSA). Overnight cultures of UPECs and all lactobacilli were prepared by inoculation of single colonies into TSB and MRS broth, respectively. The overnight cultures of each *Lactobacillus* strains were centrifuged at 4000 rpm for 30 minutes at 4°C; strains and the supernatants were collected then filtered by using 0.2 µm filter.

Effects of cell-free supernatants of Lactobacillus spp. on growth of UPECs

Initial bacterial concentrations of UPECs were arranged to 10^7 CFU/mL. UPEC strains were cultured into TSB alone (as control) or TSB supplemented with different supernatants of *Lactobacillus* (100 µL cell-free supernatants+80 µL TSB+20 µL bacteria) and

incubated at 37°C. Growths were determined by measuring the changes via spectrophotometer in absorbance (OD) at 600 nm in four hours period. The samples were tested in duplicate and each experiment was performed twice.

Effects of cell-free supernatants of Lactobacillus spp. on biofilm formation of UPECs

The biofilm formation in three out of 50 UPEC strains (which were determined previously as biofilm forming strains) were observed with crystal violet staining method.The effects of lactobacilli cell-free supernatans on three biofilm positive UPEC strains were analyzed. *E. coli* ATCC 25922 and MRSA ATCC 43300 were used as positive controls. The strains were cultured in TSB-glucose (1%v/v) for 24 h at 37 °C and diluted 1/50 in fresh TSB-glucose, yielding a final concentration of approximately 10^7 CFU/mL. 100 µL from bacterial suspension and 100 µL from cell-free supernatants of *Lactobacillus* probiotic products were added to each well of a 96-well tissue culture microtiter plate, and then incubated for 24 hours at 37°C. TSB-glucose was used as a negative control. After incubation, the waste media was gently aspirated, and the wells were washed 3× with 250 µL Phosphate Buffered Saline (PBS) solution to remove any unattached bacteria and air-dried. Then, 200 µL 99% methanol was added to each well to fixate for 15 min, then it was aspirated. Wells were stained with 200 µL 0.1% crystal violet (in water) for 5 min. Excess stain was gently rinsed off with tap water, and the plates were air-dried. The stain was re-solubilized by adding 200 µL 95% ethanol. The optical density was measured at 450nm.For the purposes of comparative analysis of test results, we introduced classification of adherence capabilities of tested strains into four categories (OD ≤ ODc non-adherent, ODc< OD ≤ 2x OD weakly adherent, 2xODc < OD ≤ 4 x ODc moderately adherent, 4xODc <OD strongly adherent) as described previously (Christensen et al., 1985).

Statistical analysis

Growth alterations were detected by using Kruskal-Wallis test. The effects of probiotic supernatants of *Lactobacillus* on biofilm formations of UPECs were determined via one-way ANOVA Tukey's multiple-comparison test. All measurements were compared to control conditions (TSB). Multiple comparisons were made at a level of $P < 0.05$.

3. Results and discussions

Effects of probiotic supernatants of Lactobacillus spp. on growth of UPECs

The direct antagonism of compounds contained in *Lactobacillus* cell-free supernatants against UPECs was monitored by turbidmetric method. Supernatants of all four *Lactobacillus* species inhibited growth of UPECs with a high rate of 99% when compared to control (TSB) (**Fig. 1**).

These results were found to be statistically significant ($p<0.0001$). To satisfy our own curiosity, we analyzed the effect of neutralized pH supernatants on the growth of randomly selected five UPEC strains and we detected that, supernatants with pH:6.8 did not alter the growth of UPECs ($p>0.05$).

Effects of cell-free supernatants of Lactobacillus spp. on biofilm formations of UPECs

All three biofilm forming strains were classified as weakly adherent according to Christensen et al., (1985) criteria. All tested cell-free supernatants of lactobacilli were shown to inhibit biofilm formation in three UPEC strains in 24 hours significantly ($p<0.05$) (**Fig. 2**).

Globally a large number of people suffer from urinary tract infections which are mostly caused by uropathogenic *E. coli* (Hacker et al., 1999; Kulkarni et al., 2009; Li et al., 2010). It is well known that *Lactobacillus* strains have antibacterial effects with their secreted compounds such as bacteriocin or exopolycacharides and organic acids (Makino et al., 2006; Hagan and Mobley 2007; Nader-Macías et al., 2008; Cadieux et al., 2009; Martín and Suárez, 2010; Stoyancheva et al., 2014). In our study we aimed to detect the inhibitory effects of four different *Lactobacillus* spp. cell free supernatants on growth and biofilm formation because of their widely usage in dairy products, fruit drinks, chewing gums and tablets which are available on market (Karska-Wysocki, 2010; Nigam et al., 2012).

Cadieux et al., (2009) have documented that some *L. rhamnosus* and *L. reuteri* strains could affect UPECs surface membrane traits. Delley et al., (2015) have shown that *L. acidophilus*, *L. rhamnosus* and *L. johnsonii* strains' cell free supernatants inhibited some UPEC strains. Similarly Tomas et al., (2011) have shown the growth inhibition of UPECs in the presence of *L. acidophilus*. Ocana et al., (1999) have also observed that some *L. acidophilus* strains (CRL 1259, CRL 1307, CRL 1320 and CRL 1324) inhibited the growth of *E. coli*, *Staphylococcus aureus*, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Klebsiella*, *N. gonorrhoeae* and *G. vaginalis*. Terraf et al., (2017) have detected that supernatants from *L. reuteri* and *L. rhamnosus* inhibited the growth of UPEC strains. Miyazaki et al., (2010) shown that supernatants of *L. casei* subspecies and *L. acidophilus* inhibited the growth of the EAggEC TN-2 strain

Many studies have suggested that in order to prevent growth of pathogenic microorganisms that cause urogenital infections, probiotic products can be used. Some researchers suggested that antagonistic effects (bacteriostatic or bactericidal) of *Lactobacillus* on growth of *E. coli* related to the presence of organic acids that are released during growth (Axe and Bailey, 1995; Diez-Gonzalez and Russell, 1997). In line with previous studies we detected that, supernatants of *L. acidophilus*, *L. plantarum*, *L. fermentum* and *L. rhamnosus* supernatants inhibited growth of UPECs.

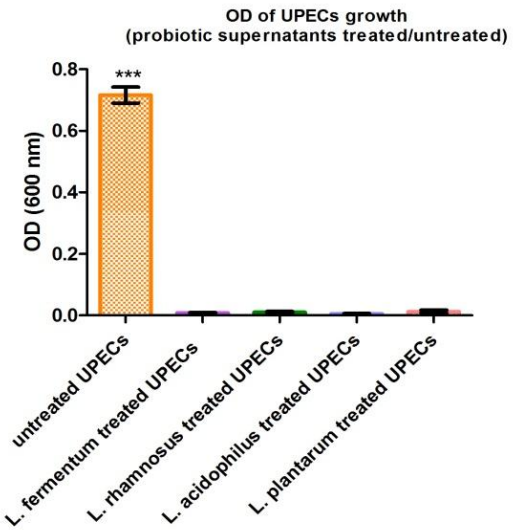

Fig. 1. Effects of different cell-free supernatants on growth in UPECs

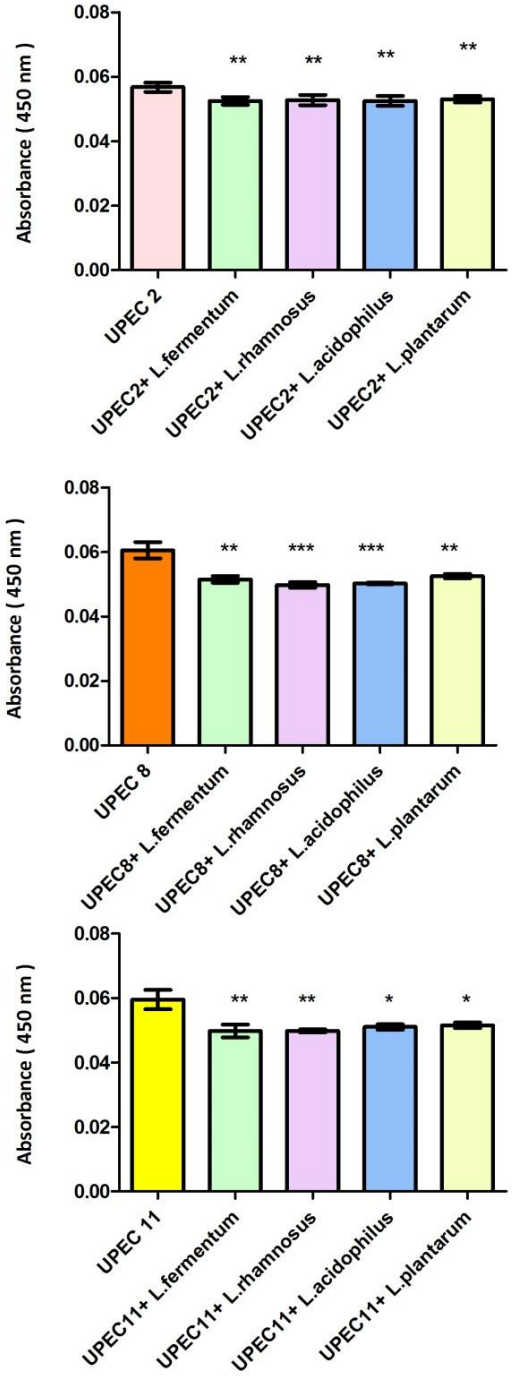

Fig. 2. Effects of different cell-free supernatants on biofilm formations in three different UPEC strains

Therefore we wanted to investigate whether the 99% of inhibition with probiotic supernatants depends on pH or not. Our results with a randomized selected five UPECs showed that, high level acidity leads the major inhibition on growth of pathogens. Evidence supports that the antagonist effects of *Lactobacillus* may be variable depending on exposure time, test microorganism, temperature as well as pH (high acidity) (Ogawa et al., 2001; Lash et al., 2005; Poppi et al., 2015).

The most known anti-biofilm activity is related to exopolysaccharides in *Lactobacillus* supernatants (Barken et al., 2008; Kim and

Kim., 2009; Wang et al., 2015). The first step of biofilm formation is adhesion to surface then multiplication of bacteria to compose extracellular polymeric matrix. Communication system which is known as quorum sensing (QS) plays an important role in adhesion and biofilm formation (Waters and Bassler, 2005; Bassler and Losick, 2006). QS provides cell to cell communication and it is important for bacterial survival and interactions in natural habitats. Previous studies have shown that there is a strong association between *Lactobacillus* supernatants and repression of the genes related with initial adhesion and chemotaxis (Balaban et al., 2007; Wang et al., 2015). It was suggested that, Lactobacilli supernatants could play role as molecules in reducing biofilm formation and quorum sensing related gene expressions (Balaban et al., 2007; Wang et al., 2015; Zamani et al., 2017). Sadri et al., (2016) have suggested that *L. acidophilus* inhibited adhesion of UPECs. Zamani et al., (2017) reported that *L. plantarum* isolated from a traditional cheese had anti-biofilm potential for *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *E. coli*. Vacheva et al., (2012) reported that *L. gasseri* Lb821, *L. plantarum* LbS11 had anti-biofilm effects on *E. coli* strains. On the other hand, Miyazaki et al., (2010) reported that the supernatants of *L. casei* and *L. rhamnosus* stimulated biofilm formation of enteroaggregative *Escherichia coli*. In our study, all the tested *Lactobacillus* supernatants were found to inhibit biofilm formation in UPEC strains in consistency with the results of many other studies (Kim and Kim., 2009; Vacheva et al., 2012; Aminnezhad et al., 2014; Zamani et al., 2017).

Conclusions

As conclusion in our study it was shown that, the growth of 50 different clinical UPEC strains were inhibited by *Lactobacillus* spp. with a rate of 99%. Besides, biofilm formations of three UPECs were also inhibited significantly in the presence of cell-free supernatant of four *Lactobacillus* strains tested. Therefore consistent with previous studies, our findings support that these four lactobacilli may be used to prevent the UTIs caused by UPEC strains, effectively.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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LEAF AND PETIOLE MICRO-ANATOMICAL DIVERSITIES IN SOME SELECTED NIGERIAN SPECIES OF *COMBRETUM* LOEFL.: THE SIGNIFICANCE IN SPECIES IDENTIFICATION AT VEGETATIVE STATE

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Abstract: Leaf and petiole samples of four *Combretum* Loefl. species which were identified in the Herbarium (IFE) were investigated anatomically in search of stable taxonomic micro-anatomical attributes to improve our knowledge of identification of members of the genus. Anatomical characters; in particular, upper and lower cuticles and epidermal structures, fibre structures, vascular architectures, petiolar outlines and trichome micro-morphology are good taxonomic tools to identify the taxa. The invariable uniseriate to multiseriate upper and lower epidermis; the absence of trichome in the petiole and the presence of branched trichome in the mid-rib region of *C. zenkeri* P. Beauv delimit the taxa. Variations in vascular architectures can be used to identify the taxa while some other anatomical features in the genus suggest great taxonomic affinities. However, the artificial key, which was constructed using stable taxonomic characters, is a reliable taxonomic tool for proper identification of the four species and which can as well be employed in separating each of the taxa from their close relatives. A detailed micro-anatomical study of leaf and petiole structures of the Nigerian *Combretum* species may provide an invaluable tool for determination and identification of the four taxa studied, thereby assisting in promoting quality assurance in the genus.

Keywords: Artificial key, *Combretum*, diagnostic, lamina, palisade parenchyma, petiole.

1. Introduction

The genus *Combretum* Loefl. is the largest in the family Combretaceae and the type genus of the family (Systma et al., 2004). The family constitutes one of the most specious families of plant in West Africa (Fontes and Guinko, 1995; Thiombiano, 2005), while sectional classification within the genus *Combretum* as first suggested by Engler and Diels (1899; 1900) was updated through the years by various authors and today comprises 46 sections. The genus *Combretum* comprises about 370 species of trees and shrubs, 300 of which are native to tropical and southern Africa, about five to Madagascar, twenty-five to tropical Asia and forty to tropical America.

However, Keay (1989) reported 25 species of the genus found in Nigeria. Despite extensive taxonomic, morphological and anatomical studies on *Combretum* in tropical, southern and West Africa (Stace, 1965; 1969; 1980a; 1980b; 1981; Exell, 1968; 1978; Verhoeven and Vander' Schijff, 1973; Oladipo et al., 2016; Akinsulire et al., 2018a; Akinsulire et al., 2020), there are still nomenclatural problems remaining. Some names are misapplied and the identity of some taxa in southern as well as western African herbaria is uncertain. Recently, the molecular work carried out by Maurin et al. (2010) dealt mostly with the subgeneric, sectional and generic delimitation of *Combretum* and has indicated that taxa boundaries need revising to reflect more accurately the phylogeny of *Combretum* and its allies.

Several species in the genus *Combretum* are used in African or Indian traditional medicine. Leaves of *C. glutinosum* Perr. ex. DC. are browsed by ruminants, and it is the preferred browse species for adult giraffes. Extracts from the bark, leaves and especially the roots produce a yellow dye. The yellowish wood is hard and extremely durable, and it is used in construction and general carpentry. It also makes good fuel wood and charcoal. Many medicinal uses have been reported using the roots, stems, leaves, bark and fruit. *Combretum* species are used in the treatment of influenza, rheumatism, intestinal worms, coughs, colic, impotence, haemorrhoids, constipation, anorexia, malaria, wounds and syphilis.

On the species' morphological examination, Akinsulire et al. (2018a) has though reported the various morphological means by which the species under study could be identified, but thorough investigation into the anatomy of leaves and petioles of *Combretum* species become imperative since plant anatomy deals with the structure, content and development of cells and tissues. It is of primary and great importance for all aspects of research in plant sciences such as morphogenesis, physiology, ecology, taxonomy, evolution, genetics, reproduction, etc. (Fahn, 1990). The macroscopic and microscopic description of a plant as employed in this study, is the first step towards establishing the identity and purity of the plant and should be carried out before any tests are undertaken (Anonymous, 1996). Correct botanical identity based on external morphology is possible when a complete plant is available (Jainab and Kensa, 2018) while anatomical characters as employed in this paper can as well help species identification when morphological features are indistinct (Cutler et al., 2008). According to Jayeola et al. (2009), identification using morphological evidences posed a threat, while scientific diagnosis calls for a very sound knowledge of anatomical structures.

Since very little or no studies have been conducted on leaf and petiole anatomy of these four species of *Combretum*, this paper therefore tends to providing useful taxonomic information to the plant identification problems especially when the reproductive morphological means of identification becomes unavailable. The systematic vegetative anatomy as carried out in this research is also aimed at relating structures particularly of vegetative organs of the genus *Combretum* to taxonomic identification and classification of the species in which the characters are exemplified. The representative species were selected for the study based on the confusion in their correct identities, paucity of information into the leaf and petiole anatomical status of each of the species and their conflicting taxonomic delimitations, their large ecological distribution, their importance in Pharmacopeia, wood fuel and other economic importance (Thiombiano, 2005). On the other hand, the research also serves a complementary purpose to previous investigations of Oladipo et al. (2016),

Akinsulire et al. (2018a) and Akinsulire et al. (2020) in the family Combretaceae.

2. Materials and methods

2.1 Study area and target species

This research was carried out in southern Nigeria. Healthy and matured plant samples of the four *Combretum* species namely: *C. platypterum* Hutch. and Dals, *C. zenkeri* Engl. and Diels., *C. racemosum* P. Beauv. and *Combretum dolichopetalum* (syn. *C. comosum* var. *dolichopetalum* (Engl. and Diels) Jongkind), were collected during regular field trips to various parts and localities in Ondo, Osun and Edo States all in southern Nigeria, while all sites of collection were geo-referenced using a GPS device (Garmin nuvi 2597LMT) (**Table 1**). Samples were identified by standard reference text (Panshin and DeZeeuw, 1964; Hutchinson and Dalziel, 1964-72) and were later used in obtaining corresponding botanical names in the Herbarium (IFE). The leaves were harvested from all parts of each of the plants, which were totally exposed to the sun and none of the plants were in shadow area. One to four accessions were collected for each of the species from different localities (**Table 1**), while ten matured leaves from different parts of each of the accessions where harvested for leaf and petiole micro-anatomical investigations.

2.2 Leaf and Petiole Anatomy

As reported by Akinsulire et al. (2018a) that the four members of the genus *Combretum* considered in this paper are subsessile, consequently, only the transverse sections of petioles of the four taxa were studied, together with their leaves. Leaf anatomy was studied using sizeable portions from the leaves which were taken from the standard median portion from each sample, while petiole anatomy of the taxa was investigated by selecting matured petiole samples from the accessions. Transverse sections of all leaf and petiole samples were obtained as still fresh, embedded in paraffin and sectioned using Reichert Microtome (Reichert Austria Nr. 367 019) and at a thickness of 8 to 10 µM. The sections were later processed, stained in Safranin O solution for 3 to 5 minutes, rinsed with 4 to 5 changes of water to remove excess stain and counterstained in Toluidine Blue solution for 3 to 5 minutes. The sections were again rinsed thoroughly with 4 to 5 changes of water and treated in series of ethanol dilution-50%, 70%, 80%, 90% and 100% to enhance dehydration process. The dehydrated sections were then transferred into absolute xylene to remove any remaining trace of water and ethanol. These made the sections clearer and prevented cloudiness of the slides. Sections were therefore mounted in 25% glycerol containing thymol crystals (to prevent fungal attack) on a clean glass slide, and covered with microscope cover slip, for light microscopy (Sonibare et al., 2014; Ogundare and Saheed 2015; Akinsulire et al., 2018b; Jainab and Kensa, 2018; Priya and Hari, 2018).

2.3 Light Microscopy

For each species, micro-anatomical parameters such as thickness of upper cuticle, thickness of upper epidermis, thickness of palisade layer, thickness of spongy layer, thickness of lower epidermis as well as thickness of lower cuticle and other micro-anatomical characteristics were investigated and documented both quantitatively and qualitatively while thirty replicates (n = 30) were taken for each of the quantitative anatomical character (including trichomes) investigated. Descriptive terminologies were also carried out while list of microscopic features were also made.

All slides were observed (at magnification of X40) using light microscope (Leica Galen

III) equipped with calibrated ocular eyepieces (square and linear micrometres) to enable the measurement of leaf and petiole cell parameters and to enable recording cell and tissues dimensions.

Photomicrographs of all anatomical and diagnostic features were made with the aid of Accu-scope Trinocular Microscope (Accu-scope 33001 LED Trinocular Microscope fitted with 3.2 MP CMOS Digital Camera).

2.4 Statistics

All quantitative parameters were measured using ocular micrometer and the measurements were converted to microns using the stage micrometre and ocular constant with respect to the objective with which the measurements were taken. The data were computed and were analyzed using One Way Analysis of Variance (ANOVA), while means were separated using Tukey's Honestly Significant Difference (HSD). Multivariate Statistical Analysis, Principal Components Analysis (PCA) and Cluster Analyses were also carried out on the data using Paleontological Statistics Software Package for Education and Data Analysis (Hammer et al., 2001).

2.5 Dichotomous Key

An artificial key for the identification of members of the genus was constructed using taxonomic information generated from the leaf and petiole micro-anatomical investigations.

Table 1. Sites of collection and coordinates of the four species of *Combretum*

Species/Accession	Site of Collection	Collector(s)	Coordinate
C. platypterum	International Secondary School, Road 7, OAU, Ile-Ife, Osun State, Nigeria	B.E Omomoh and O.P Akinsulire	N07˚31.205′ E004˚32.086′
C. platypterum	Imole-Ayo Street, Ajagbale Road, Oka – Ondo, Ondo State, Nigeria	O.P Akinsulire	N07˚4.769′ E004˚49.9642′
C. platypterum	Beside Asikolaye lodge, Apata II, Ile-Ife, Osun State, Nigeria	O.P Akinsulire	N07˚27.364′ E004˚33.512′
C. platypterum	Prince Olu-Adegbite Street, Oka-Ondo, Ondo State, Nigeria	O.P Akinsulire	N07˚3.9667′ E004˚50.25
C. zenkeri	International Secondary School, Road 7, OAU, Ile-Ife, Osun State, Nigeria	B.E Omomoh and O.P Akinsulire	N07˚31.238′ E004˚31.958′
C. zenkeri	Beside New Bukka, OAU, Ile-Ife, Osun State, Nigeria.	O.P Akinsulire	N07˚31.027′ E004˚30.811′
C. racemosum	Biological Garden, OAU, Ile-Ife, Osun State, Nigeria	B.E Omomoh and O.P Akinsulire	N07˚31.351′ E004˚31.410′
C. racemosum	Awo Community, Ejigbo Road, Ede, Osun State, Nigeria	B.E Omomoh and O.T Oladipo	N07˚46.265′ E004˚24.320′
C. racemosum	Adeyemi College of Education Road, Lipakala, Ondo, Ondo State, Nigeria	O.P Akinsulire	N07˚4.7206′ E004˚49.4362′
C. dolichopetalum	Uromi Town, Edo State, Nigeria	O.T Oladipo, B.E Omomoh and A.J Akinloye	N07˚46.26′ E004˚24.32′

OAU: Obafemi Awolowo University

3. Results

Qualitative results are presented below with reference to corresponding figures and photomicrographs while a list of important qualitative leaf and petiole micro-anatomical characters are presented in **Table 2**, as **Table 3** shows the species' significant differences in cells and tissues dimensions of the leaves and petioles with Tukey's (HSD). **Table 4** provides the information regarding the Principal Components Analyses (PCA) Factor Loadings.

Table 2. Qualitative leaf and petiole anatomical characters of four species of *Combretum*

Species Characters	***C. platypterum***	***C. zenkeri***	***C. racemosum***	***C. dolichopetalum***
Leaf Anatomy (Lamina)				
Leaf	Bifacial	Bifacial	Bifacial	Bifacial
Upper Cuticle	Thin	Thin	Thick	Thin
Upper Epidermis	Uniseriate	Uniseriate / Biseriate	Uniseriate	Uniseriate
Palisade Mesophyll	1-layered	1-layered	1-layered	1-layered
Lower Epidermis	Uniseriate	Uniseriate / Biseriate / Multiseriate	Uniseriate	Uniseriate
Lower Cuticle	Thick	Thin	Thin	Thin
Trichome/ Trichome Type	-	+ (SUNT, BTR)	-	-
Leaf Anatomy (Midrib)				
Fibre	Absent	Absent	Present	Absent
Vascular Bundle	Collateral	Collateral	Collateral	Collateral
Vascular Architecture	Falcate	Falcate	Falcate	Falcate
Petiole Anatomy				
Outline	Oval/ Grooved	Triangular/ Grooved	Oval/ Grooved	Oval/ Grooved
Vascular Bundle	Collateral	Collateral	Collateral	Collateral
Vascular Architecture	Falcate / Open adaxial	Triangular/ Dissected	Circular/ Open adaxial	Oval/ Dissected
Trichome Presence / Type	+ (SUNT)	-	+ (SUNT)	+ (SUNT)

SUNT=Simple Unicellular non-glandular Trichome; BRT=Branched Trichome, + = Presence of a Character, - = Absence of a Character, Bi/Multiser=Biseriate/Multiseriate

3.1. *Combretum platypterum* (**Fig. 1A** and **B**; **Fig. 2A** and **B**)

The transverse section of the leaf was examined and it was revealed that leaf in *C. platypterum* is bifacial with thin upper cuticle and uniseriate epidermis (**Fig. 1A**). The cells of the upper epidermis are majorly rectangular or square, with straight or slightly undulating periclinal walls (**Fig. 1A**). Palisade mesophyll cells are 1-layered and are highly chlorophyllous. The cells are cylindrical in shape and are compact in arrangement, occurring on the adaxial region (**Fig. 1A**). Spongy mesophyll layer is distinguishable from the palisade cells, the cells are largely irregular in shape and are arranged with small to large intercellular air spaces, the cells are generally circular, oval, polygonal or spindle-shaped (**Fig. 1A**).

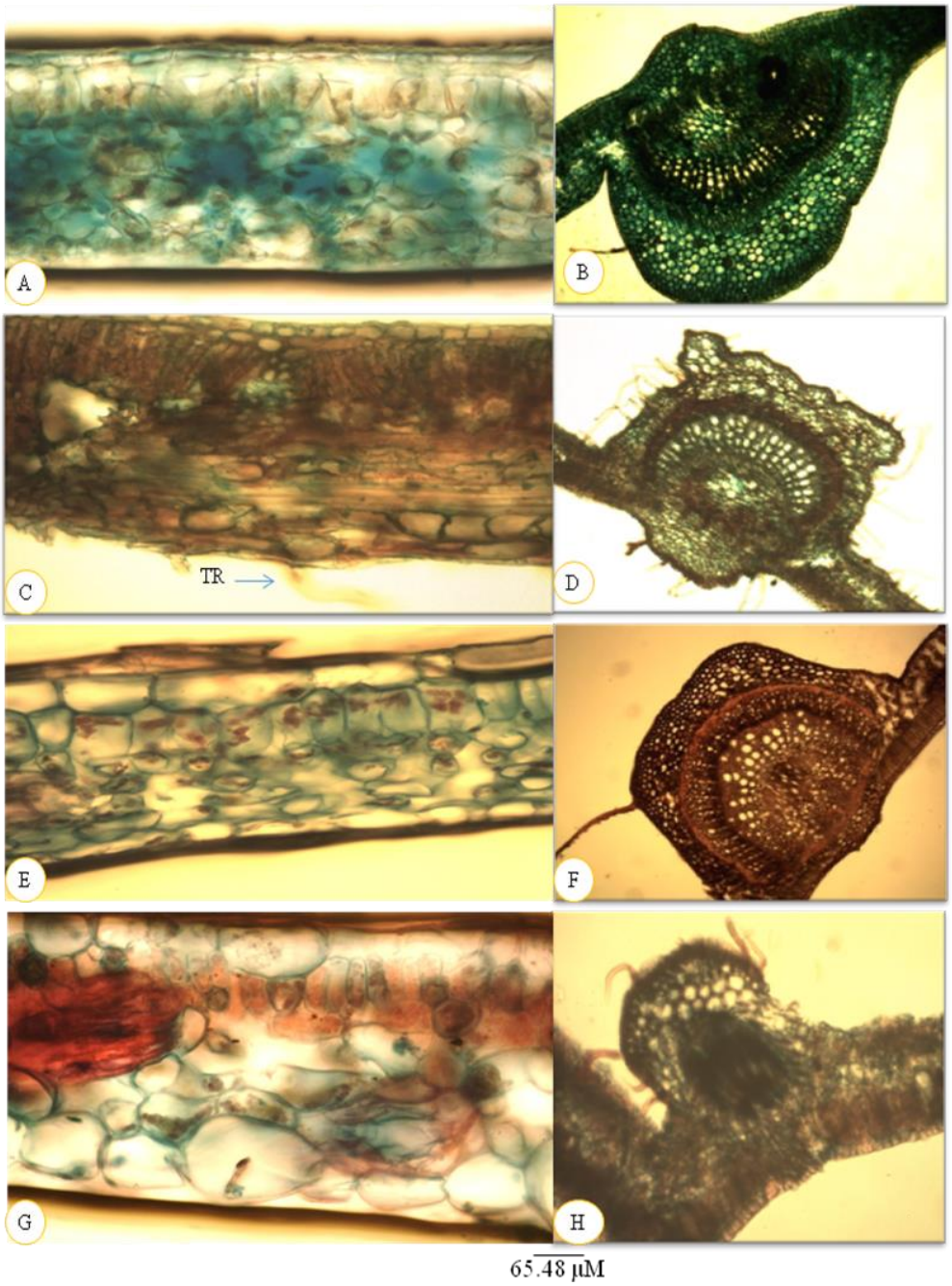

Fig. 1. Leaf anatomy of four species of *Combretum*
A and B = Photomicrographs of lamina and midrib of *C. platypterum*
C and D = Photomicrographs of lamina and midrib of *C. zenkeri*
E and F = Photomicrographs of lamina and midrib of *C. racemosum*
G and H = Photomicrographs of lamina and midrib of *C. dolichopetalum*

Lower epidermis is uniseriate, the cells are rectangular with straight or undulating periclinal walls, lower cuticle is thick (**Fig. 1A**). In the midrib region (**Fig. 1B**), the epidermis is darkly stained and composed of uniseriate row of cells, the vascular bundle is collateral and the vascular architecture is falcate (**Fig. 1B**). Simple, unicellular, non-glandular and unbranched trichomes present on the midrib region but sparsely distributed, trichome density is 0 to1 mm^2 (**Fig. 1B**).

Table 3. Dimensions of cells and tissues of the leaves in four *Combretum* species with Tukey's Honestly Significant Difference (HSD ^{a})

Species	*C. platypterum*	*C. zenkeri*	*C. racemosum*	*C. dolichopetalum*
TUC (µM)	12.00±0.56^{a}	12.00±0.56^{a}	18.00±0.56^{b}	12.25±0.56^{a}
TUE (µM)	34.00±1.12^{a}	52.00±3.21^{c}	45.00±1.15^{b}	44.00±1.12^{b}
TPL (µM)	55.50±1.02^{b}	38.63±0.82^{a}	67.13±1.27^{c}	54.75±0.65^{b}
TSL (µM)	110.00±2.18^{d}	46.35±0.98^{a}	100.69±1.91^{c}	65.70±0.78^{b}
TLE (µM)	24.00±1.12^{a}	59.50±2.35^{c}	24.50±1.14^{a}	34.50±1.14^{b}
TLC (µM)	20.00±0.00^{c}	14.75±0.99^{b}	12.00±0.56^{a}	10.00±0.00^{a}

Values are expressed as mean of thirty replicates (n=30) ± S.E.M (Harmonic mean sample size-30.00)

Values in each row with different superscripts are significantly different ($p<0.05$)

TUC=Thickness of Upper Cuticle; TUE=Thickness of Upper Epidermis; TPL=Thickness of Palisade Layer; TSL=Thickness of Spongy Layer; TLE=Thickness of Lower Epidermis; TLC=Thickness of Lower Cuticle; µM=Micrometer

Table 4. Principal Components Analysis (PCA) factor loadings of some important leaf anatomical characters of species of *Combretum*

	PCA 1	**PCA 2**	**PCA 3**	**PCA 4**
Upper Cuticle Thickness	0.518	0.754	0.404	-
Upper Epidermis Thickness	-	0.567	-	-
Palisade Layer Thickness	0.895	0.437	-	-
Spongy Layer Thickness	0.974	-	-	0.801
Lower Epidermis Thickness	-	-	-	-
Lower Cuticle Thickness	-	-	0.424	-

Petiole (**Fig. 2A and B**): The petiole is oval in sectional outline (**Fig. 2A**) with no wings and slightly grooved. The ground tissues form the outer and the central portions, consisting of thin-walled polygonal parenchymatous cells (**Fig. 2B**). The vascular bundle is collateral and single, surrounded by a thin layer of phloem and a single parenchymatous bundle sheath. The xylem elements are in compact parallel lines with four to six cells in each row (**Fig. 2B**). The vascular system of the petiole is central and the vascular ring is falcate (**Fig. 2A**), embedded in rounded or polygonal conjunctive tissues. Simple unicellular, non-glandular trichomes are present but are sparse in distribution, 0 to 2 mm^2 (**Fig. 2B**).

3.2. *Combretum zenkeri* (**Fig. 1C** and **D**; **Fig. 2C** and **D**)

It was examined in the transverse section of the leaf of *C. zenkeri* that the leaf is bifacial with thin upper cuticle (**Fig. 1C**). Upper epidermis is mostly biseriated, occasionally uniseriated and laterally compressed with straight to undulating periclinal walls and the cells are majorly rectangular or square.

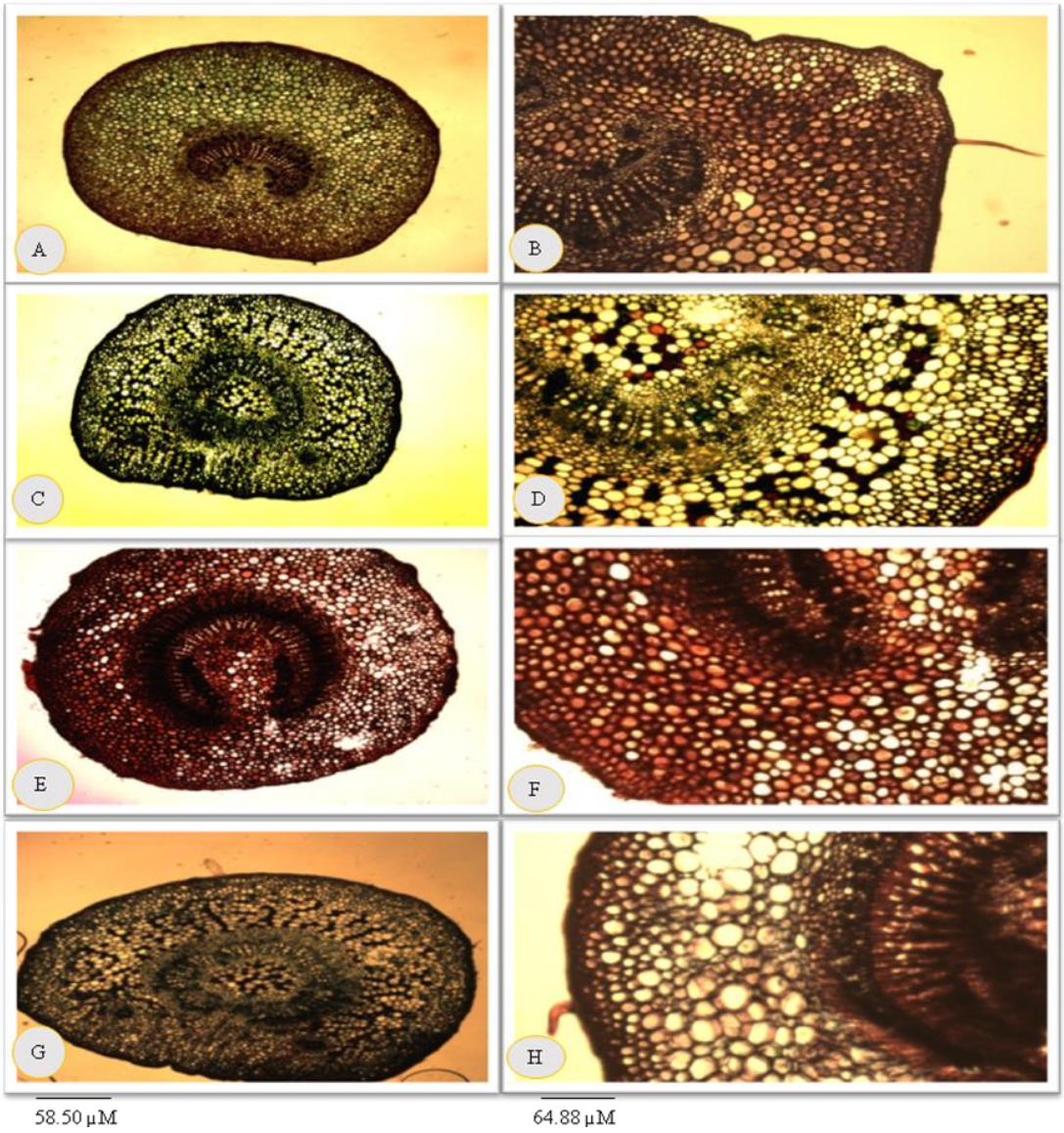

Fig. 2. Petiole anatomy of four species of *Combretum*
A and B = Photomicrographs of petiole of *C. platypterum*
C and D = Photomicrographs of petiole of *C. zenkeri*
E and F = Photomicrographs of petiole of *C. racemosum*
G and H = Photomicrographs of petiole of *C. dolichopetalum*

Palisade mesophyll cells are 1-layered, cylindrical and compactly arranged, occurring in slanting rows and occurred on the adaxial region (**Fig. 1C**). Spongy mesophyll cells are largely irregular in shape and arranged with small to large intercellular air spaces, spongy mesophyll cells are either polygonal or spindle-shaped, lower epidermis is uniseriated, biseriated and multiseriated, the cells are rectangular or polygonal in shape, lower cuticle is thin, simple unicellular trichomes attached to the lower epidermal region (**Fig. 1C**). Anatomical examination of the midrib region (**Fig. 1D**) revealed the darkly stained epidermal cells to compose of small barrel-shaped cells, vascular bundle is collateral, vascular architecture is falcate.

Simple, unicellular, non-glandular trichome present, trichome density is 0 to 6 mm^2, slightly branched trichome also present but very scanty, density is 0 to 1 mm^2 (**Fig. 1D**).

Petiole (**Fig. 2C** and **D**): The petiole is slightly triangular or oval in sectional outline (**Fig. 2C**) with no wings; the epidermal cells are small and darkly stained. The ground tissues consist of about five to seven thick walled cells that are compact in arrangement; other ground tissues also form the outer and the central portions consisting of thin-walled polygonal parenchymatous cells. The vascular bundle is collateral (**Fig. 2D**). The xylem elements are in compact parallel lines with five to seven cells in each row. The vascular system of the petiole is central while the vascular ring is continuous and in the form of a prominent triangular-shape and which is dissected adaxially and embedded in rounded or polygonal conjunctive tissues (**Fig. 2C**). Simple unicellular non-glandular trichomes are present, 0 to 2 mm^2 (**Fig. 2D**).

3.3 *Combretum racemosum* (**Fig. 1E** and **F**; **Fig. 2E** and **F**)

Transverse section of the leaf shows that leaf in *C. racemosum* is bifacial and it was noted that the upper cuticle is thick while the lower cuticle is thin (**Fig. 1E**). The adaxial and abaxial epidermal cells are periclinally elongated. Both surfaces compose of uniseriate rows of cells (**Fig. 1E**), palisade mesophyll cells are 1-layered; cells are highly chlorophyllous and are slightly elongated with cylindrical and compactly arranged cells (**Fig. 1E**). Spongy mesophyll cells are largely irregular in shape and are arranged with large intercellular air spaces, the highly chlorophyllous spongy cells are circular, oval or polygonal in shape (**Fig. 1E**). In the mid-vein region (**Fig. 1F**), epidermal cells are darkly stained, vascular bundle is collateral, vascular architecture is largely falcate, simple unicellular non-glandular trichomes are present on the midrib, trichome density is 1 to 2 mm^2 (**Fig. 1F**).

Petiole (**Fig. 2E** and **F**): The petiole is slightly circular or oval in sectional outline (**Fig. 2E**) with no wings; the epidermal cells are small and darkly stained. The ground tissues consist of about five to seven thick walled cells that are compact in arrangement (**Fig. 2F**); other ground tissues form the outer and the central portions consisting of thin-walled polygonal parenchymatous cells. The vascular bundle is collateral, vascular architecture is single arched, surrounded by a thin layer of phloem and a single parenchymatous bundle sheath (**Fig. 2F**). The xylem elements are in compact parallel lines with four to eight cells in each row. The vascular system of the petiole is central while the vascular ring is continuous and in the form of a prominent circular shape with an open adaxial (**Fig. 2E**), embedded in rounded or polygonal conjunctive tissues. Simple unicellular non-glandular trichome present, trichomes are sparsely distributed, density is 0 to 2 mm^2 (**Fig. 2F**).

3.4 *Combretum dolichopetalum* (**Fig. 1G** and **H**; **Fig. 2G** and **H**)

Cross-sectional examination of the leaf shows that its upper and lower cuticles are thin (**Fig. 1G**), while upper and lower epidermal cells are largely rectangular in shape and occur in uniseriate rows. The cells of upper and lower epidermis are periclinally elongated (**Fig. 1G**), Palisade mesophyll cells are1-layered and occur on the adaxial region (**Fig. 1G**). The layer composed of compactly arranged cells that are generally cylindrical in shape, containing a large mass of chloroplasts (**Fig. 1G**). Spongy mesophyll cells are largely irregular in shape with small intercellular air spaces (**Fig. 1G**). In the mid-vein region (**Fig. 1H**), the barrel shaped or slightly rectangular

epidermal cells are darkly stained and are uniseriated. Vascular bundle is collateral (**Fig. 1H**), vascular architecture is falcate; structure is almost closed but narrowly opens out adaxially (**Fig. 1H**).

Petiole (**Fig. 2G** and **H**): in cross-sectional outline, the petiole has a broadly oval shape (**Fig. 2G**) with even and smooth outline except on the abaxial region which is somewhat flattened and slightly arched, or slightly ridged and grooved (**Fig. 2G**). The epidermis is uniseriate and darkly stained, consisting of small sized barrel-shaped cells with outer thick walls (**Fig. 2H**). The cortex consists of outer angular collenchyma of three to five layers and inner parenchyma, the parenchyma are rounded or polygonal in shape and are four to eight layered (**Fig. 2H**). The vascular bundle is collateral while the pith comprises of rounded to polygonal parenchyma cells. Vascular ring is slightly oval but dissected and discontinuous with two to three solitary vascular bundles together with the main bundle all along the same plane (**Fig. 2G**), simple unicellular non-glandular trichome present, density 0 to 1 mm^2 (**Fig. 2H**).

4. Discussions

Anatomical line of evidence has always been used in the classification and identification of different plant taxa (Metcalfe and Chalk, 1979; Aguoru and Okoli, 2008; 2012; Ajuru and Okoli, 2013; Ekeke and Mensah, 2015; Agogbua et al., 2015; Ekeke et al., 2015; Ekeke and Agbawa, 2016; Ekeke et al., 2016; Oladipo et al., 2016; Ekeke et al., 2017; Akinsulire *et al.*, 2018a; Akinsulire et al., 2018b; Akinsulire et al., 2018c; Akinsulire et al., 2020). Close affinities based on investigated leaf and petiole anatomical characters were however observed in the four *Combretum* species. It should be noted that collateral vascular bundles observed in the leaves midrib (**Fig. 1B, D, F, H**) and petioles in the four species (**Fig. 2B, D, F, H**) suggest a generic character, useful in the identification of the studied genus.

Other generic features include 1-layered palisade cells (**Fig. 1A, C, E, G**) on the lamina. Meanwhile, simple unicellular non-glandular trichomes on the midribs are classificatory and as well diagnostic for each of *C. platypterum*, *C racemosum* and *C. dolichopetalum* (**Fig. 1B, F, H**) which can as well be employed in species identification purpose. The lamina and midrib anatomy of the four species also showed some clear variations in the natures and arrangements of upper and lower epidermal cells (**Table 2**), thickness of both upper and lower cuticles (**Table 3**), as well as vascular architectures of the midrib (**Fig. 1B, D, F, H; Table 2**) which are of great taxonomic significance and are reported in this plant group for the very first time.

In the lamina, the uniseriate or superimposed biseriate/multiseriate upper and lower epidermis as well as branched trichome in addition to seemingly generic simple unicellular trichomes found in *C. zenkeri* (**Fig. 1C**) is diagnostic and are good taxonomic tools which can be employed in the identification of the taxa as the epidermis in both surfaces in other three species remain uniseriated, while their midrib trichomes remain only simple unicellular (**Fig. 1A, E, G**), hence useful in grouping the taxa.

In the vascular area of the midrib, falcate vascular architecture seemed to be generic and can be used for grouping the taxa (**Fig. 1B, D, F, H**; **Table 2**). On the petiole, triangular and grooved petiole outline and the absence of trichome are diagnostic for *C. zenkeri* (**Fig. 2C**) while other three species can be classified on the basis of their oval and grooved petiolar outlines as well as their possession of trichomes (**Fig. 2A, D, E**; **Table 2**).

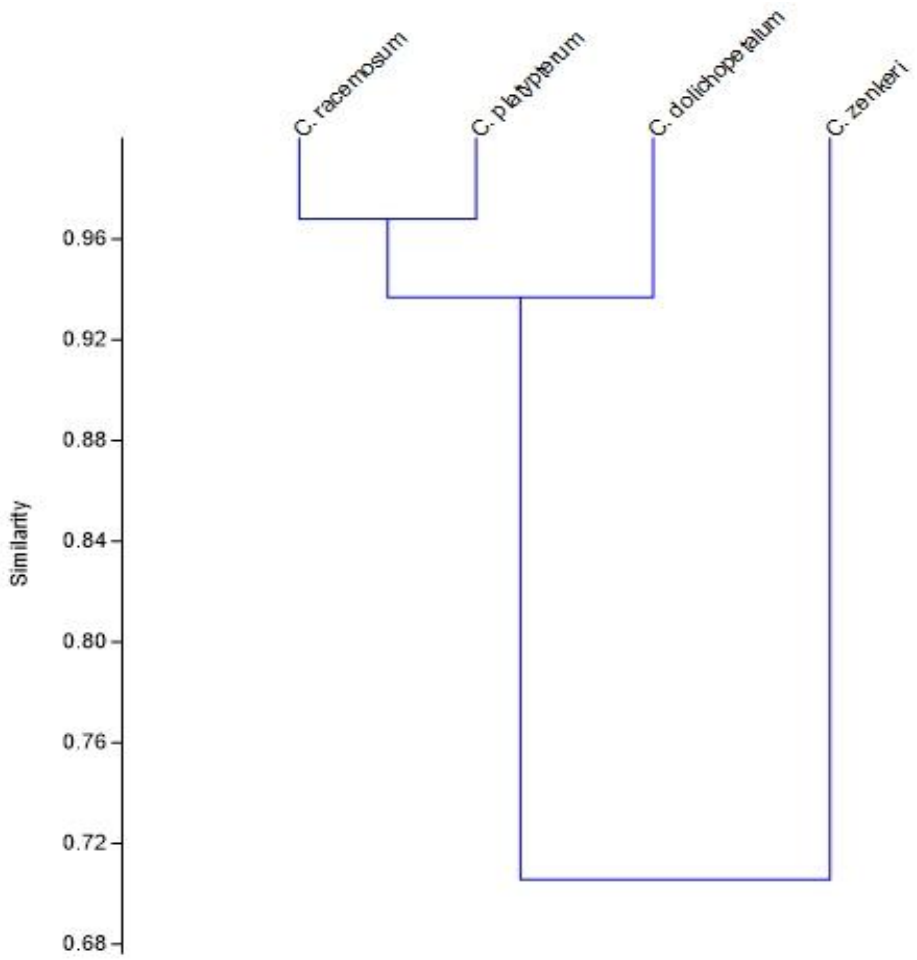

Fig. 3. Cluster analysis (Dendogram) of the *Combretum* species

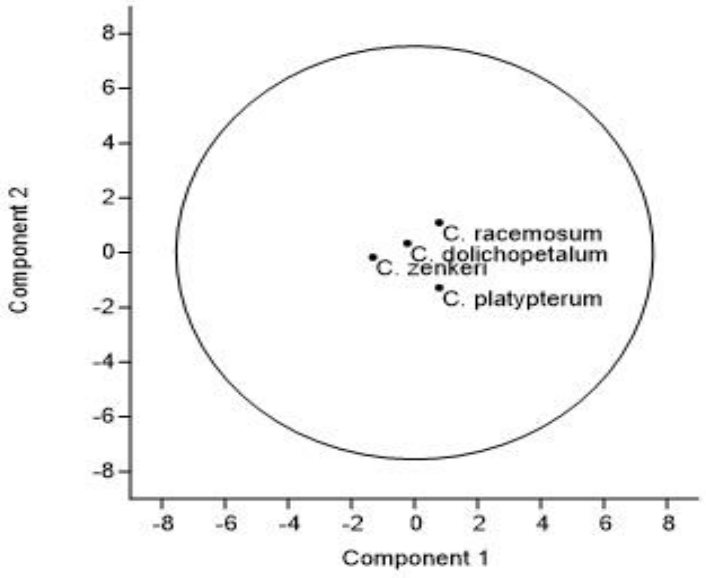

Fig. 4. Scatter plot of four *Combretum* species showing relationships and distances

This revelation partly agrees with recent investigations carried out by Oladipo et al. (2016) using information from wood anatomy in classifying the *Combretum* species under study, but strongly agrees with Akinsulire et al. (2018a) who as well classified members of this same genus using vegetative and reproductive morphological information. Each of the petiolar vascular architectures is distinct per species (**Table 2**) as it is falcate in *C. platypterum*, triangular in *C. zenkeri*, circular in *C. racemosum* and oval in *C. dolichopetalum*, and is therefore useful taxonomic character useful in identifying each of the species.

Considering the quantitative illustrations and analyses (**Table 3**), *C. racemosum* was revealed to have the thickest upper cuticle and palisade layer among the four taxa, *C. platypterum* possesses the thickest spongy mesophyll layer and lowest cuticle thickness (Table 2), while the highest level of thickness of upper and lower epidermis was documented in *C. zenkeri* (**Table 2**), hence diagnostic for each of the four species respectively, and are good taxonomic tools useful in species identification.

Quantitatively, the relationship between members of the genus and anatomical means by which individual members of the genus could be separately identified were also revealed as significant differences were observed in each of the anatomical characters considered (**Table 3**). However, the dendogram (**Fig. 3**) shows that *C. racemosum* and *C. platypterum*, which formed a clade and were clustered to the highest level, have greater similarities, and thus more closely related while *C. dolichopetalum* and *C. zenkeri* must have shared common ancestry with *C. racemosum* and *C. platypterum*, or both belong in the same haplogroup, hence their generic classification (Hutchinson and Dalziel, 1954).

The scatter plot (**Fig. 4**) revealed *C. racemosum*, and *C. dolichopetalum* to have more generic affinity to *C. zenkeri* than to *C. platypterum*, which is also a good systematic tool for the identification of members of the genus. The PCA factor loadings of the leaf quantitative anatomical characters revealed that characters like thickness of upper cuticle, thickness of upper epidermis, thickness of palisade layer and thickness of spongy layer are all important in separating the four *Combretum* species studied as they had higher loadings (**Table 4**). Moreover, principal components 1, 2 and 4 were important in bringing out the variations in the taxa as other components were increasingly uninformative.

A taxonomic (dichotomous) key based on leaf and petiole micro-anatomical characters of the species of *Combretum* examined in this research, and which is of great taxonomic usefulness in species identification and in separating the species even from their close relatives, is presented below:

1a. Upper epidermal cuticle thick, lower epidermal cuticle thin……..*C. racemosum*

1b. Upper epidermal cuticle thin, lower epidermal cuticle thick

2a. Upper epidermis uniseriate, biseriate or multiseriate…….*C. zenkeri*

2b. Upper epidermis uniseriate

3a.Vascular architecture falcate/open on adaxial side.....…*C. platypterum*

3b.Vascular architecture oval/ dissected/closed on adaxial side……...*C. dolichopetalum*

Conclusions

Investigations into the leaf and petiole anatomy of the species revealed great taxonomic affinity and a range of dissimilarities within the genus as well as unveiling diagnostic characters that can serve delimitation purposes, thereby enhancing the species identification. Considering their anatomical resemblances as even evident in

previous researches into the species' morphology and wood anatomy, the results strengthen the fact that these species belong in the same haplogroup and should be maintained as separate species placed in the same genus. Thus, leaf and petiole anatomical characters as investigated in this study should be taken as important lines of evidence in the identification and classification of these species. The close anatomical affinities observed in these investigations revealed that the species are closely related and therefore, their placement in one genus (Hutchinson and Dalziel, 1954) is strongly supported.

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Dedication

This work is dedicated to Late (Chief) Gabriel Ibhanesebhor, the former curator of IFE Herbarium who played a highly significant role in the morphological identification of the *Combretum* species.

Conflict of interest

The authors have declared that there is no conflict of interest.

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COVID-19: A CENTENNIAL PANDEMIC FROM ORIGIN TO CLINICAL TRIALS

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Abstract: In December 2019, an unexpected interaction of coronavirus with human's occurred for the third time in history after Severe Acute Respiratory Syndrome (SARS) in 2002-2003 and Middle East Respiratory Syndrome (MERS) in 2012. Soon the virus was confirmed as SARS-CoV-2, and the severity of its transmission lead the World Health Organization to declare it as World Pandemic. Due to its highly contagious nature, new methods like social distancing, self-hygiene and quarantine were being adopted by many countries to halt the transmission. Due to the dearth in specific therapeutics and/or vaccines against Coronavirus Disease (COVID-19), a significant thrust in drugs and vaccine discovery was ratified by all the nations. The current review comprehensively details about the emergence and molecular pathogenesis with an interesting timeline which notes all the major events during this crisis. Given the potential general readers and health workers, the symptoms and diagnostic approaches were simplified. Emphasis was given to therapeutic approaches and clinical trials section to support the translational research and to cope up with the viral outbreak.

Keywords: COVID-19, Coronavirus, Clinical trials, Pandemic, Infection.

1. Introduction

It all started with the rise in pneumonia-like cases from the hospitals located in Wuhan, Hubei province, China. The transmission of this was identified from a local Huanan Seafood wholesale market, which is notoriously known for its selling of live and wild animals which were considered a delicacy in many Chinese houses. The market was closed and the researchers soon identified the novel coronavirus. The suspension of Chinese The other coronavirus family members, which caused well-known diseases, are the Middle East Respiratory Syndrome (MERS) and Spring Festival by the authorities and quarantining the Wuhan city and later the entire Hubei province has helped to stop the further transmission of viral disease. On February 11th 2020, the pandemic was named as Coronavirus Disease 2019 (COVID-19 Disease) by the WHO which was also classified as "SARS-CoV-2" (Severe acute respiratory syndrome-coronavirus-2 virus) by the International Virus Classification Commission (Zhou et al., 2020). Severe Acute Respiratory Syndrome (SARS) (Wit et al., 2016). By June 9th 2020, at least 188 countries and territories reported COVID-

19 cases with worst-hit countries being the United States of America, Brazil, Russia, Spain, UK, India, Italy, Peru and Germany and the numbers dynamically changing day by day (Worldometer Corona, 2020). Being a developed nation with a highly efficient health care system, the USA recorded the highest cases and fatalities and is leading. It is followed by Brazil, Russia, Spain and UK (**Fig. 1**) (Worldometer Corona, 2020). Although, China has managed to reduce the number of active COVID-19 patients by following stringent quarantine measures, it is facing wide criticism for the projected number of infected patients. In this review, we attempted to explain the origin, evolution and molecular pathogenesis of the virus, with more emphasis on treatment strategies and clinical trials. The below information is based on the recent advancement in the research on COVID-19.

Origin

By the late November 2019, the preliminary reports about COVID-19 were noticed from the local hospitals in Wuhan, China. Several pulmonologists identified the disease as virus-initiated pneumonia based on clinical symptoms and lung radiographs. Preliminary epidemiological studies linked all the cases to their exposure to local Huanan seafood market.

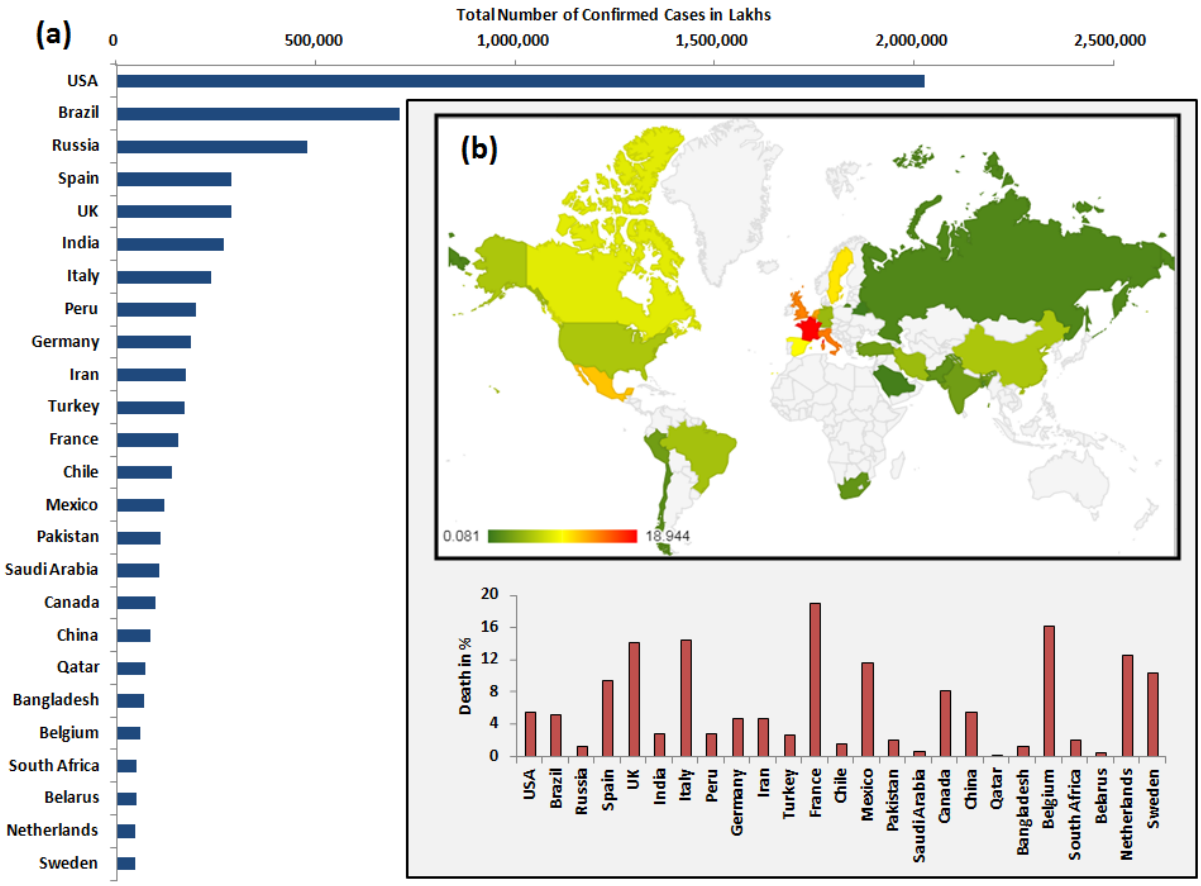

Fig. 1. Representation of world's top 25 worst hit countries by SARS-CoV-2 as of May 15, 2020. (a) Bar diagram showing the total number of cases. (b) World map (upper panel) and bar diagram (lower panel) representing the % of death in top 25 countries (Worldometer Corona, 2020)

This link was confirmed by the Chinese Centre for Disease Control and Prevention, which could successfully isolate and identify the SARS-CoV-2 from the environmental samples of Huanan seafood market. Few different strains of coronavirus were previously identified a few months earlier from the same market (Xiong et al., 2020). The bronchoalveolar lavage fluid was first used to isolate SARS-CoV-2 from three patients with clinical symptoms from Wuhan Jinyintan Hospital (Zhu et al., 2020) and the virus was identified after its genome sequencing and evolutionary tree analysis (Zhou et al., 2020). According to the recent classification developed by *Coronaviridae* Study Group (CSG) of International Committee on Taxonomy of Viruses, there are two subfamilies five genera, 27 subgenera and 39 species which comes under the family of *Coronaviridae* with suborder, order and realm, *Cornidovirineae*, *Nidovirales* and *Riboviria* respectively (Gorbalenya et al., 2020). The positive sense single stranded RNA, SARS-CoV-2 belongs to the genus Betacoronavirus and subgenus Sarbecovirus (Gorbalenya et al., 2020). Genome-wide phylogenetic analysis revealed the sequence similarity between the coronaviruses with the recent SARS-CoV-2. It almost shares 79.5% similarity with SARS-CoV and 50% similarity between MERS-CoV (Lu et al., 2020; Zhou et al., 2020; Zhu et al., 2020).

The highest sequence similarity of 94.6% between the SARS-CoV-2 and SARS-CoV was observed in seven conserved replicase domains of ORF1ab which indicates that SARS-CoV-2 belongs to the Sarbecovirus of β-CoV's (F. Wu et al., 2020). The genomic size of SARS-CoV-2 virion is about 29.9 kb (Guo et al., 2020). The genomic RNA, along with phosphorylated nucleocapsid, is surrounded by the phospholipid bilayers. Two different spike proteins – the spike glycoprotein trimmer (S) and hemagglutinin-esterase (HE) are embedded in the phospholipid bilayer. The S protein is present in all coronaviruses, and HE exists in only a few kinds of CoVs. The envelope (E) protein and membrane (M) protein exist along with spike protein (F. Wu et al., 2020).

Evolutionary Insights

Bats are the known natural hosts for all the coronaviruses discovered until now, which infect humans (Vijaykrishna et al., 2007). During 2002 SARS pandemic, civets were pointed out to be natural suspects of human transmission, but later they were known to be intermediate hosts. A vast reservoir of genetically diverse coronaviruses was identified in Chinese rhinolophid bats in Yunnan Province, China. This led to the conclusion that bats are the natural hosts for many coronaviruses including SARS-CoV-2 which showed high sequence similarity of 96% with RaTG13 bat coronavirus (Lau et al., 2005; Ge et al., 2013; Corman et al., 2018; F. Wu et al., 2020). It may be either transmitted due to the eating habits of few people or through an intermediate animal host which is in close contact with humans. The SARS-CoV might undergo mutation or recombination in these animal hosts in order to survive and transmit among humans. At whole-genome level, the sequence similarity between SARS-CoV-2 and Pangolin CoV is 72% which designates the status of pangolins as intermediate hosts of COVID-19 (Lam et al., 2020).

Further studies on tracking the sequence identity of CoV's between multiple species will help us in better understanding of the disease. The initial genome sequencing from the clinical samples of 10 subjects revealed no variation with 99.98% sequence similarity (Lu et al., 2020; Zhou et al., 2020). The significant change in variation was observed by Tang et al. (2020a) after examining and comparing 103 genomes of SARS-CoV-2.

He concluded that the SARS-CoV-2 was evolved into L and S types due to severe selective pressure. He also highlighted that the L type might be much aggressive that S type as it underwent higher selective pressure than L type (Tang et al., 2020b). The comparative crystal and atomic binding studies on spike protein in complex with the Angiotensin Converting Enzyme 2 (ACE2) receptor revealed that SARS-CoV2 has higher affinity towards ACE2 receptor than SARS-CoV due to key residue. This is one of the important evolutionary steps which support increased pathogenicity in COVID-19 (Lan et al., 2020).
In SARS-CoV-2, a 380 amino acid substitutions was also observed in various proteins when compared to SARS-CoV, but their significance and impact on pathogenesis is still unclear (A. Wu et al., 2020b). He concluded that the SARS-CoV-2 was evolved into L and S types due to severe selective pressure. He also highlighted that the L type might be much aggressive that S type as it underwent higher selective pressure than L type (Tang et al., 2020b). The comparative crystal and atomic binding studies on spike protein in complex with the Angiotensin Converting Enzyme 2 (ACE2) receptor revealed that SARS-CoV2 has higher affinity towards ACE2 receptor than SARS-CoV due to key residue. This is one of the important evolutionary steps which support increased pathogenicity in COVID-19 (Lan et al., 2020). In SARS-CoV-2, a 380 amino acid substitutions was also observed in various proteins when compared to SARS-CoV, but their significance and impact on pathogenesis is still unclear (A. Wu et al., 2020b).

Epidemiology and Statistics

On December 31st 2019, the WHO was informed about the cluster of cases with pneumonia-like symptoms at Wuhan City, Hubei Province, China. On January 7th 2020, Chinese authorities confirmed the new type of coronavirus which they called novel coronavirus. Other pathogens like SARS, MERS, adenoviruses and other influenza viruses were ruled out for causing pneumonia-like symptoms in Wuhan citizens. On 12th January, WHO with the National Health Commission indicated that the evident outbreak is associated with exposures in one seafood market in Wuhan. The entire city of Wuhan was quarantined on January 23rd, and the lockdown was further extended to other parts of the country and the world. On March 11th 2020, WHO declared COVID-19 as world pandemic but by that time, almost 100 countries with more than 100,000 cases were reported (**Fig. 2**). Furthermore, as of 9th June 2020, a total of 7,238,307 cases and 409,634 fatalities were reported (WHO, 2020). The percentage of death also varied between top 25 leading countries. It shows a minimum of 0.081% in Qatar to 18.94% in France (Worldometer Corona, 2020). This variation observed is governed by various factors like age, sex and other comorbidities a patient exhibit during infection. Other reasons, like the evolution of the virus into a more virulent form, cannot be ruled out (**Fig. 1**).

Epidemiological investigation revealed that the outbreak started from Wuhan seafood market. The early COVID-19 patients in China had a history of visiting the Wuhan's seafood market and later the transmission was through their respiratory droplets (Neeltje van Doremalen et al., 2020). The SARS-CoV-2 viral proteins were also detected from blood and faeces of the infected subjects (W. Wang et al., 2020), but no significant transmission was identified (Aylward, Bruce (WHO); Liang, 2020). According to the recent study, asymptomatic people can also infect the population (Hoehl et al., 2020).

This poses a great threat as the asymptomatic carrier might not know that he/she is the carrier of the novel coronavirus but still can infect others. Once a person's immune system comes in contact with the virus, the body starts showing the symptoms within 2 to 14 days after the onset of infection. Children have the majority of the chances of getting asymptomatic infections; the assessed risk percentage for this is almost close to 15.5%.

The average incubation period in adolescent and younger adults is eight days, and 50% of their family members showed symptoms in 1.4 days after exposure (Liao et al., 2020; D. Wang et al., 2020).

Pathogenesis

Viral infections which belong to Category A and B of influenza viruses are usually responsible for seasonal epidemics causing lung infections (Boktor & Hafner, 2020). Flu viruses are declared as one of the potential bioweapons by Journal of Royal Society of Medicine (Madjid et al., 2003). The spread and severity of coronaviruses are dependent on various indirect factors like error susceptible replicase inducing mutations and multiple recombination episodes in the genetic material of virus (Cui et al., 2019). In case of coronavirus, as soon as it enters the host body, the epitope of S-Glucoprotein (spike protein) attaches to the ACE2 receptors of various organs cells, but primarily with epithelial lining of the upper respiratory tract and then it proceeds towards lower respiratory tract (Xiao et al., 2020).

Fig. 2. Timeline showing the most important events occurred in the world from novel coronavirus outbreak in China untill June 09th 2020 (Original, 2020)

The viral spike protein has two subunits; S1 which determines the host selectivity and cellular tropism through receptor binding domain (RBD) and S2, which mediates viral envelop and cell membrane fusion by heptad repeat (HR)1 and HR2 (Xia et al., 2020). Recent studies also showed that the virus enters through CD147, a novel entry point (Su et al., 2020). ACE2 receptors are enzymatic receptors (Hamming et al., 2004), which are also reported to be present in cells of various vital body parts like stomach linings, kidneys, lungs, renal tissues, etc. So, the cells which have the ACE 2 receptors are more susceptible to the infection. A recent report also showed the presence of virus in testis, which might affect the fertility of patients in their young age (Fan et al., 2020).

After the attachment, the bound ACE2 receptor will be cleaved by transmembrane protease serine 2 enzyme (TMPRSS2) which facilitates the viral entry into the epithelial cells. The viral genome will be uncoated, and the translation machinery of host synthesizes two polypeptides, pp1a and pp1ab (Guo et al., 2020). They encode replicase and other non-structural proteins forming replication-transcription complex which synthesizes subgenomic RNA's encoding viral proteins. The newly synthesized genomic RNA, envelop, and capsid proteins will be assembled, and the virions are released out of the cell via ER and golgi apparatus (Hussain et al., 2005) (**Fig. 3**). This rapid replication creates an enormous interferon and cytokine storm by the host triggering inflammatory response leading to tissue injury and acute respiratory distress syndrome (ARDS) further driving to coma or sudden death (Tetro, 2020).

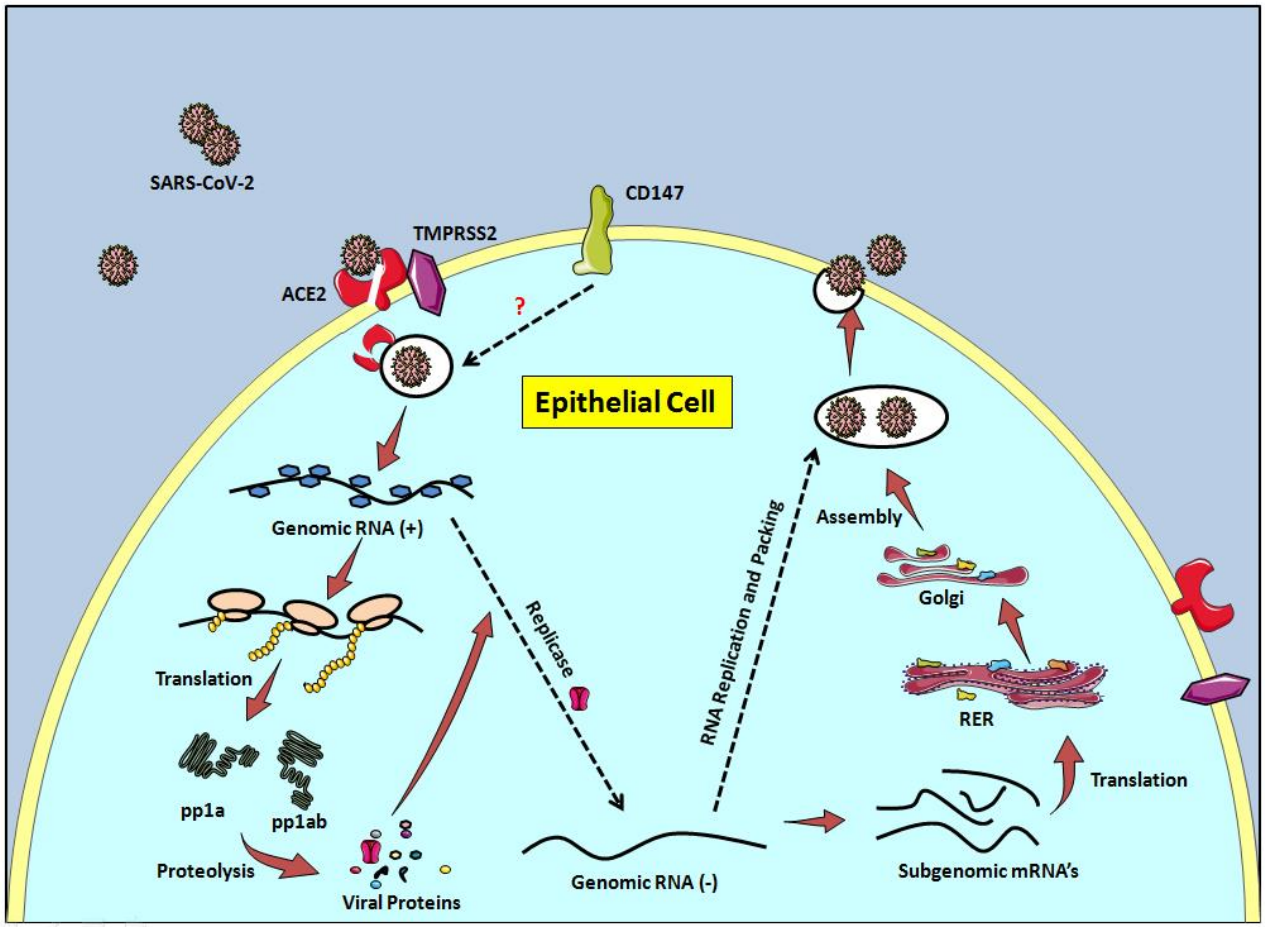

Fig. 3. Graphical representation of life cycle of SARS-CoV-2 in epithelial cells (Original, 2020)

Symptoms

SARS CoV infected around 8000 people worldwide and reported to cause 774 deaths (Cherry, 2004). All the cases reported flu-like symptoms, dry cough, and heaviness in breathing. MERS CoV infection cases were reported in 2012 in Saudi Arabia, and the patients died soon due to respiratory failure (Alraddadi et al., 2016). Novel Coronavirus reported to have much higher efficiency to replicate in the human host with high rate of transmission. It enters the lung cells by crossing the upper respiratory tract and attaches to the ACE2 receptors of host cell using its spike proteins (Tai et al., 2020). Its penetration into the lungs causes pneumonia of unknown etiology. Viruses of this family are known to cause various diseases like enteric, respiratory and neurological illness in different animals as well (Perlman & Netland, 2009; Chen et al., 2020). The disease spectrum varies from asymptomatic condition to clinical conditions like respiratory system failures, which in severe cases needs ventilators leading to fatalities. The mild symptoms include fever, dry cough and dyspnea and atypical pneumonia (Huang et al., 2020). Usually, the disease initiates with difficulty in breathing and chest heaviness. Though the mortality rates are as low as 2-3%, the death is mostly accountable to the patient's hyper response to the viral invasions driving to sepsis (Gyawali et al., 2019). The chances of sepsis and pneumonia increase with pre-existing co-occurring conditions like heart disease, lung infection, cancers and diabetes (Hotchkiss et al., 2016). The foremost reason behind the high risk of a novel coronavirus in these patients is that they already possess a weaker immune system. According to the study on 138 patients of Zhongnan Hospital, Wuhan city, almost 98.6% showed fever, 69.6% showed fatigue and 59.4% were with dry cough. Lymphopenia and higher lactate dehydrogenase levels were also observed in 70.3% and 39.9% patients, respectively (**Fig. 4**) (D. Wang et al., 2020).

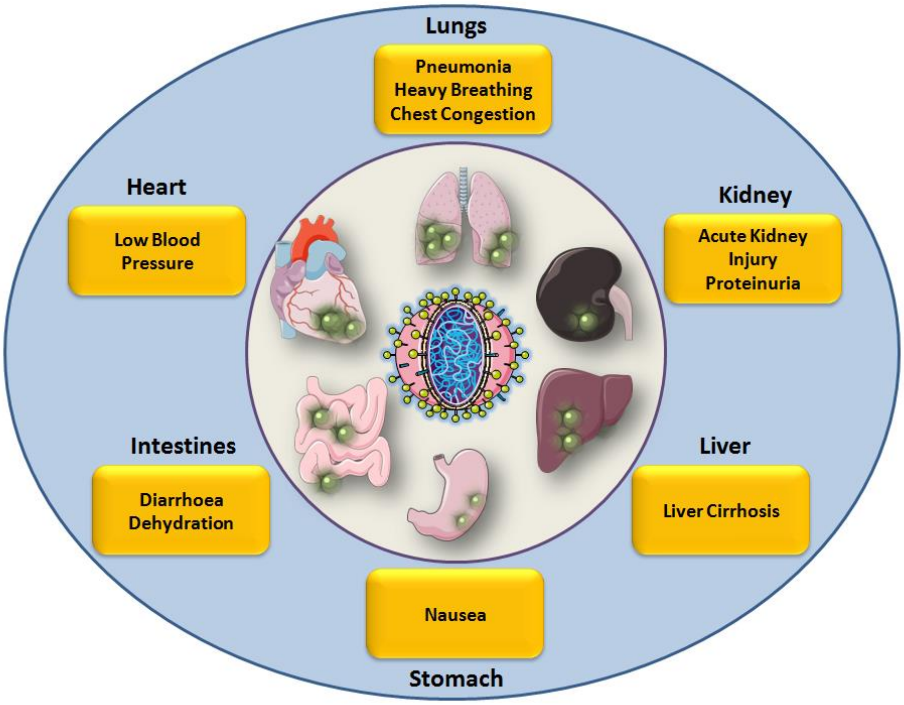

Fig. 4. The pathogenic alterations exhibited by SARS-CoV-2 on various organs and its related symptoms (Original, 2020)

In a conclusive report of CDC (Centre for Disease Control and Prevention, US) about China, the symptoms were divided into the following ways, i.e. mild disease phase (MDP), severe disease phase (SDP) and critical disease phase (CRP). In MDP, the infected person shows the symptoms associated with fever, dry cough and no pneumonia to mild pneumonia in most of the cases. In many cases, diarrhoea and severe stomach ache are reported as the first signs of virus infection (Pan et al., 2020). In the second phase, SDP, the patients acquire health deteriorating symptoms like dyspnoea, higher respiratory frequency of ≥ 30/min, low blood oxygen saturation of ≤ 93%, reduced P/F ratio of ≤ 300mm Hg, and lung infiltrates of > 50% in 48 hrs. This occurred in 14% of the patients admitted in Wuhan hospital by February end. Patients with low immune responses proceed to the third phase (CRP). They undergo respiratory failure, low blood pressure and multiple organ failure due to sepsis/septic shock. This phase was confirmed only in 5% of the admitted cases (Z. Wu & McGoogan, 2020). These patients exhibited a unique pattern of immune deregulation comprising macrophage activation syndrome or depletion of CD4 lymphocytes and NK cells (Evangelos et al., 2020).

Post-mortem samples of heart, lung and liver showed the bilateral diffuse alveolar damage with cellular fibromyxoid exudates. Desquamation or peeling of pneumocytes and hyaline membrane formation indicated ARDS (Acute Respiratory Distress Syndrome). Lung tissues showed pulmonary oedema along with interstitial inflammatory lung infiltrates. Multinucleated syncytial cells with large nuclei indicate viral cytopathic-like changes (Aylward, Bruce (WHO); Liang, 2020).

Diagnosis

After the analysis and careful observation of the symptoms, it is essential to diagnose the disease accurately for confirmation. After proper identification of symptoms like dry cough, fever, chest heaviness and difficulty in the breathing, the specimen (nasopharyngeal and oropharyngeal swabs) are collected for diagnosis from upper respiratory tract. From lower respiratory tract, expectorated sputum, endotracheal aspirates, bronchotracheal lavage were collected for analysis (Aylward, Bruce (WHO); Liang, 2020).

Chest X-ray and CT imaging were used to examine the extent of pneumonia in the chest region. The chest X-ray helps in understanding the status of interstitial lung disease, presence of lung opacity, the accumulation of liquid in lungs, thickening of the pleural membrane which later leads to calcification of pleural membrane, hilar lymphadenopathy which is observed in some of the patients. Mediastinal strips and the cardiac silhouette are also identified by chest X-ray. CT scan helps in assessing the common findings like ground glass opacity, crazy paving in the right upper lobe of the lung, lateral segment of the middle lobe and lesions in the superior and posterior-basal segments of the right lower lobe (Albarello et al., 2020). A molecular technique that is widely used for identification of the viral genome in the infected person is RT- PCR. It is not widely appreciated as the test accuracy is greatly dependent on the experience of the lab personnel, sterile lab environment and type and condition of the specimen being tested (Centers for Disease Control and Prevention, 2020). Due to this limitation, the CFDA has approved a few sequencing and nucleic acid testing kits which depend on quantitative fluorescence. This kit found to be much more accurate than RT-PCR but prone to false-negatives. To address this, rapid nucleic acid test papers for detection within a few minutes was developed. Serological assays include ELISA and microneutralization test (Centers for Disease Control and Prevention, 2020), which mainly

detects the presence of viral protein were also developed (**Fig. 5**). The other test like colloidal gold solution test, CRISPR/Cas13 System test, which was widely used for ZIKA and DENGUE infections (Myhrvold et al., 2018) also gave a result for Corona viral proteins. However, they are currently for the research and surveillance purpose only.

Therapeutic interventions

Treatment for many viral diseases is difficult, and presently, there are no specific drugs to treat this COVID-19 pandemic. There are various effortful treatment strategies proposed all over the world. The most popular treatment is with hydroxychloroquine which was under consideration by the USA. Here we summarize potential treatment options for COVID-19 (**Fig. 6**).

Small Molecules

Currently, the drug which is widely used in clinical trials and also in clinics is hydroxychloroquine. The exact mode of action of this anti-malarial drug is not yet elucidated in the viral scenario, but through the laboratory studies, it is believed to change the pH of the host cell surface halting the viral entry.

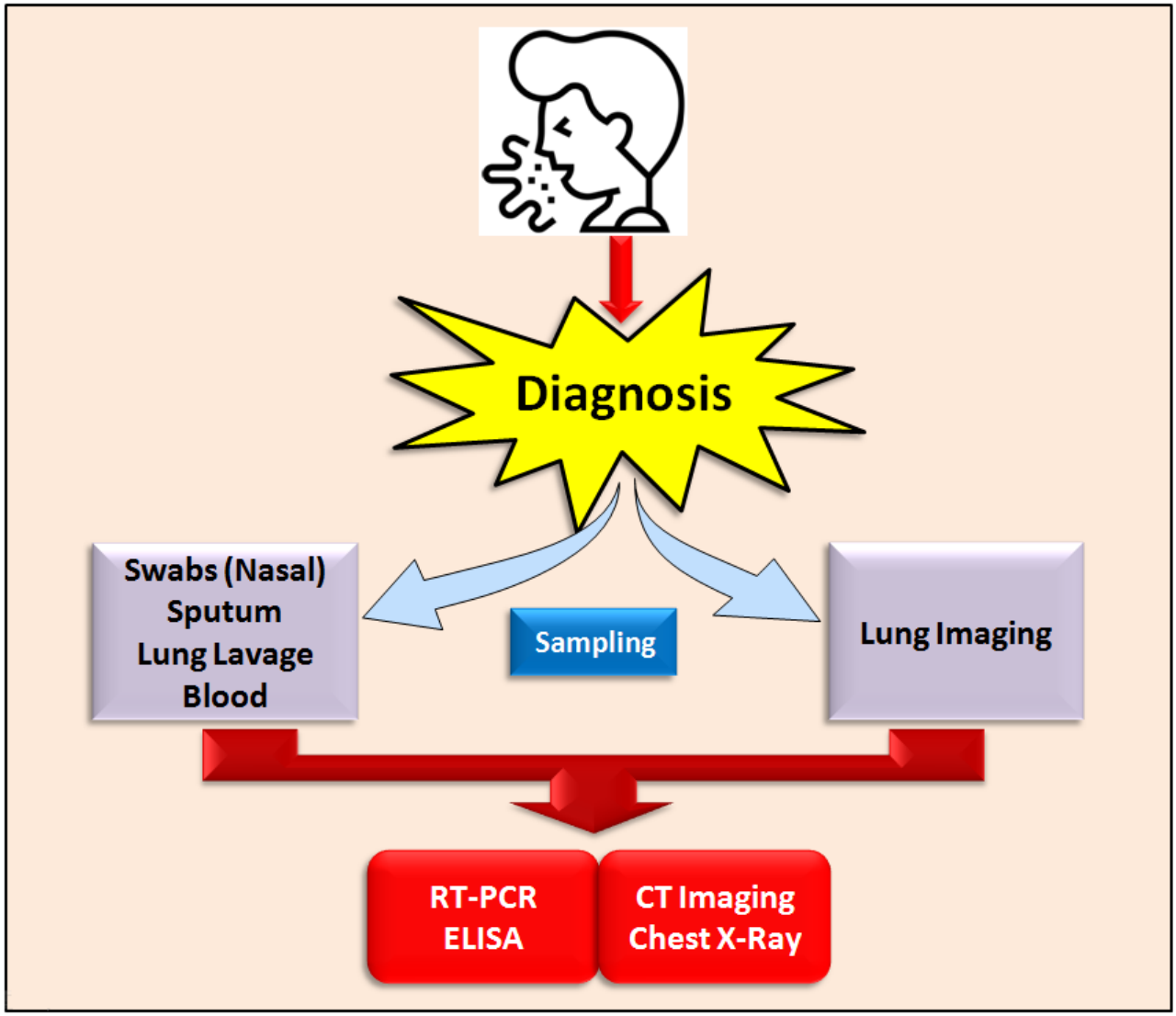

Fig. 5. Representation of current diagnostic techniques used in clinics to detect COVID-19 (Original, 2020)

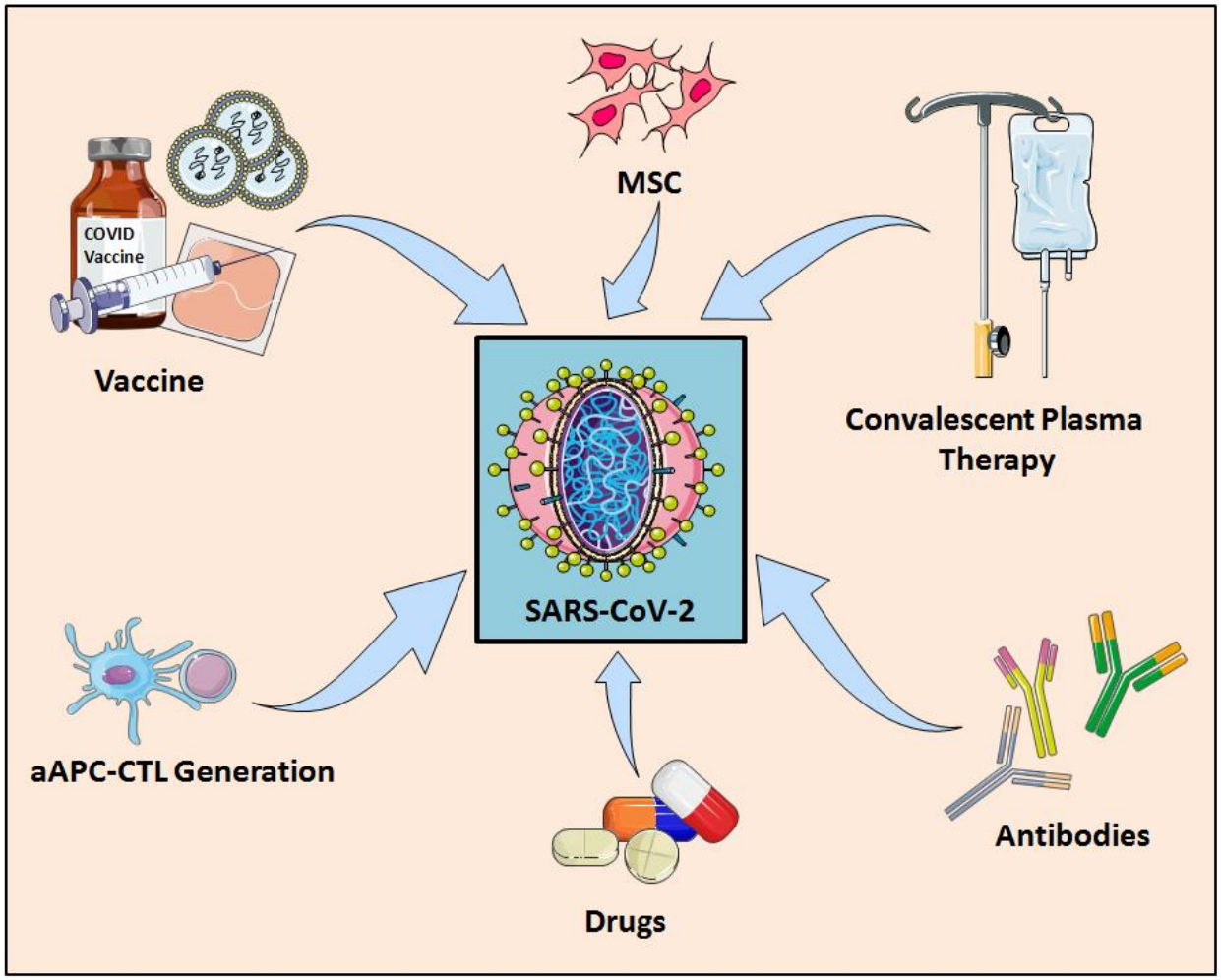

Fig. 6. Graphic representation of various therapeutic intervention methods currently in clinical trials and clinics to treat COVID-19 (Original, 2020)

This is also meant to restriction of viral transcriptomes by inhibiting the nucleic acid replication, thus imparting its antiviral properties (Fox, 1993). Few studies also pointed out its role in controlling cytokine storm in the infected host by its immunomodulatory effect (Yao et al., 2020). Many antiviral compounds which were used during SARS and MERS pandemic were again being considered to treat COVID-19 by many nations across the world. For example, in vivo experiments with remdesiver which has broad spectrum antiviral properties showed its restraining capability on SARS-CoV. It is a nucleotide analogue which inhibits viral RNA polymerases (Grein et al., 2020). Few in vitro studies also showed its antiviral activity in SARS-CoV-2, leading to its usage as a prime drug to the first COVID-19 patient in the USA (Agostini et al., 2018; Sebastian Hoehl et al., 2020; M. Wang et al., 2020). A further efficacy and safety studies should be carried out for its constant usage. A combination treatment of ritonavir and lopinavir is greatly used in the treatment of HIV, which also proved to be effective in MERS and SARS (Chu et al., 2004; Kim et al., 2016). Furthermore, a clinical trial was already started to check its effect on SARS-CoV-2. Ribavirin was widely used during the SARS outbreak in Hong Kong, which can inhibit SARS-CoV replication in vitro synergistically along with IFN-β (Wenzel & Edmond, 2003; Morgenstern et al., 2005).

A serious consideration regarding the usage of ribavirin and IFN-β combination should be done for the SARS-CoV-2 infection also. Chloroquine interferes with ACE2 receptor of host and thereby blocks the viral entry. In vitro studies also showed its effect on viral replication which stands out it as a potent SARS-CoV-2 inhibitor (Yamamoto et al., 2004; M. Wang et al., 2020). Arbidol and its derivative arbidolmesylate are broad-spectrum antiviral agents which are proved to be effective in inhibiting HIV, SARS-CoV and SARS-CoV-2 in vitro, which entered into clinical trials (Jin et al, 2020). Another vital molecule is Nelfinavir which is a selective HIV protease inhibitor. It also showed potent inhibition of SARS-CoV (Yamamoto et al., 2004), which notifies its therapeutic value in COVID-19. As S protein binds to ACE2 receptor of host for the viral entry, the blockade of it and downstream signalling helps in viral restriction. ACE2 is involved in Renin-angiotensin system (RAS) and so ACE1 and AT1R, RAS inhibitors can be potential therapeutic molecules for treating COVID-19. Another drug, danoprevir, also called as Ganovo acts as HCV protease inhibitor. This drug is given in combination with ritonavir which acts as a Cytochrome P450 3A4 (CYP3A4) inhibitor, which in turn increases the plasma concentration of Ganovo. The principal reason behind using this combination of the drug is that the chymotrypsin-like protease of SARS CoV-2 exhibits similarities with HCV and HIV proteases thereby following the similar inhibitory trend. This combination is given to the patient in the presence or absence of interferon nebulisation (Hongyi et al., 2020). When the drug favipiravir, a new RNA-dependent RNA polymerase (RdRp) inhibitor is present in the infected host, it gets converted into active phosphoribosyl molecule. It is recognized as a substrate by viral RNA polymerase, hence inhibiting RNA Polymerase activity (Furuta et al., 2017). Repurposing of few viral RNA synthesis inhibitors like emtricitabine, tenofovir, disoproxil were also under consideration due to their known Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) processes (Harrison, 2020). Another drug which is under the trials is thiolanox, given in high doses to treat patients with ARDS. The controlled trials are being conducted in both paediatric and adult patients to improve their lung oxygen and pulmonary artery pressure. Another fascinating drug, DAS181 (Fludase) is known to cleave the host cell receptors (sialic acid) which support for viral entry. In-vitro studies show immense success against human and Avian flu viruses (Triana-Baltzer et al., 2009). Anakinra (IL-1 inhibitor), in combination with Siltuximab (Anti-IL6 antibody), reduce the cytokine storm syndrome, which occurs in corona patients due to hyperactivity of antibodies (Mehta et al., 2020). Sildenafil, a common Viagra drug is under clinical trials for COVID-19 to open a tiny blood vessel that helps in drawing blood and oxygen in the lungs, thus can get better in respiratory distress (Healy, 2020). Fingolimod, when phosphorylated forms fingolimod-phosphate, which is similar to naturally occurring sphingosine 1-phosphate. It has both anti-inflammatory and anticancerous properties (Lankadasari et al., 2018). It is also under clinical trials for COVID-patients (**Table 1**).

Convalescent plasma therapy:

Another way of treating the COVID-19 is by using the convalescent serum or plasma. The serum is collected from the patients who recovered from the infection and after careful scrutiny for any traces of the virus; the antibodies are extracted and injected into the current patients of the novel coronavirus.

Even though it is still on the trial run but similar attempts was earlier made in the outbreak of 2009–2010 H1N1 influenza, 2013 West African Ebola epidemic (Casadevall & Pirofski, 2020). Earlier in 2003, the utility of convalescent serum was tested in Hongkong on 80 SARS patients who were proved to be the most effective than any other medication (Cheng et al., 2005). Recently many clinics started trying this therapy when a COVID-19 patient is critically ill. So, there is a tremendous hope that even this time, the use of the convalescent may stand out.

Monoclonal antibodies

The monoclonal antibodies (mAbs) are efficient by blocking syncytia formation during SARS-CoV infection (J. Duan et al., 2005). Tocilizumab is an IL-6 antagonist, used in rheumatoid arthritis and it is in clinical trials along with DAS181 to inhibit the SARS-CoV-2 infection. Sarilumab and siltuximab also target the IL-6 pathway by binding and blocking the IL-6 receptor. Scientists are suggesting that it might play an essential role in severe respiratory infections and in combating cytokine release storm (Hodgson, 2020). The antibody, bevacizumab, is currently being used in various cancer treatments. It is a vascular endothelial growth factor (VEGF) blocker which is being studied in COVID-19 patients to deal with the conditions like hypoxia. Interferons are the primary immune regulators during viral infection. The hypersecretion during the viral attack hyperactivates immune system resulting in tissue injury. Emapalumab, an anti-interferon-gamma antibody is used in hemophagocytic lymphohistiocytosis is also in clinical trials with anakinra to restrain the organ failure due to hyperimmune activation (Lounder et al., 2019). The spike protein of SARS-CoV-2 also binds with CD147 to invade the host cell. Usage of meplazumab, an anti-CD147 antibody in the treatment of COVID-19 blocks the viral entry into the cells (Lythgoe & Middleton, 2020) (**Table 1**).

Vaccine

The most effective way to completely eradicate the virus infection is through immunizing people by vaccination. Presently, a race in finding the novel coronavirus vaccine was initiated by many nations. Attention was drawn towards the Bacille Calmette-Guérin vaccine (BCG vaccine) as it presented a clear correlation between the severities of COVID-19 in the BCG vaccinated countries. It is under clinical trials presuming its role in activating immune system during SARS-CoV-2 infection. The usage of messenger RNA (mRNA) molecules to induce their transcription after their delivery into the host cell is also in careful consideration. A clinical trial was initiated already by exploiting the synthetic viral spike protein mRNA (mRNA-1273) encapsulated with nanoliposomes for its safe delivery (Mishra & Carnahan, 2020). Various targets like S protein-RBD structure, adenoviral vector-mediated and epitope-based vaccines are proposed and under clinical trials (Abdelmageed et al., 2020; Xie et al., 2020) (**Table 2**).

MSC Therapy

Mesenchymal stem cells (MSC) can be easily extracted from various sources like UCMs (Umbilical Cord Matrix) cells, adipose tissues and bone marrow. After injecting the MSC's into the patient, they reach the damaged tissue and initiate the repair and regeneration. It has anti-inflammatory and immunomodulatory responses and also helps in immune response regulation towards the infected cell by antigen (Peroni & Borjesson, 2011). This technique is under careful consideration in COVID-19 patients, as most of them show lung injury (**Table 2**).

Table 1. List of ongoing and completed clinical trials of drugs on COVID-19

S. No	Curative Agent	ClinicalTrial.gov ID	Phase	Sponsor/Collaborator
1.	Hydroxychloroquine	NCT04261517	III	Shanghai Public Health Clinical Center
2.	Ganovo + ritonavir +/- Interferon Nebulisation	NCT04291729	IV	The Ninth Hospital of Nanchang, Ascletis Pharmaceuticals Co., Ltd.
3.	Favipiravir	NCT04336904	III	Giuliano Rizzardini
4.	Lopinavir/ritonavir	NCT04330690	II	Sunnybrook Health Sciences Centre,AbbVie
5.	Emtricitabine/tenofovir, disoproxil, Hydroxychloroquine	NCT04334928	III	Plan Nacional sobre el Sida (PNS), Effice Servicios Para la Investigation S.L.
6.	Thiolanox	NCT03331445	II	University of British Columbia, Mallinckrodt
7.	Tocilizumab	NCT04315480	II	Università Politecnica delle Marche Azienda Ospedaliera Ospedali Riuniti Marche Nord
8.	Deferoxamine	NCT04333550	I and II	Kermanshah University of Medical Sciences
9.	DAS181	NCT04324489	NA	Renmin Hospital of Wuhan University Ansun Biopharma, Inc.
10.	Losartan	NCT04335123	I	University of Kansas Medical Center
11.	Tocilizumab Injection	NCT04317092	II	National Cancer Institute, Naples
12.	Sarilumab	NCT04315298	II and III	Regeneron Pharmaceuticals
13.	Anakinra+ Siltuximab+ Tocilizumab	NCT04330638	III	University Hospital, Ghent Belgium Health Care KnowledgeCentre
14.	Sildenafil citrate tablets	NCT04304313	III	Tongji Hospital
15.	Sargramostim	NCT04326920	IV	University Hospital, Ghent Flanders Institute of Biotechnology
16.	Tocilizumab	NCT04331795	II	University of Chicago
17.	Hydroxychloroquine	NCT04333225	II	Baylor Research Institute
18.	Tocilizumab + Azithromycin + Hydroxychloroquine	NCT04332094	II	Fundació Institut de de la Santa Creu i Sant Pau, Instituto de Salud Carlos III
19.	Remdesivir	NCT04292899	III	Gilead Sciences

20.	Bevacizumab	NCT04305106	NA	Qilu Hospital of Shandong University
21.	Fingolimod	NCT04280588	II	First Affiliated Hospital of Fujia Medical University
22.	Dexamethasone	NCT04325061	IV	Dr. Negrin University Hospital Li Ka Shing Knowledge
23.	Methylprednisolone	NCT04273321	NA	Beijing Chao Yang Hospital
24.	Plaquenil	NCT04326725	NA	Istinye University
25.	Emapalumab and Anakinra	NCT04324021	II and III	Swedish Orphan Biovitrum
26.	Camostat Mesilate	NCT04321096	I and II	University of Aarhus
27.	Methylprednisolone	NCT04263402	IV	Tongji Hospital
28.	Baricitinib	NCT04320277	III	Hospital of Prato
29.	Methylprednisolone	NCT04323592	II and III	University of Trieste
30.	Recombinant human interferon Alpha-1b	NCT04320238	III	Shanghai Jiao Tong University School of Medicine
31.	Escin	NCT04322344	II	University of Catanzaro
32.	RoActemra iv, Kevzara sc, RoActemra sc	NCT04322773	II	Marius Henriksen Lars Erik Kristensen
33.	Colchicine	NCT04322682	III	Montreal Heart Institute
34.	Darunavir and Cobicistat	NCT04252274	III	Shanghai Public Health Clinical Center
35.	Meplazumab for Injection	NCT04275245	I and II	Tang-Du Hospital

Table 2. List of ongoing and completed clinical trials of vaccines and biological molecules on COVID-19

S. No	Vaccine	ClinicalTrial.gov ID	Phase	Sponsor/Collaborator
1.	Recombinant Novel Coronavirus Vaccine (Adenovirus Type 5 Vector)	NCT04313127	I	CanSino Biologics Inc., Institute of Biotechnology, Academy of Military Medical Sciences. PLA of China,Tongji Hospital
2.	Allogeneic human dental pulp stem cells	NCT04336254	I	Renmin Hospital of Wuhan University Beijing SH Bio-Tech Corporation,
3.	WJ-MSCs	NCT04313322	I	Stem Cells Arabia
4.	BCG Vaccine	NCT04328441	III	UMC Utrecht, Radboud University
5.	MSCs	NCT04288102	II	Beijing 302 Hospital
6.	Pathogen-specific aAPC	NCT04299724	I	Shenzhen Geno-Immune Medical Institute
7.	LV-SMENP-DC vaccine and specific CTLs	NCT04276896	I and II	Shenzhen Geno-Immune Medical antigen-Institute
8.	CAStem	NCT04331613	I and II	Chinese Academy of Sciences
9.	UC-MSCs	NCT04269525	II	ZhiYong Peng Tuohua Biological Technology Co. Ltd
10.	mRNA-1273	NCT04283461	I	National Institute of Allergy and Infectious Diseases (NIAID)
11.	NK Cells	NCT04280224	I	Xinxiang medical university

aAPC therapy

Re-educating immune cells to the viral proteins before the infection is one the method being tested under clinical trials. In this process, the critical and conserved viral genes like protease and structural genes will be engineered as minigenes. They will be transfected into antigen-presenting cells (APC) such as dendritic cells to generate artificial APCs. They will be used to activate cytotoxic T cells in vitro or will be directly injected into the patient after inactivating their proliferative capacity (**Table 2**).

Conclusions

"The word pandemic must be used very carefully as it creates the fear amongst the populations of the world"- A statement by WHO when SARS-CoV-2 was declared as a pandemic. Today, with all the highly sophisticated scientific knowledge, we are stuck dealing with an novel coronavirus, which invaded almost all the parts of the world. The initial screenings on bronchoalveolar lavage of three infected patients identified it as SARS-CoV-2, which was closely related to its β subfamily members, i.e. SARS-CoV and MERS-CoV. They are known to exhibit a similar kind of response to the vital organs of the host body. Chinese government initially managed to conceal its zoonotic nature from the rest of the world, but in a few days, it infected a large number of populations in Wuhan, China. Sooner, due to migration of the people including asymptomatic carriers, it reached other countries, triggering a pandemic. On further investigation, scientists suggested that the transmission is through respiratory droplets of the infected subjects.

The role of the immune system in COVID-19 is inevitable. The stronger the immune system, the quicker the patient heals, but, if a person is suffering from any preoccupied disease or smoking, the infection might complicate. The patient might suffer from a sudden lowering of blood pressure, ARDS and in some cases causing diarrhoea like conditions. The genetic drift promotes the evolution of the virus and tracking the viral strains by genome sequencing in all counties help us in understanding the progression and severity of the disease. Presently, the confirmation of the virus was done using RT-PCR and CT scan of lungs which are mostly unavailable in rural parts of the developing and underdeveloped nations. Multiple ongoing research and development projects should be encouraged at the lab scale to come up with diagnostic tools to detect the disease much earlier, cheaper and accurate. Usually, chest X-ray facility is available rurally, and instant results can be obtained in a short span, but manual analysis by radiologists creates ambiguity. The exploitation of artificial intelligence and machine learning for robust analysis of X-ray data should be coded and made available free to achieve precise results (Mei et al., 2020). If this is achieved, a screening program should be initiated by the government from urban to local community level to test all the citizens and separate those who show symptoms.

Currently, as there is no specific treatment for COVID-19, the first line of treatment is to quarantine the patient. Various drugs like hydroxychloroquine and monoclonal antibodies which halt the viral activity are on clinical trials. A deep understanding of the viral pathogenesis helps us in discovering new pathways which can be targeted using drug repurposing should be encouraged. A balanced promotion of basic research to understand the virus along with the translational work is necessary at this critical time to combat the disease.

Ultimately, transparency in reporting cases, self-hygiene and social distancing are crucial in controlling COVID-19 as per WHO guidelines.

Conflict of Interest

The research was conducted in the absence of any commercial or financial relationships. The authors declare no competing interest.

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DEVELOPMENT AND VALIDATION OF AN LC-MS/MS METHOD FOR PROTODIOSCIN IDENTIFICATION AND QUANTIFICATION IN HERBAL SUPPLEMENTS WITH *TRIBULUS TERRESTRIS* L.

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Abstract: *Tribulus terrestris* L. (TT) became a very popular plant due to its effects in sexual disorders. The pharmacological actions seem to be related with its content in sterolic saponins. Protodioscin has been reported to be the main compound from TT products with anabolic effects. Because of the popularity of the plant, numerous herbal supplements are consumed around the world. Quantification of steroidal saponins through HPLC methods is sometimes difficult. Several methods have been proposed but not all are reproducible. The aim of this study was to validate a LC MS/MS method for quantification of protodioscin in herbal supplements. Two herbal supplements found on the Romanian market have been chosen for this study. The compounds were separated on a C18 column with ammonium acetate buffer and acetonitrile as mobile phase. The ionization was performed in electrospray negative mode and detection of protodioscin was made by monitoring the sum of ions *m/z 737.41*, *m/z 739.42* and *m/z 755.42*. In one of the herbal supplements, protodioscin was under the limit of quantification. In conclusion a simple and rapid method is proposed for the quantification of protodioscin in herbal supplements.

Keywords: *Tribulus terrestris*, protodioscin, LC-MS/MS.

1. Introduction

Tribulus terrestris L. (Zygophillaceae family) is a popular plant that received great interest in the last decade. Because of its content in steroidal saponins the extracts from *Tribulus terrestris* (TT) are consumed as anabolic and sexual enhancers (Zhu et al., 2017). Protodioscin is thought to be responsible for the main pharmacologiocal actions of TT products, although contradictory results have been obtain in the recent years regarding the pharmacological effects, especially the anabolic effect (Neychev & Mitev, 2016). This suggest either that the herbal drugs collected from different regions contain significantly different compounds, or that the experiments were performed with extracts with different organs from TT.

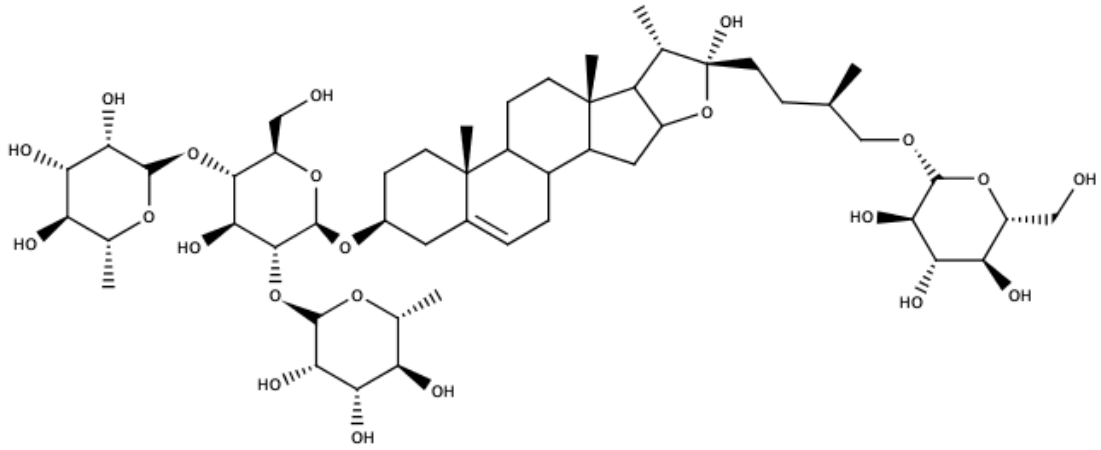

Fig. 1. Chemical structure of Protodioscin

However, due to the geographical influences, the chemical composition of TT products varies in large limits (Dinchev et al., 2008). As a response to the high demand of TT extracts, the availability and variability of herbal supplements increased with the risk of quality diminishment. Standardization and quality controls are necessary for the safety and phytoequivalence of the supplements. Today, LC–MS/MS is the most used technique in bioanalysis, and offers numerous possibilities for sensible detection of chemical compounds found in very low concentrations. Although there are some published papers with validated HPLC-DAD methods for protodioscin identification, these are hardly reproducible (Ganzera et al., 2001).

The aim of this present study was to develop a new, simple and rapid LC MS/MS method for protodioscin quantification in herbal supplements.

2. Materials and Methods

Plant material

Tribulus terrestris products were bought from a local pharmacy. The first product had its origin in Poland, and the second one was from China. The voucher specimens were deposited at the herbarium of Pharmacognosy and Phytotherapy, George Emil Palade University of Medicine, Pharmacy, Science and Tehnology of Târgu Mureș, Romania (TT-1.2-17 and TT-2.2-17).

Chemicals and reagents

Protodioscin was obtained from Cayman Chemical. HPLC grade Acetonitril was obtained from Scharlau. Water was obtained from a Millipore Direct-Q water purification system (Millipore, Bedford, MA, USA). Other reagents used were of analytical grade.

LC MS/MS analysis

LC MS/MS analysis was performed using a QTOF 4600 (AB Sciex), UHPLC Flexar FX-10 (Perkin Elmer). Chromatographic separation of protodioscin was performed on a Phenomenex Luna C18, 4.6 x 100mm, 3 µm column. The mobile phase consisted of a mixture of 70% 1mM ammonium acetate buffer (phase A) and 30% acetonitrile (phase B) and the flow rate was set at 0.600 mL/min. Sample injection volume was 5 µl. The analysis time was 10 minutes for the samples and 3 minutes for the standard solutions. Ionization of analytes was performed in electrospray negative mode and the source parameters were set as follows: Spray voltage: -4200V, vaporizer temperature: 600 ^{o}C, Ion Gas Source 1: 40, Ion Gas Source 2: 30, Curtain Gas: 25, Declustering Potential: -300,

Ion Release Delay: 100, Ion Release Width: 10. Protodioscin detection was performed by monitoring the sum of ions *m/z 737.41*, *m/z 739.42* and *m/z 755.42* resulted from *m/z 1047.7* at a collision energy of 65V.

Preparation of standard solutions

Two stock solutions of 200 µg/mL and 20 µg/mL protodioscin in HPLC grade acetonitrile were prepared. These stock solutions were used to prepare the calibration standard solutions with concentrations of 0.5, 1, 3, 5, 6, 8, 10 and 15 µg/mL protodioscin by diluting the appropriate amount of stock solution with a solvent made up of acetonitrile and ultrapure water, at a ratio of 3:7.

Preparation of sample solutions

From the herbal drug (origin China) extracts were prepared using ultrasound assisted extraction. The herbal drug was accurately weighted and was extracted twice with water (in a ratio of 1:5) at 60 °C for 60 minutes. The extract was filtered through a 0.45 µm microporous cellulose syringe filter, and an aliquot of this solution was used for the analysis.

The capsules (origin Poland) of finished product were analyzed using sample solutions prepared by accurately weighting to 10 mL volumetric flasks the powder equivalent to one capsule (550mg) from a larger pooled sample for assay testing, and the entire content of one capsule for uniformity of content testing. Samples were further diluted with 3 mL HPLC grade acetonitrile and 5 mL ultrapure water. The solution was placed in an ultrasonic water bath, sonicated at room temperature for 60 minutes, and was further diluted up to the mark with ultrapure water. Before analysis the samples were filtered through a 0.45 µm microporous cellulose syringe filter.

Accuracy evaluation was performed by the standard addition method and the recovery was calculated.

Preparation of placebo solutions

In order to check the existence of any interference from the excipients contained in the capsules, placebo solutions were prepared. Approximately 5mg microcrystalline cellulose and 0.15 mg magnesium stearate were accurately weighted in a 10 mL flask and 3mL acetonitrile and 5mL ultrapure water were added. The samples were sonicated for 60 minutes and then were diluted up to the mark with water. The solution was passed through a 0.45 µm microporous cellulose syringe filter and transferred to a HPLC vial.

3. Results and discussions

Eight calibration standard solutions in a concentration range of 0.5 - 15.0 µg protodioscin/ mL were prepared and injected for linearity testing. The average calibration curve was plotted using a 1/ y2 weighting and the accuracy of each calibration standard was calculated. Not more than two calibration standards (and not the lower or upper limit of quantification) of a calibration curve failed accuracy testing, the recalculated concentration being within ± 15% compared to the nominal concentration. The calibration curve plotted had a correlation coefficient (R) of >0.99 and is thus considered linear throughout the interval of 0.5 - 15.0 µg/ mL.

The lower limit of quantitation was set as the smallest concentration in the standard calibration curve (0.5 µg/mL) (**Table 1**).

Table 1. Table appearance

Linearity	R = 0.9969
Concentration range	0.5-15.0 µg/mL
Lower limit of quantification (LLOQ)	0.5 µg/mL

The representative MS spectrum of protodioscin under negative ionization mode is illustrated in **Fig. 4** protodioscin was identified by comparing the mass spectrum, extracted ion chromatogram and retention time with reference standard. Protodioscin was detected at 2.24 min (**Fig. 3**).

HPLC-UV analysis usually has a number of drawbacks compared to LC-MS techniques, related mostly to the interference of other compounds when analyzing complex matrices, as sometimes it is not possible to chromatographically separate compounds or due to low concentrations not detectable with conventional UV-Vis detectors (Ganzera et al., 2001). In our previous research, we concluded that protodioscin was not detectable in using UV-Vis detection; therefore, an LC-MS method was developed. Although there are published papers were methods for quantitative and qualitative determinations of protodioscin in HPLC-UV were developed, HPLC-UV analysis of sterolic saponins is usually very difficult because these compounds do not have a chromophore and show a very weak UV absorption at very unspecific wavelengths (Lozano et al., 2017). Pavin et al. (2018) determined the protodioscin concentration using a HPLC method with the mobile phase consisted of 0.025% acetic acid in water (solvent A) and acetonitrile (solvent B) with a flow rate of 1.0 mL/min, DAD detection at 250 nm and protodisocin was detected at aprox 43 min. Shishovska et al. (2015) detected protodioscin at 200 nm, using a HPLC-DAD method with acetonitrile and water as mobile phase and a flow rate of 1.0 mL/min. The characteristic fragmentation patterns of protodioscin were investigated using ESI-MS in negative mode. Due to ionization of protodioscin being performed in negative mode ammonium acetate, which helps deprotonation of compounds, was used as aqueous mobile phase.

The assay of the powder pooled from 10 capsules was 92.63 µg/550mg powder (equivalent to content of one capsule). The uniformity of protodioscin content in capsules was tested on 5 separate capsules, the average concentration of protodioscin was 78.12 µg/capsule and the relative standard deviation (RSD) was 11.51% (**Table 2**). Thus, it can be concluded that there are large variations among the capsules from the herbal supplement. Analysis of placebo samples revealed that the sample do not interfere with the detection of protodioscin.

It has been demonstrated that the content in steroidal saponins of TT preparations influences the pharmacological effects (Gauthaman et al., 2002). Different phytochemical profiles are linked with different outcomes in *in vitro* and *in vivo* experiments (Semerdjieva & Zheljazkov, 2019). Protodioscin was not identified in the herbal drug from China, and the results are in concordance with Dinchev et al. (2008), who reported great variations in the content of TT different organs collected from different countries.

Table 2. Uniformity control of protodioscin content in capsules

Sample	Protodioscin concentration µg/capsule	Min-Max (µg)	Average	RSD
1	67.45	67.45 – 91.70	78.12	11.51
2	91.70			
3	75.24			
4	81.00			
5	75.19			

RSD – Relative standard deviation

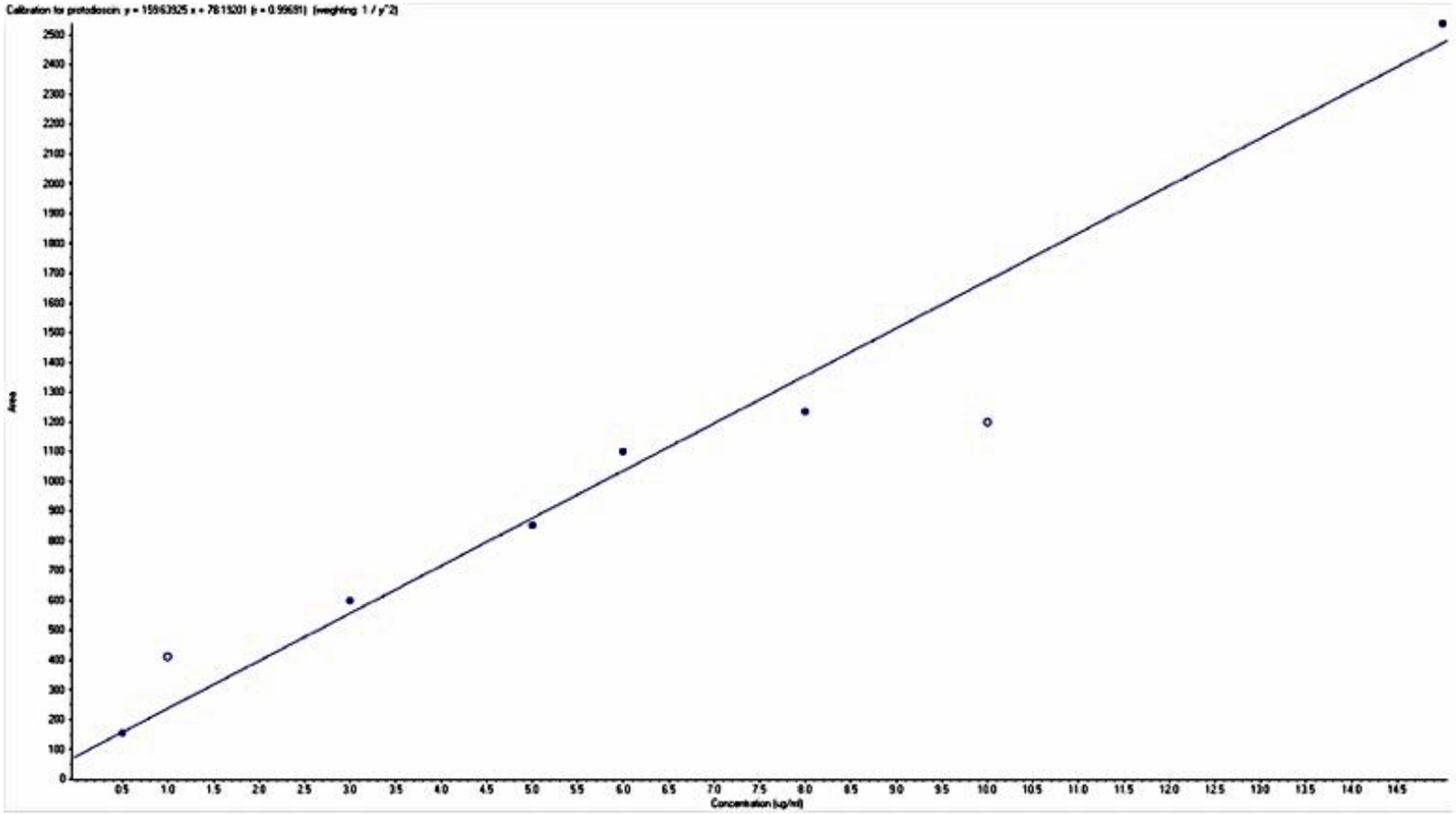

Fig. 2. Calibration curve

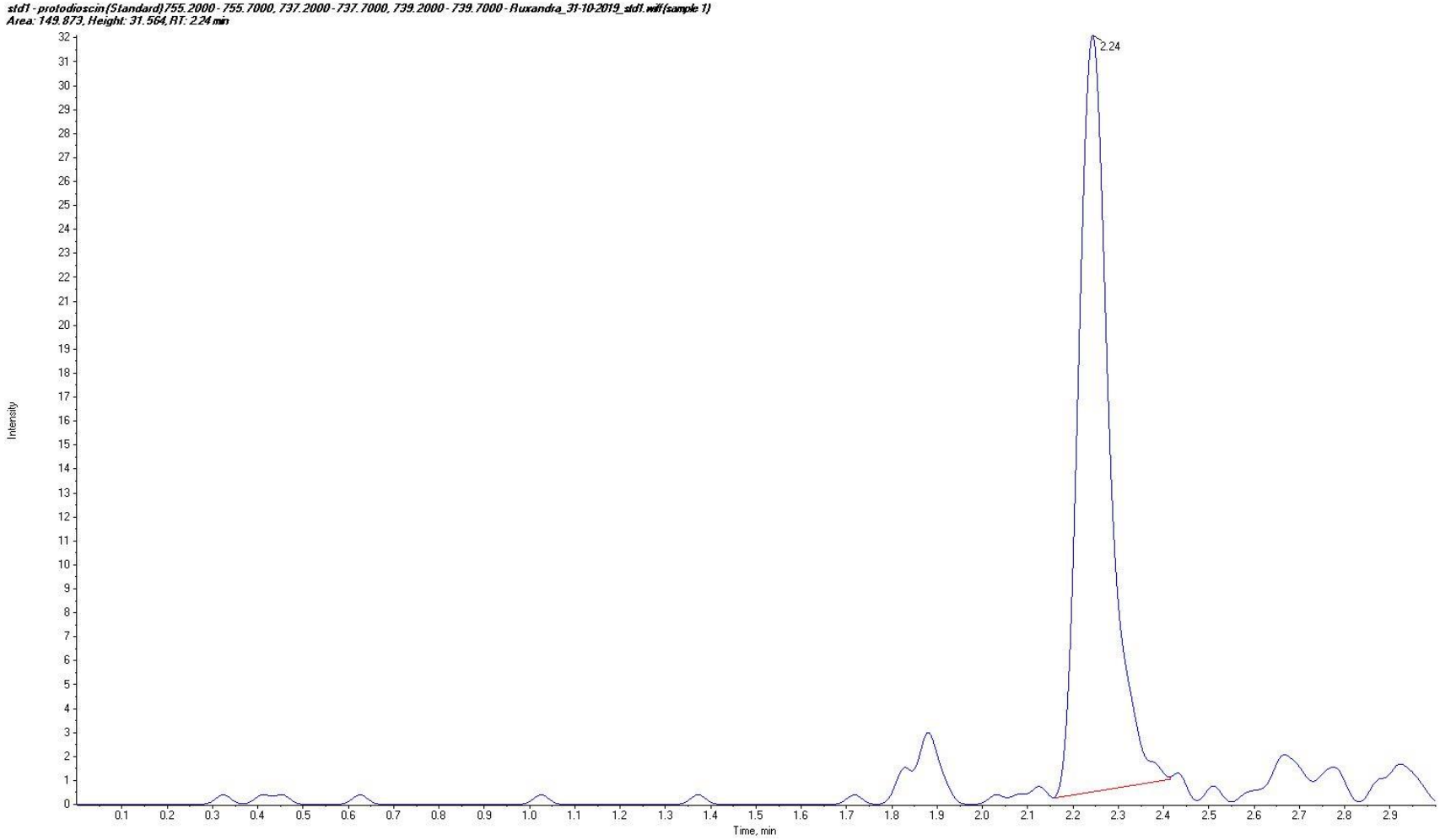

Fig. 3. Chromatogram of LLOQ protodioscin solution

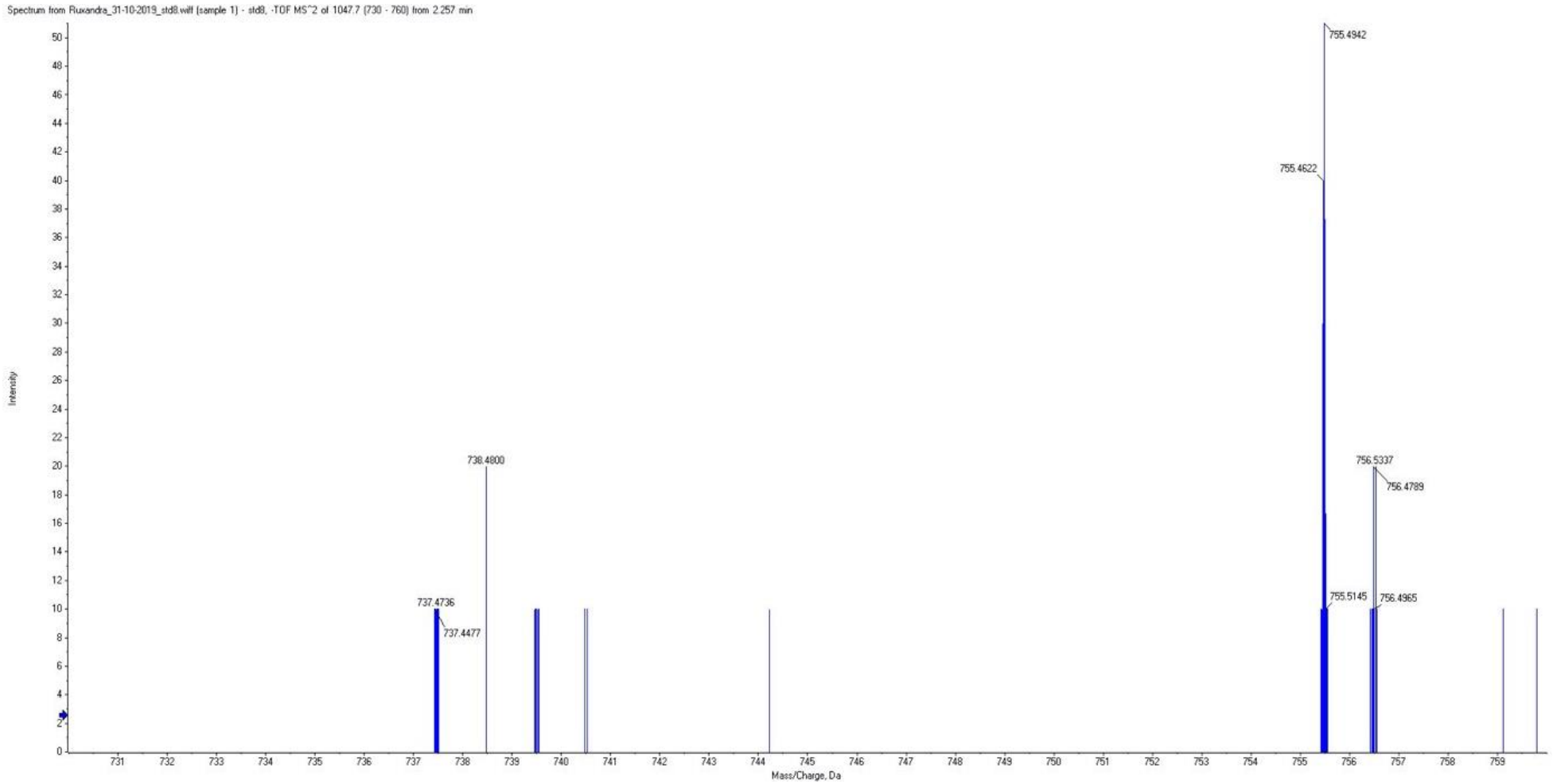

Fig. 4. MS/MS mass spectra of protodioscin

The content of protodioscin, for example, was lower in the Asian countries and the highest content was determined in the samples from Europe.

The industry of food supplements and therefore their consumption has increased constantly in the recent years. Because the quality control of supplements is usually minimal, and the standardization processes are scarce, the effects are in the best case

Although it was not the primary aim of this study, we have demonstrated that two different supplements with TT found on the Romanian market, have significantly different concentrations.

These findings demonstrate that the same outcome is impossible to achieve after the consumption of these supplements.

Conclusions

A rapid and reproducible LC-MS method was developed, which can be applied to other food supplements based *on Tribulus terrestris* extracts. As previously reported great differences are in the marketed supplements with *T. terrestris*, therefore therapeutic efficacy it is rightly questioned.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

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PHYTOCHEMICAL CHARACTERIZATION OF TRANSILVANIAN *PRUNELLA VULGARIS*

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Abstract: *Prunella vulgaris* L. grows in spontaneous flora of Romania in wet places, fields, meadows, unpopulated areas, both in the sun and in the shade. The plant is rich in phenolic acids (caffeic acid, rosmarinic acid), pentacyclic triterpenic compounds (ursolic, oleanolic, betulinic acid) and flavonoids (rutoside, quercetin). *Prunella vulgaris* L. has shown numerous pharmacological actions: antioxidant, anti-allergic, antimicrobial, immunostimulatory activities. The aim of the study was to evaluate the phytochemical and pharmacological profile of the leaves and spike inflorescence of *Prunella vulgaris* L. collected from Romania. The polyphenol content in leaves was found to be 63.78 ± 2.01 mg GAE/g dry weight in the methanolic extract and 45.73 ± 13.87 mg GAE/g in the aqueous extract. In the spike inflorescence, total polyphenol content was 36.44 ± 6.73 mg GAE/g in the methanolic extract and 26.49 ± 2.97 mg GAE/g in the aqueous extract. The results from the antioxidant assays (DPPH and ABTS) were not significantly different between the two herbal drugs. Further studies are needed in order to quantify the active compounds.

Keywords: *Prunella vulgaris*, total polyphenols, antioxidant, Lamiaceae.

1. Introduction

Prunella vulgaris L. (PV) is one of the herbs having a long history of traditional use in Chinese medicine (Pinkas, 1994). According to the empirical evidence, the infusion of *Prunella vulgaris* L. was used to treat diseases of the mouth, migraines, skin conditions (Zdařilová et al., 2009; Vostálová et al., 2010; Sârbu et al., 2013). It has a monography in the latest edition of the *European Pharmacopoeia* (Eur. Ph. 8.0, 2011). *Prunellae spica* represents an important source of active compounds: phenolic acids, flavonoids, pentacyclic triterpenic compounds,

sterols and polysaccharides (Morteza-Semnani et al., 2006; Mahboubi et al., 2015). According to the published reports, there is a direct correlation between the concentration of phenolic compounds in herbal drugs and their antioxidant activity (Shan et al., 2005). Medicinal properties attributed to *Prunella* sp. include anti-allergic, anti-inflammatory (Ryu et al., 2000), immunostimulatory (Han et al., 2009; Hwang et al., 2013a), antihyperglycemic (Zheng et al., 2007), and antihypertensive (Fu et al., 2011). Ethanolic and aqueous extracts of the species induce apoptosis, thus having a link between antioxidant action and phenolic content (Hwang et al., 2013b). Also, the literature indicates that the extracts exhibit antiviral and antimicrobial actions (Komal et al., 2018; Li et al., 2019). *Prunella vulgaris* can be found in the wild flora of Romania, but there is a lack of information regarding the phytochemical profile of the herbal drug. Being a widespread species in Romania, the defined objectives of the present study are the analysis of the plant product by chromatographic methods and the evaluation of the antioxidant action. Because most of the natural treatments indicate the preparation of plants as an infusion, a study of the *Prunella vulgaris* species is required for a comparative analysis depending on the extraction yield to obtain an extract with the richest concentration in active principles.

2. Materials and Methods

Plant material

Prunella vulgaris L. leaves and spike inflorescence were harvested from Mureș County, Romania. Harvesting was done at the end of the flowering period, when the fruit develops, in August. The herbal drug was dried in the shade and kept in laboratory conditions until analysis. Voucher specimens of all samples (PV-L-19, PV-SI-19) are deposited at the Department of Pharmacognosy and Phytotherapy, Faculty of Pharmacy, George Emil Palade University of Medicine, Pharmacy, Science, and Technology of Târgu Mureș, Romania.

Preparation of PV extracts

Aqueous extract (PV-AQ): 5g of dried and crushed plant product (leaf (L) or spike inflorescence (SI)) were extracted with 50 mL of distilled water for one hour at 60 °C on an ultrasonic water bath followed by filtration.

Methanolic Extract (PV-Me): 5g of dried and crushed plant product (leaf - L or spike inflorescence - SI) were extracted with 50 mL methanolic solution (70:30 *v/v*, methanol: water) for one hour at 60 °C on an ultrasonic water bath followed by filtration.

Thin-layer chromatography (TLC)

The solutions (30 µL of the methanolic extract from spike inflorescence) were spotted on TLC silicagel plates (ALUGRAM Xtra SIL G Macherey- Nagel Düren, 10 x 20) as 1 cm bands, using a Hamilton syringe. Plates were developed in an ascending mode in a saturated chamber using ethyl acetate-methanol-water (40:5.4:4, *v/v/v*) as a mobile phase. Rosmarinic acid, caffeic acid, betulinic acid, quercetin, rutozide were used as standards. Following development, the plates were dried and spayed with diphenylboroyloxymethylamine and PEG 400 5% solution in MeOH for fluorescence intensification and stabilization. The chromatograms were observed in UV/Vis before and after pulverization. An UV lamp 254/365nm Vilber Lourmat was used for compounds identification at 365 nm.

Total polyphenol content (TPC)

The total polyphenol content was determined spectrophotometrically using Folin Ciocalteu reagent (Singleton et al., 1999). A standard curve for gallic acid was prepared with five points ranged between 0.002-0.02

mg/mL (R^2 = 0.9865). The results were expressed as mg of gallic acid equivalents/ 1 g of the dried herbal drug.

DPPH radical scavenging activity

Free radical scavenging activity was performed using DPPH method. To 2,5 mL methanol solution of DPPH 0,1 mM, different volumes of samples (50 µL, 100 µL, 200 µL, 300 µL, 400 µL, 500 µL) were added. The absorbance was measured at 517 nm (using a UV-VIS spectrophotometer) after 30 minutes. The % inhibition was calculated using the following formula:

% inhibition = [(A control - A sample) / (A control)]*100

The IC_{50} (the concentration of the sample that scavenge 50% of DPPH free radical) values were determined. Ascorbic acid was used as positive control (Shen et al., 2010; Fazal et al., 2016; Nayak et al., 2018).

ABTS radical scavenging activity

The $ABTS^{\bullet+}$ radical cation was generated by reacting 10 mg ABTS and 2.45 mM potassium persulfate, followed by incubation at room temperature, in the dark for 12 hours. The $ABTS^{\bullet+}$ solution was diluted with ethanol. To 2.5 mL $ABTS^{\bullet+}$ solution, different extract volumes (10 µL, 60 µL, 90 µL , 150 µL , 300 µL)were added and the absorbance was measured after 6 minutes at 734 nm (using a UV-VIS spectrophotometer). The controls contained the extraction solvent instead of the test sample. The % inhibition was calculated using the following formula: % inhibition = [(A control - A sample) / (A control)]*100, where A0 is the absorbance of the control and A1 is the absorbance of the test samples.

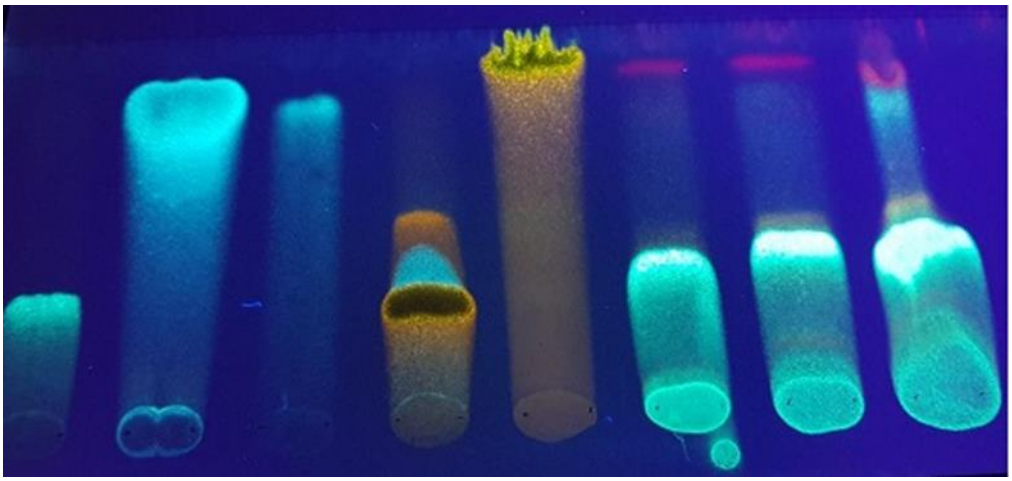
Results were expressed as IC_{50} (µg/mL). Ascorbic acid was used as positive control (Zheleva-Dimitrova et al., 2010; Chew et al., 2011).

Data analysis

Statistical analysis was performed using Graph Pad Prism 8. All measurements were made in triplicate, and the results were expressed as mean ± SD. Analysis of variance (ANOVA) was performed, followed by Tukey's multiple comparison Test at $p < 0.05$.

3. Results and discussions

Qualitative TLC analysis was performed in order to identify the main polyphenolic compounds found in the *Prunella* inflorescence. TLC is widely used in quality control of natural products and food supplements, as the procedure is a rapid, cheap and straightforward method. TLC of sample and reference compounds were analyzed before and after spraying, in daylight, and at UV_{365} nm. Before revelation, two yellow spots were observed, corresponding to the 2 flavonoids used as standard compounds: rutozide (Rf = 0.9) and quercetin (Rf = 0.26). After revelation with NEU reagent, in daylight, yellow-green spots corresponding to rosmarinic acid were observed, yellow-colored spots corresponding to rutozide and caffeic acid. In UV light (**Fig. 1**), polyphenolic compounds with specific fluorescence are observed. In *Prunellae spica* extracts, rosmarinic acid (Rf = 0.28) with greenish-blue fluorescence was identified on the basis of the retention factor, in comparison with the reference compounds used. Analyzing the chromatographic plate, we can observe that rosmarinic acid is the major compound considering the intensity of the spot. The presence of an orange spot was also observed, indicating the presence of a flavonozide. Due to the fact that caffeic acid and betulinic acid had the same retention factor using this mobile phase, it is not possible to determine which of these compounds are present in the extract.



RA	CA	BA	Rut	Q	PVE

Fig. 1. Chromatograms of *Prunella vulgaris* inflorescence extract after spraying with NEU / PEG reagent, in UV 365 nm. From left to right: rosmarinic acid, caffeic acid, betulinic acid, rutozide, quercetin, *Prunella vulgaris* extracts

Although the TLC analysis offers only qualitative information, we can conclude that the main polyphenolic compound found in *Prunella vulgaris* flowers is rosmarinic acid. The results are consistent with the reported data (Shekarchi et al., 2012; Wagner et al., 2016).

The methanol extract of *Prunellae spica* are characterized in the upper Rf range by rosmarinic acid - RA (Rf=0.28), caffeic acid - CA (Rf=0.8), betulinic acid - BA (Rf=0.8), rutozide - Rut (Rf=0.26), quercetin - Q (Rf=0.9) (**Fig. 1**).

Polyphenols are plant secondary metabolites, considered to be a valuable class of natural compounds with a highly satisfactory therapeutic effect.

Many researches aiming to characterize the spike of *Prunella vulgaris* have been published in recent years in China and other countries, however, the herbal drug collected from Romania is poorly characterized (Collins et al., 2009; Liu et al., 2017).

Our results are very similar to the results obtained by Aybastier et al. (2011) with the plant collected from Turkey. Although most researches were focused on the extracts obtained from PV spike, the TPC was significant higher in the extracts obtained from the leaves. The moment of harvesting is probably responsible for the lower quantity of polyphenols in the spike. It is known that the content of secondary metabolites (rosmarinic acid, caffeic acid) in herbal drugs varies considerably through the plant development and subsequently their antioxidant action depends on the moment of harvest (Chen et al., 2019). We have chosen to collect the herbal drug at the end of the flowering period, when the fruit develops, because *Prunella vulgaris* fruits contain mainly oleanolic and usolic acids (Du and Chen, 2009). These pentacyclic triterpenic acids are known to have great pharmacological actions like anti-diabetic, anti-hyperlipidemic, antiinflamatory and hepatoprotective effects (Kashyap et al., 2016; Ayeleso et al., 2017; Ding et al., 2018). The extraction solvent plays an important role regarding the final concentrations of active principles in the obtained extracts. As an extraction solvent, methanol is often used because it assures high extraction yields than other polar solvents.

Table 1. TPC and antioxidant activity

Sample	TPC (mg GAE/g DW)	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)
PV – L - MeOH	63.78 ± 2.01[a]	35.31 ± 0.68	1.29 ± 0.12
PV – L - AQ	45.73 ± 13.87[ab]	61.35 ± 0.99	2.23 ± 0.11
PV – SI - MeOH	36.44 ± 6.73[b]	53.89 ± 4.93	1.53 ± 0.11
PV – SI - AQ	26.49 ± 2.97[b]	56.64 ± 2.81	2.32 ± 0.15

DW – dry weight of the herbal drug; all tests were performed in triplicate; different letters in columns mean statistically significant differences

For an optimal extraction of polyphenolic compounds, studies have revealed that the percentage of methanol should be varied between 50% and 80% (Gupta, 2010).

Our findings (**Table 1**) are in line with data from earlier studies. TPC values measured for *Prunella vulgaris* extracts varied has been reported to be between 8.05 – 63.75 mg GAE / g dry plant (Aybastier et al., 2011; Singh et al., 2015; Chen et al., 2019; Shanaida et al., 2018). TPC was expressed in mg GAE/g DW, all of the determinations being made three times. Dosing total polyphenols at an early stage of the study is important because there are numerous studies that compare the antioxidant action to biological activity (Mahboubi et al., 2015). Although the concentration of polyphenolic compounds is higher in the methanolic extracts, our study revealed that there are no significant differences (at $p<0.05$) between methanolic and aqueous extracts from the same herbal drug. The main criteria underlying solvent selection are its yield and toxicity. In a study carried out in Iran, the TPC from the dried parts of *Prunella vulgaris* L. species were determined. The results showed that the herbal drug harvested from Iran contains 2 times higher concentrations of total polyphenols (115-156 mg GAE/g) than the product used in the present study (Mahboubi et al., 2015).

The pedo-climateric influence over the phytochemical profile of plants is well documented in the scientific literature. According to Sárosi et al. (2015) the herbal products obtained from *Prunella vulgaris* that grows in sunny exposure contain higher quantities of polyphenolic compounds than the plants that grow in shade.

The DPPH assay is based on the reduction of DPPH radical, resulting a discoloration of the violet solution. This is a simple and quick method to determine the antioxidant action by means of a suitable dilution of hydroalcoholic extracts of *Prunella vulgaris* species) (Sochor et al., 2010; Lee et al., 2015).

The ABTS radical scavenging assay is based on the formation of ABTS radical resulting in a blue solution and the subsequent reduction of the cation by the antioxidant compounds, resulting a discoloration of the solution. Feng et al. (2010) demonstrated the antioxidant action of different hydroalcoholic extracts from *Prunella vulgaris*. Positive correlations between the total polyphenol content and antioxidant activity were reported in their work. We can assume that rosmarinic acid found in high quantities is the main compound responsible for the antioxidant action (Cao et al., 2005). The results obtained in the two radical scavenging assays suggest the scientific basis for its folkloric use in some pathologic conditions.

Conclusions

On the basis of our results, we can conclude that both herbal drugs obtained from *Prunella vulgaris* are an important source of polyphenolic compounds with antioxidant

potential and thus we can continue further researches regarding on the therapeutic potential of this species. Giving the obtained results, we suggest that aqueous extracts may be further used in preclinical studies in order to prevent the toxicity of methanol. Further detailed phytochemical screenings are needed in order to observe the influence of Transylvanian specific pedo-climateric conditions.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

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