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Research Article

The Level of Saliva Glutathione in Moderate Gingivitis Patients Increases After Gargling with 5% Cosmos (*Cosmos caudatus*) Extract

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KEYWORDS

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reactive oxygen species

ABSTRACT

Introduction: Gingivitis is an inflammation of the gums caused by bacterial plaque accumulation producing reactive oxygen species (ROS). Reactive oxygen species is a harmful by-products from aerobic metabolisms of mitochondria, that when accumulated can cause large variety of diseases. Antioxidants can counter ROS activities. Oxidative stress may occur due to an imbalance between ROS and antioxidants. Glutathione (GSH) is an antioxidant that protects cells against oxidative damage. *Cosmos caudatus* is rich in antioxidants due to its flavonoid and phenolic contents. **Objective:** The aim of this study was to detect the concentration of saliva GSH in moderate gingivitis patients after gargling with a solution containing 5% Cosmos (*Cosmos caudatus*) extract. **Methods:** Twenty subjects with moderate gingivitis were divided into two groups—the treatment and control group—by drawing lots in a randomized controlled trial. The treatment group gargled with 5% Cosmos extract whereas the control group gargled with Chlorhexidine 0.1%. Each subject was required to gargle for 60 seconds every morning and night, for five days consecutively. The GSH level was measured before and after gargling on the sixth day after treatment using a spectrophotometer with wavelength of 412 nm. Data was analyzed using an independent T-Test ($p < 0.05$). **Results:** The study showed there was significant difference between the saliva GSH level of each group before and after gargling; moreover, after gargling with the solutions, no significant difference of saliva GSH was found when compared between the treatment group and the control group. **Conclusion:** The level of saliva GSH increases after gargling using 5% Cosmos extract and has the same effect with Chlorhexidine 0.1%.

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INTRODUCTION

Gingiva, commonly referred to as the "gums" consists of masticatory mucosa that covers the alveolar processes of the jaw and surrounds the neck of the teeth. Gingiva surrounds the tooth like a collar and is firm, resistant, and can be tightly adapted to the tooth and bone. The gingiva can be demarcated based on clinical observation into free marginal gingiva—the attached gingiva which varies in width—and also the interdental gingiva.[1] Healthy gingiva appears pink in color; however, there may be a varying brownish pigmentation. Gingiva may also exhibit varying consistency and is non-mobile upon the underlying bone. The keratinized surface of the gingiva may be firm and thick with heavy or light visible stipples.[1]

There are two important factors that relate to one another associating with periodontal pathophysiological development like gingivitis: the activation of the immune system and the production of radical oxygen molecules and related metabolites. An increase in the production of radical oxygen causes oxidative stress, which is involved in many periodontal diseases.[2] During the inflammation of the gingiva, migration of polymorphonuclear neutrophils (PMNs), alteration of the coronal aspect of the junctional epithelium, loss of collagen around blood vessels, accumulation of lymphocytes, and altered fibroblasts occurs, which then causes higher production of reactive oxygen species (ROS).[3]

Glutathione (GSH) is a natural protein which is produced within the body and functions as an antioxidant in the immune system as it has the ability to directly scavenge free radicals. It is able to act as a co-substrate in GSH peroxidase catalyze to reduce toxic oxygen species such as hydrogen peroxides and lipid hydroperoxides making GSH a key to defense mechanisms in opposition to cellular oxidative stress and helps in the regeneration of cells. When the immune system weakens or when there is presence of inflammation, the production of GSH in the body will decrease. Glutathione present in saliva is an indicator of a necessity to provide antioxidants.[4] The change on the antioxidant enzyme and the concentration or level of GSH present in the saliva can be used to determine the prognosis, the evolution of a disease, and the oral manifestation.[5]

Anti-inflammation medicines are naturally made by the herbal medicine industries and Indonesia is one of the countries with major potential to be used as a base for this industry's further development.[6] One of the herbal medicinal plants that can be further developed into an anti-inflammation medicine is *Cosmos caudatus*,[7,8] commonly known as 'kenikir' in Indonesia, which has a high anti-inflammatory property. Cosmos originates from the lands of America. However, it is now widely spread to tropical countries and has been commonly used in cooking because of its high level of antioxidants. The leaves of the plant contain saponin, flavonoids, polyphenols, and essential oils that have many health benefits. Cosmos contains phenolic compounds that contribute to the color, antioxidants, and anti-carcinogenic properties of the plant.[8] Cosmos is also said to protect the effects of antioxidants due to the presence of hydroxyl group of flavonoids found in Cosmos against lipid peroxidation and therefore is able to scavenge free radicals.[9] Furthermore, Cosmos has a high flavonoid content, such as catechin, epicatechin, and quercetin.[10] These properties may be responsible in preventing further bone loss and protecting the bone from oxidative stress. Radman et al. found that Cosmos might have tumor-inhibitory effects, traditionally used for reducing body heat, improving blood circulation, anti-aging, promoting fresh breath, strengthening bone marrow, and treating infections caused by pathogenic microorganisms, antimutagens, and antifungal properties.[11] Another study has proven that this plant has high antioxidant capacity of more than 2500 mg/L ascorbic acid equivalent antioxidant capacity (AEAC) per 100 g of fresh samples.[12]

According to Rasdi et al. ethanol extract exhibits a high degree and broad spectrum of antimicrobial activity.[13] Concentration effects were also observed at the highest concentrations used to test on bacterial strains. The highest antimicrobial activity that ethanol extract exhibited is 50 mg/ml, which is at a 5% concentration of Cosmos extract. Studies by Ajaykumar et al. and Mediani et al., has been done on the effects of Cosmos extract as an antioxidant and anti-inflammatory agent; however, none have been conducted on its antioxidant effect towards gingivitis and salivary GSH levels.[7,14] Hence, in order to fully manipulate the potential of the Cosmos extract, research on the effects of the extract as a mouthwash on saliva GSH levels in moderate gingivitis patients should be carried out as it may be able to provide an alternative mouthwash. Based on the above theory, it can hypothetically be deduced that the level of saliva GSH in moderate gingivitis patients will increase after gargling with 5% Cosmos extract. Therefore, with the research gap in mind, the aim of this study was to determine the concentration of saliva GSH in patients with moderate gingivitis after using Cosmos extract as a mouthwash.

MATERIALS AND METHODS

The Dental Health Research Ethics Commission from the Faculty of Dentistry Universitas Gadjah Mada (UGM) gave ethical clearance with No. 0445/KKEP/FKG-UGM/EC/2015. Cosmos extract was obtained from the Pharmacology Laboratory Faculty of Pharmacy,

UGM Yogyakarta. Saliva samples were collected from subjects at their residences, which were close to the Faculty of Dentistry, UGM. Saliva samples were stored in the Research Laboratory, Faculty of Dentistry, UGM. Saliva GSH activity was measured in the Research Laboratory, Faculty of Dentistry, UGM. Determination of the plant morphology was carried out using expert determination and plant recognition method to ensure that the plant used in this research was Cosmos. The determination of the plant species was carried out by Laboratory Division II Department of Biology Pharmacy Faculty of Pharmacy, UGM.

Cosmos caudatus Mouthwash with 5% Concentration

The Cosmos plant was obtained from a stall that sells fresh vegetables in Pasar Kranggan, Yogyakarta. The plant sample—in the form of leaves and petioles free from diseases and insects or any kind of pollutant—was cleaned with running water, placed on a tray, and covered with a cloth which was then left to dry, avoiding direct contact with sunlight until it was completely dried. The dried samples were then crushed with a mortar and pestle until achieving a powdered form. The powder was then diluted with 70% ethanol and by using the maceration method, then shaken and soaked for 24 hours. The product was filtered with a filter paper. The filtrate was taken and evaporated under vacuumed pressure of 40° - 50° C in order to evaporate the 70% ethanol and to obtain the crude extract of the plant. The extract obtained was then diluted by using sterile aquades until a 5% concentration was obtained. All materials were provided by the Pharmacology Laboratory Faculty of Pharmacy, UGM, Yogyakarta.

Gingival Index Status Examination

A gingival indices examination was carried out by inspecting and probing the buccal, lingual, mesial, and distal regions of the teeth at the subjects' residences and also at the 3rd Floor of Dental Hospital of Prof Soedomo, Dental Faculty, UGM. The gingival index score is based on Löe and Silness[15] modification, which are: Score 0: Healthy gingiva, with no sign of inflammation, well-adapted with teeth, normal consistency; Score 1: Slight inflammation, slight changes in texture, no bleeding upon probing; Score 2: Moderate inflammation, redness of gingiva, oedema, shiny, bleeding upon probing; and Score 3: Chronic inflammation, apparent redness, oedema, ulceration, spontaneous bleeding. The total examination score is divided with the total region examined resulting in the gingival indices. The criteria of gingival index are: 0.1-1.0: mild gingivitis; 1.1-2.0: moderate gingivitis; and 2.1-3.0: chronic gingivitis.

Mouthwash Usage Instructions and Saliva Collection

Twenty participants were chosen among students studying in UGM with the following criteria: moderate gingivitis and currently not consuming para-sympathomimetic medications (pilocarpine, anti-cholinergic, anti-asthma, antibiotic, and anti-inflammation), no usage of any type of mouthwash, and those who are non-smokers, non-alcoholic and do not have any systemic conditions such as diabetes mellitus and hypertension. Sample size was determined using Yamane (1967) formula. Subjects were instructed to gargle twice a day after brushing their teeth in the morning and night, for five consecutive days. Each gargle uses 10 ml of mouthwash for a duration of 60 seconds. Subjects were then instructed to not consume any food or beverage for 30 minutes after gargling.

Unstimulated saliva sampling was carried out twice on each subject on the first day before the usage of mouthwash and on the sixth day after the usage of Cosmos extract mouthwash or Chlorhexidine 0.1% (Hexadol®, PT. Otto Pharmaceutical Industries). Saliva sampling was carried out in the morning after a minimum of six hours fasting or after only consuming water. The saliva sampling involved draining and spitting where patients were instructed to hold the saliva at the bottom of the mouth for five minutes in a sitting position at their respective residence in their living room. The saliva was collected in a container and kept in ice packs throughout the journey from the residence to the laboratory. Collected saliva was then transferred into a test tube and kept at -21°C.

Production of Glutathione Standard

Pure GSH powder was obtained from the Faculty of Pharmacy, UGM. Standard GSH solution was made by mixing 2 mg/ml reduced GSH (µL), Buffer phosphate 0.1 M pH 8 (µL), 200 µL Trichloroacetic acid (TCA) 5%, and 25 µL 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) (Table 1). The tubes were then incubated at room temperature for an hour, and the solutions were read using a spectrophotometer at a wavelength of 412 nm which was then calculated and determined the GSH level using GSH standard curve. Production of GHS standard was carried out with materials from and at the Research Laboratory, Faculty of Dentistry, UGM.

Determining GSH Gingival Concentration

A saliva sample of 1.5 ml was centrifugated at 3500 rpm for 10 minutes at a temperature of 4◦C until supernatant (a clear liquid that lies above the solid residue after centrifugation). The supernatant was then

Table 1. GSH standard solution (standard curve)

Standard Concentration (mg/ml)	GSH Standard 2 mg/ml (µL)	Buffer Phoshate 0.1 M pH 8 (µL)	TCA 5% (µL)	DTNB (µL)
0	-	2000	200	25
1	1	1999	200	25
2	2	1998	200	25
4	4	1996	200	25
5	5	1995	200	25
10	10	1990	200	25

transferred into another microcentrifuge tube. Next, the protein present in the supernatant was removed by adding 200 µL TCA 5%, then centrifugated. The supernatant was divided into three reaction test tubes of 400 µL, respectively, then added with 1600 µL Phosphate Buffer Solution (PBS) 0.1M pH 8.0 into each tube and vortexed for 30 seconds. The DTNB 25 µL solution was then added into each tube, vortexed for 30 seconds, and left at room temperature for one hour. Every sample made was done in triplicate and each absorption reading shown by the spectrophotometer was recorded. The reaction between saliva GSH and DTNB produces 5-thio 2-nitrobenzoate (TNB) acid, which is yellow in color. Solution absorption was read using a spectrophotometer at a wavelength of 412 nm. The increase in saliva GSH concentration was obtained by calculating the difference between the concentration of GSH before and after five days of gargling.

Data Analysis

Data obtained from before and after gargling was analyzed using a homogeneity and normality test. Then an unpaired T-test ($p < 0.005$) was used to determine the difference between the treatment group and control group.

RESULTS

Based on the research carried out, there was an increase in the saliva GSH level on moderate gingivitis patients after gargling either with 5% concentration Cosmos extract or Chlorhexidine 0.1%. The average mean and standard deviation of saliva GSH level before and after gargling in both groups can be seen in Figure 1. Results of independent T-test analysis ($p < 0.05$) are shown in Table 2. When analyzed it is known that the significance, p value is 0.139. Thus, from the value of p, it is determined that there is no significant difference between the extract group and the control group. Hence, it can be indicated that 5% Cosmos extract and Chlorhexidine 0.1% as a mouthwash have the same effect in increasing saliva GSH levels in patients with moderate gingivitis.

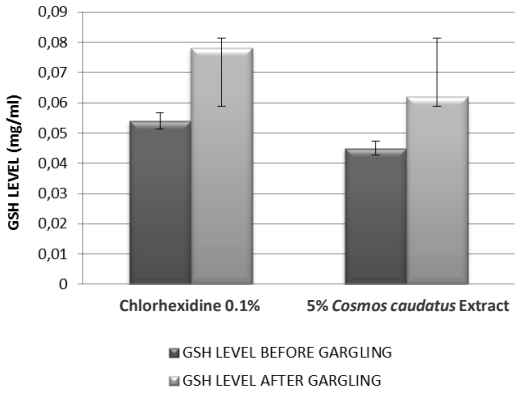

Figure 1. Mean and standard deviation of saliva GSH towards the cosmos extract group and chlorhexidine 0.1% group. This shows that there is an increase in saliva GSH level after gargling for both groups

Table 2. Results of independent T-test saliva GSH level after gargling based on groups ($p < 0.05$)

	N	t	df	p
Cosmos extract	10	-1.549	18	0.139
Chlorhexidine 0.1%	10			

DISCUSSION

This research is carried out to determine the changes in the level of saliva GSH in moderate gingivitis patients after gargling with 5% cosmos extract and Chlorhexidine 0.1%. However, there were three saliva samples of the moderate gingivitis patients removed from the study due to the presence of blood in the saliva. According to Kamodyova et al.,[16] if there is 1% of blood contamination in the sample, this results in a biased salivary oxidative stress markers concentration and hence the sample was removed from the analysis, without having the need to detect or to test any blood constituents in the sample. Glutathione is a naturally occurring tripeptide with nucleophilic and reducing properties in metabolic pathways and also in the antioxidant system of most aerobic cells.[17] Results obtained from this research showed that, in moderate gingivitis patients, the level of saliva GSH before gargling is lower when compared to after gargling using either 5% cosmos extract or Chlorhexidine 0.1%. This result showed that there is an increase in the level of saliva GSH. However, there is no significant difference between the level of saliva GSH between the responding and control groups. This indicates that the increased level of saliva GSH produced by both groups is the same.

Free radicals such as lipid peroxidase are known as indicators for oxidative damage that is more reactive than oxygen molecules themselves causing an impairment of oxidant-antioxidant balance in saliva. Saliva GSH is proven to be reduced by periodontal diseases, such as gingivitis.[18] Oxidative stress develops when the generation of ROS is higher than the production of the antioxidant protection, in this case, GSH. An increase in GSH level shows an increase of antioxidants in the body that enables a faster healing process. A rise in GSH level after patients gargled with the Cosmos extract of 5% concentration is due to the presence of antioxidants in the Cosmos extract that have the ability to scavenge free radicals. As stated by Mustafa et al., cosmos extract is rich in antioxidants and has high antioxidant activity, which relates to the ability of the antioxidant to inhibit free radicals.[10] This further supported the theory that Cosmos extract is a good source of antioxidants and, at a concentration of 5%, it exhibits a high degree of antimicrobial activity.[13,19]

Cosmos is also rich in phenolic compounds, such as flavonoids, flavones, and flavanones, and showed strong antioxidant activity.[10] According to Mohamed et al., cosmos contains phenolic compounds that contribute to the color, antioxidant, and anticarcinogenic properties of the plant.[8] Furthermore, the results of this research are supported by stating that plants that contain high phenolic and flavonoid properties make a good source of naturally produced antioxidant.[20]

Phenolic compounds present in Cosmos play an important role in the antioxidant activity of the plant by scavenging electrophiles and active oxygen species, slowing down the nitrosation process of chelating metal ions to limit auto-oxidation, and increasing the ability to adjust some enzyme actions.[14] Antioxidant activity is determined by the structure of the phenolic molecules. Phenolic molecules are found to show antioxidant activity by deactivating free radicals and inhibiting the degradation of hydrogen peroxides which results in free radicals. Natural medications containing phenolic compounds are able to reduce lipid peroxidation, prevent oxidative DNA molecule damage, and prevent free radical formation, such as superoxides, hydrogen peroxides, and radical hydroxyl groups.[21] The antioxidants in Cosmos exist as dimers, such as hexamers, quercetin glycosides, chlorogenics, neo-chlorogenics, crypto-chlorogenic acid, and (+)-catechin.[22] The major flavonoid components of Cosmos are quercetin and rutin.[23] Quercetin is a component that is in the leaves of the Cosmos plant and acts as an antioxidant and prevents cell damage due to free radicals; thus, it is able to increase GSH level.[24]

Flavonoids show various biological activities, such as antioxidant activity and the ability to control enzymatic activities.[25] Flavonoids are able to inhibit various enzymes—such as lipoxygenase, cyclooxygenase, and prostaglandins—and influences detoxification functions, like GSH S-transferase a detoxifying enzyme.[26] Cyclooxygenase and lipoxygenase are inflammatory mediators that release arachidonic acid which triggers the early inflammation process.[27] According to Durgo, flavonoids have the potential to increase GSH levels.[25] Flavonoid is an antioxidant that has a direct reaction towards free radicals; specifically, radicals that can be oxidize by flavonoids. As a consequence, more stable and less reactive radicals are formed and thereafter indirectly increase GSH levels. Flavonoids that are oxidized by radicals, such as oxygen radicals, result in less reactive radicals according to the following equation:[28]

$$\mathbf{FOH + R' \dashrightarrow FOH' + RH}$$

where FOH represents flavonoid, R´ is free radical, and FO´ is less reactive free radical.

Flavonoids stabilize ROS by reacting with the compound of the radical, whereby the high reactivity of the hydroxyl group of the flavonoids makes radicals inactive.[29] This stability hence increases GSH level. The increase of the GSH level is possible because the body naturally produces it, whereas ROS decreases due to the presence of antioxidants from the Cosmos extract.

Flavonoids increases GSH levels due to their ability to modulate cell-signaling pathways. The protective effect of flavonoids against xenobiotics' toxicity are attributed to the modulation of these enzymes which is gamma-glutamylcysteine synthetase, an endogenous antioxidant that is involved in GSH synthesis,[30,31] the mechanism of flavonoid action. According to Watson, low concentrations of flavonoids can increase the intracellular level of GSH.[32] Cosmos extract also shows antibacterial activity which plays a role in the healing process of the gingiva in moderate gingivitis patients; this can be indicated by the increase of GSH level after gargling and reduces inflammation, as gingivitis causing bacteria population had decreased. Based on research carried out by Lee, ethanol extract of Cosmos was found active against several strains of human pathogenic bacteria, such as *Salmonella sps, Proteus mirabilis, Staphylococcus aureus*, and also *Vibrio cholera.*[33]

Further findings by Rasdi et al. show ethanol extract exhibits a high degree and broad spectrum of antimicrobial activity.[13] The effects of different Cosmos extracts against five microbial strains, two Gram-positive

bacteria—*Bacillus subtilis* and *Staphylococcus aureus*—two Gram-negative bacteria, such as *E. coli* and *Pseudomonas aeruginosa*, and a fungus—*Candida albicans*—by disk diffusion method showed that ethanolic Cosmos extract had significant inhibitory activity towards these strains. The results of the research also indicates that both polar molecules and non-polar molecules of the Cosmos extract have low affinity towards microbes and fungus.

Nazihah et al. further prove that the leaves of the Cosmos extract that contain phytochemicals such as terpenoids, fatty acids, flavonoids, alkaloids, tannins, and saponin possess antimicrobial activity.[34] Yusoff et al. make further statements regarding the antimicrobial property of Cosmos extract.[35] Cosmos at a 5% concentration exhibit high levels of antimicrobial activity when tested on *Bacillus cereus, E. coli,* and *Staphylococcus aureus* on raw chicken meat.

The control used in the research is Chlorhexidine 0.1%. Based on the research, it is found that Chlorhexidine 0.1% can increase the saliva GSH level in patients with moderate gingivitis. Neto et al. proved that using lower concentrations of Chlorhexidine, such as Chlorhexidine 0.1%, is effective in lowering plaque and gingivitis scores and also diminishing adverse effects.[36] Türkoğlu et al. also mentioned that Chlorhexidine as a mouth rinse is effective in plaque control and could be useful in managing plaque-associated gingivitis.[37] Chlorhexidine mouthwash can also provide an important adjunct to the prevention and control of gingivitis, especially with the regular personal oral hygiene procedures.[38] Furthermore, it is known that Chlorhexidine is a most widely and commonly used antiplaque and antigingivitic agent.

An increase in the saliva GSH level in moderate gingivitis patients after gargling with Chlorhexidine 0.1% is assumed due to the antibacterial strength of the Chlorhexidine itself. Haffajee et al. supported this, finding that Chlorhexidine 0.1% shows low minimum inhibitory concentration when tested with *Actinomyces spp.*; periodontal pathogens, such as *Eubacterium nodatum, Tannerella forsythia,* and *Prevotella spp.*; as well as the cariogenic pathogens, such as *Streptococcus mutans.*[39] Chlorhexidine has also been reported effective against *Candida albicans* in vitro and in vivo. There is a broad range of susceptibility of both Gram-positive and Gram-negative bacterial strains towards Chlorhexidine.[40] A low dosage of Chlorhexidine causes the cellular transport of the bacterial cell to be damaged with the creation of pores in the cellular membrane whereas, at a higher dosage, the solution penetrates the bacterial cells leading to cell death.[36]

Antibacterial properties of the Cosmos extract and Chlorhexidine 0.1% are able to destroy the pathogenic microorganism and inhibits the development of the bacterial biofilm which contributes to the main reason gingivitis occurs.[13,36] The destruction of the bacteria causes a decrease in the ROS production released by the PMN leukocytes. Hence, when the amount of bacterial plaque decreases, there is a decrease in ROS production as well, which will then lead to a decrease in the inflammation of the gingiva. Lower ROS increases the amount of GSH present in the saliva.[29] This is possible because the production of GSH occurs naturally in the body, whereas the decrease in ROS until the GSH activity scavenges ROS will reduce as well; hence, there is an increase of GSH saliva after gargling with the mouthwash.

Gargling with 5% Cosmos extract and Chlorhexidine 0.1% can increase the saliva GSH level in moderate gingivitis patients. Both mouthwashes may have the same effect in increasing the rate of healing gingivitis even though the mechanism for each is different. The mechanism for the 5% Cosmos extract is due to the presence of antioxidants and antibacterial effect whereas the mechanism for Chlorhexidine 0.1% is due to the antibacterial strength that reduces the accumulation of plaque-induced gingivitis. It is hoped that both mouthwashes will be able to be used for anti-inflammation purposes that contribute to increase the rapid healing processes of gingivitis which can be seen when there is an increase of saliva GSH.

However, in this study, there are limitations, such as the fact that it used a sample size of 20 subjects. Therefore, increasing the sample size could produce a more accurate result and better significance value. Obtaining the best young Cosmos leaves was also a limitation as there are many varieties of Cosmos plants that may influence the results. Besides that, this study was conducted for those with moderate gingivitis, and a more accurate result could have been achieved for chronic gingivitis patients. Furthermore, this study has not yet been carried out to verify the hypersensitivity of mucous membranes.

CONCLUSION

Based on the result of this study, it can be concluded that saliva GSH can increase after gargling with 5% Cosmos extract mouthwash. The effect of the Cosmos extract is the same with Chlorhexidine 0.1%. Further study can occur regarding the application of Cosmos leaves in order to increase GSH during the healing processes of gingivitis and be more user-friendly. Hypersensitivity test regarding the effect of Cosmos

extract towards the systemic condition of an individual can also be carried out, so that an optimum manipulation of the plant could be obtained.

Conflict of interest

There is no conflict of interest

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