Research Paper

In vitro Antimicrobial Activity and Phytochemical Screening of *Clematis* Species Indigenous to Ethiopia

S. HAWAZE, H. DETI* AND S. SULEMAN
School of Pharmacy, College of Public Health and Medical Sciences, Jimma University, P. O. Box 378, Jimma, Ethiopia

The leaves extracts of two indigenous plants of Ethiopia: *Clematis longicauda* steud ex A. Rich. and *Clematis burgensis* Engl. are used in Southwestern Ethiopia to treat otorrhoea and eczema. Antimicrobial activity and MIC of crude extracts were determined by disk diffusion and broth dilution. Phytochemical screening was performed on the extracts. The methanol and petroleum ether extracts of both plants showed antibacterial and antifungal activity. Sensitivity of reference strains was concentration dependent. Methanol and petroleum ether extracts of *C. burgensis* leaves exerted greater inhibitory effects than *C. longicauda* extracts whereas aqueous extracts of both plants were inactive. The MIC study revealed a concentration of 0.78 mg/ml on bacteria and 3.125 mg/ml on fungi for methanol extract and 1.56 mg/ml on both fungi and bacteria for petroleum ether extract. Phytochemical screening results indicated the presence of proteins, fixed oils, carbohydrates, tannins, saponins, flavonoids, and steroids. Preliminary chromatographic investigation showed fluorescing spots with R_f values that ranged from 0.05 to 0.96 for phenolic compounds and saponins. As the study is one of the first reports on the two indigenous species of *Clematis*; isolation, purification and characterization of the different primary and secondary metabolites may further yield alternative options to the microbial chemotherapy.

Key words: ***Clematis longicauda, Clematis burgensis,* phytochemical screening, antimicrobial activity, ethiopia**

The use of medicinal plants as sources of medicine have been documented in all cultures[1]. The use of traditional medicine (TM) has expanded globally and is gaining popularity in the last decade. It has continued to be used not only for primary health care of the poor in developing countries, but also in countries where conventional medicine is predominant in the national health care system[2].

About 80% of the world's population relies on herbal medicines to meet the health needs. This is quite significant for millions of people in the vast rural areas of developing countries[3]. In Ethiopia, traditional remedies represent not only part of the struggle of the people to fulfil their essential drug needs but also they are integral components of the cultural beliefs and attitudes[4].

Ethiopia has an enormous source of plant species, which are used in traditional medicine. More than 80% of the population has been relying on traditional medicine and greater than 95% of medicinal preparations are of plant origin[5]. It is also estimated that the flora of Ethiopia contain between 6,500 and 7,000 species of higher plants which have been used by traditional healers for the treatment of human as well as animal diseases[6,7] . Despite its significant contribution to society, TM has received very little attention in modern research and development and less effort has been paid to upgrade the traditional health practices in the country. But, the long history of use of medicinal plants in Ethiopia and its huge biotic riches can be of paramount importance in future research and drug discovery[8].

In this study, two indigenous species of plants, namely: *Clematis longicauda* steud ex A. Rich. and *Clematis burgensis* Engl. (Ranunculaceae), having traditional claims for the treatment of ear and skin disorders were investigated for their antimicrobial activities on bacterial and fungal strains. In addition, we conducted a phytochemcial screening on the methanol and petroleum ether extracts.

Plants in the genus Clematis are climbing or trailing shrubs. The leaves are opposite, pinnate or bipinnate with petioles, petiolules and rachis capable of twining

***Address for correspondence**
E-mail: habtewold.deti@ju.edu.et

round supports. *Clematis longicauda* steud ex A. Rich. grows in open montane forest and forest borders, along roads, streams and on fences and in woodland associations. It is endemic and widely distributed in different parts of Ethiopia at an altitude of 1300-1500 m. *Clematis burgensis* Engl. is associated with shrubs near a dry river bed; and grows in some parts of Ethiopia up to an altitude of 1700 m[9]. Leaves of *C. longica*uda (Vernacular name: *Fitti*) and *C. burgensis* (Vernacular name: *Fitti*) are used by people in Southwestern Ethiopia, to treat Otorrhoea *(Dhukuba Gurra)* and Eczema *(Chiifee, Abiyatoo)*.

As the plants are endemic and there is no previous studies on the plants as far as our knowledge is concerned, obtaining scientific data from literature is difficult. Therefore, this investigation was undertaken with the objective to provide scientific evidence on the traditional claim of antimicrobial effect of these plants and lay down basis for the search for new chemotherapeutic agents.

MATERIALS AND METHODS

The leaves of *C. longicauda* and *C. burgensis* (fig. 1) were collected on November, 2010 from Unquuree and Caala (fig. 2), Sekoru district, Southwestern Ethiopia. The areas lie at an altitude range of 1693–1740 m above sea level and have a dry and hot climate with a mean annual temperature of 19.2° and annual rainfall that varies from 1300–1800 mm. Clay soil with a thin layer of humus top soil is the main soil type over the area and evergreen montane thickets and shrubs are characteristic vegetation types of the area. Botanical identity of collected plants leaves were confirmed in Jimma University Herbarium by Dr. M. Remesh (Taxonomy and Ethnobotany specialist); and voucher specimen was deposited as #00104 and #00103 respectively.

Fig. 1: Pictures of Clematis species.
Picture of *Clematis longicauda* steud ex A. Rich (a) and *Clematis burgensis* Engl. (b) photographed in *Unquuree* and *Caala*, Sekoru district, Jimma zone, South western Ethiopia on November 2010

Extraction:
The collected leaves were washed in running tap water, shed dried, powdered and used for extraction. 70 g of powder was soxhelet extracted with petroleum ether (BDH® chemicals ltd., Pool, England) and methanol (analytical reagent grade, Applichem GmbH, Germany); and it was macerated with distilled water, in order of increasing polarity. Each extract was collected and concentrated under reduced pressure using rotary vacuum evaporator (Bibly Sterilin ltd., Staffordshire, England) at 40° and the semi-solid mass was dried in an oven (Memmer GmbH co.KG, Germany) at 40° for 10 days; and then physical properties were recorded and the percentage yield was calculated.

Microbial reference strains and culture preparation:
Staphylococcus aureus ATCC® 25923, Pseudomonas aeruginosa ATCC® 27853, and *Candida albicans* (clinical isolate) were used for the investigation of antimicrobial activity. All reference strains were obtained from Ethiopian Health and Nutrition Research

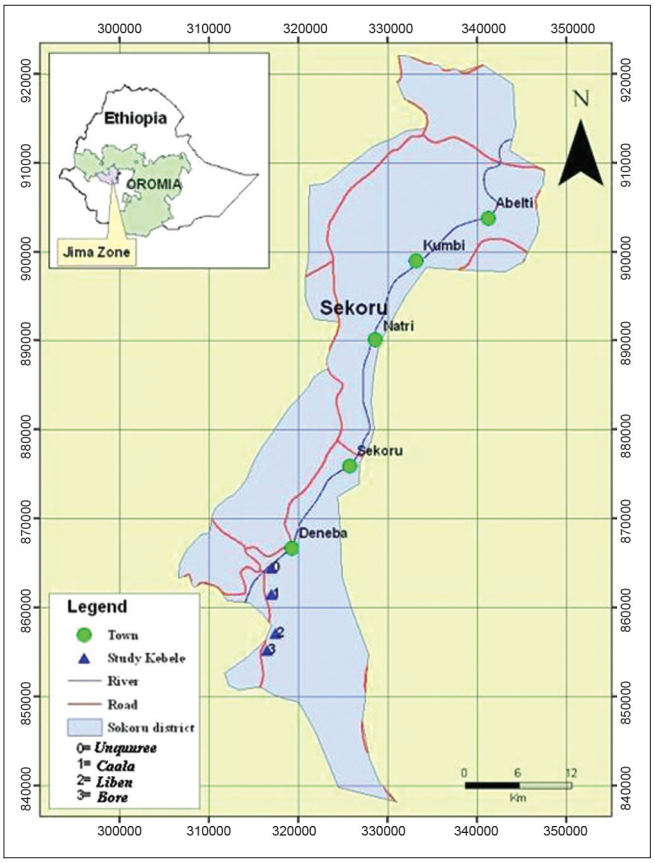

Fig. 2: Map showing sites from where the plant materials to be studied were collected in Southwestern Ethiopia.
(Adapted from 'Traditional medicinal plant knowledge and use by local healers in Sekoru District, Jimma Zone, Southwestern Ethiopia' by Yinger H. and Yewhalaw D.[8])

Institute (EHNRI). Nutrient broth, Mueller Hinton agar and Sabouraud dextrose agar (Hi-media laboratories Pvt. ltd, Mumbai, India) were prepared according to manufacturer's instruction. Inoculates were prepared using the direct colony suspension method[11]. Well isolated colonies of bacteria (from overnight growth) or fungi (from 72 h old culture) were transferred into test tube containing 5 ml of nutrient broth. The amount of bacteria or fungi needed to undertake the study was determined using UV/Vis spectrophotometer (CECIL instruments, Cambridge, England) at 625 nm so that the absorbance of the suspension was held between 0.08 and 0.1 which was assumed to contain 1-2×10^8 CFU/ml.

Controls and standard disks:
Solvent impregnated disks; standard disks of gentamicin and vancomycin (Oxoid Ltd, Hampshire, England) and ketoconazole (prepared in the laboratory from ketoconazole powder, working standard, Cipla ltd, Mumbai, India) were used in the course of the study.

Antimicrobial activity test:
Paper disk diffusion method described by Choudhury *et al* was used[12]. From 100 mg/ml stock solutions of extracts, different concentrations (250, 500 and 1000 µg) were loaded onto sterilized paper disks (Whatman filter paper, 6 mm in diameter). The crude extract loaded disks and solvent impregnated disks were dried in an oven (Memmer GmbH co. KG, Germany) at 37º for 6 h. The crude extract impregnated disks, solvent impregnated disks (negative controls), and standard disks (positive controls) were placed inverted by their bioactive face on the seeded plates inside microbiological safety cabinet (Labcaire systems ltd., England). Each disk was pressed down to achieve utter contact with the agar surface. The centers of the disks put on the agar surface were at least 24 mm apart[13]. The media were incubated at 37º (for bacteria) for 24 h and at 25º (for fungi) for 48 h and the test was carried out in triplicates. Evaluation of antimicrobial activity was done by observing the clear zone around disks and measuring the diameter of the zone of inhibition around disks in mm by sliding digital vernier calliper.

Determination of minimum inhibitory concentration (MIC):
The Vollekova *et al*. method modified by Usman *et al*. was employed[14,15]. Each inoculum was prepared in nutrient broth and density of bacteria/fungi was adjusted as described above. In this method, the broth dilution technique was utilized where the plant extract was prepared to concentration of 100 mg/ml (stock solution) in respective extracting solvent and serially diluted (two-fold) to a working concentration ranging from 0.195 to 25 mg/ml using nutrient broth and later inoculated with 0.2 ml suspension of reference strain. After incubation, the test tubes were observed for turbidity and the least concentration where no turbidity was observed was noted as MIC value.

Preliminary phytochemical screening:
Extracts were tested for the presence of primary metabolites and secondary metabolites using different color tests by Oguyemi and Debela[16,17].

Thin layer chromatography:
Separation of phenolic compounds and saponins from methanol extracts of both plants was carried out by TLC using pre-coated silica gel 60 F254 plates Whatman ltd., Kent, England after the methanol extracts were fractionated into Solution A (lipophilic substances fractionated with chloroform/acetic acid; 99:1), Solution B (lipophilic and slightly hydrophilic substances fractionated with methanol/chloroform/acetic acid; 49.5:49.5:1), and Solution C (hydrophilic substances fractionated with methanol/water; 1:1). Solvent system for saponins [petroleum ether/chloroform/acetic acid (7:2:1), ethyl acetate/pyridine/water (3:1:3), and n-butanol/acetic acid/water (4:1:5)] and solvent system for phenolic compounds [benzene/methanol/acetic acid (45:5:8), n-butanol/acetic acid/acetone (17:1:2), and hexane/ethyl acetate (3:1)] were used to resolve components as described by Debela[17]. The detection of spots was performed in light-tight UV cabinet with UV lamp (St. John's innovation center, Cambridge, England) under UV light (365 nm). Rf value of each spot was calculated as R_f=Distance travelled by the solute/Distance travelled by the solvent on the TLC.

RESULTS AND DISCUSSION

The physical characteristics and percentage yield of the gradient extracts are summarized in Table 1. As indicated in the table, there were differences in the yield of extraction products which might be due to polarity difference of solvents used for extraction, solubility of various ingredients, and type of extraction method used[18]. The highest yield by methanol gradient

is indicative of its extracting ability and the polarity of compounds found in the plants and furthermore, these methanol gradient extracts have prominent antimicrobial activity.

The concentration of the extracts and inhibitory effects is indicated in Table 2. The study revealed that the inhibitory effects appeared at concentrations >250 µg for methanol and petroleum ether gradient extracts of both plants. The aqueous gradient extract of both plants were found to be inactive.

The petroleum ether gradient extracts of both plants were more effective against bacterial strains while methanol gradient extracts were more active against fungus. This indicates presence of more than one active principle. It is observed that a single plant is known to contain several active principles of biological significance[19]. The Gram-positive bacterium *(S. aureus)* was more sensitive to methanol and petroleum extracts of both plants than the Gram negative (*P. aeruginosa*). The higher sensitivity of Gram-positive bacteria could be attributed to their outer peptidoglycan layer which is not an effective permeability barrier. Gram-negative bacteria having an outer phospholipid membrane carrying the structural lipopolysaccharide components make the cell wall impermeable to lipophilic solutes while porins constitute a selective barrier to hydrophilic solutes with an exclusion limit of 600 Da[20]. The sensitivity of reference strains to both plants for their methanol and petroleum ether gradient extracts was concentration dependent. Both methanol and petroleum ether gradient extracts of *C. burgensis* leaves exerted greater inhibitory effects than that of *C. longicauda*. This might be due to higher yields from *C. burgensis.*

The gradient extracts which showed antibacterial and antifungal activity were selected further to determine MIC. The MIC of the methanol and petroleum ether extracts of *C. longicauda* and *C. burgensis* leaves against *S. aureus, P. aeruginosa,* and *C. albicans* is presented in (Table 3).

Qualitative phytochemical screening showed presence of primary metabolites and secondary metabolites (Table 4). Steroids and fixed oils were only present in the petroleum ether gradient extracts while proteins, carbohydrates, and flavonoids were present in all extracts of both plants. Saponins and tannins were present only in the methanol and water gradient extracts of both plants. The secondary

TABLE 1: PHYSICAL CHARACTERISTICS AND PERCENTAGE YIELD OF EXTRACTS FROM THE LEAVES OF *C. LONGICAUDA* AND *C. BURGENSIS*

Plant species	Type of extract	Texture	Color	Odor	Percentage yield (w/w)
C. longicauda	Methanol	Non sticky	Blackish green	Peculiar	24.71
	Water	Sticky	Brownish	Peculiar	22.71
	Petroleum ether	Oily	Dark green	Peculiar	2.14
C. burgensis	Methanol	Non sticky	Blackish green	Peculiar	27.85
	Water	Sticky	Brownish	Peculiar	24.43
	Petroleum ether	Oily	Dark green	Peculiar	2.57

TABLE 2: ANTIMICROBIAL ACTIVITY OF THE GRADIENT EXTRACTS OF *C. LONGICAUDA STAUD* AND *C. BURGENSIS LEAVES*

Type of paper disk	Amount (µg/disk)	*C. longicaudaA Steud*			*C. burgensis*		
		Zone of inhibition (Mean±SD)			Zone of inhibition (Mean±SD)		
		Sa	*Pa*	*Ca*	*Sa*	*Pa*	*Ca*
Methanol extract impregnated disks	250	2.30±0.07	1.74±0.07	6.74±0.08	3.18±0.07	2.47±0.05	7.45±0.05
	500	3.13±0.07	3.00±0.05	8.42±0.06	5.42±0.06	4.82±0.07	12.90±0.08
	1000	3.98±0.06	3.74±0.06	13.01±0.07	7.25±0.08	5.93±0.07	15.32±0.06
Petroleum ether extract impregnated disks	250	5.86±0.05	4.89±0.05	3.45±0.06	5.91±0.05	4.94±0.07	4.44±0.05
	500	7.14±0.08	6.69±0.06	6.14±0.07	7.23±0.06	7.11±0.05	7.81±0.08
	1000	9.11±0.07	9.49±0.07	8.53±0.05	9.15±0.05	9.65±0.05	10.55±0.07
Methanol impregnated disks	30	0±0	0±0	0±0	0±0	0±0	0±0
Petroleum ether impregnated disks	30	0±0	0±0	0±0	0±0	0±0	0±0
Gentamicin impregnated disks	10	16.46±0.08	10.67±0.06		16.46±0.08	10.67±0.06	
Vancomycin impregnated disks	30	11.08±0.07	8.72±0.05	Not Done	11.08±0.07	8.72±0.05	Not Done
Ketoconazole impregnated disks	20	Not Done		22.17±0.08	Not Done		22.17±0.08

Ca -Candida albicans; Pa- Pseudomonas aeruginosa; Sa- Staphylococcus aureus

TABLE 3: MINIMUM INHIBITORY CONCENTRATION OF DIFFERENT EXTRACTS OF *C. LONGICAUDA AND C. BURGENSIS*

	Microorganisms	Type of extract	Concentration (mg/ml)							
			0.195	0.39	0.78	1.56	3.125	6.25	12.5	25
C. longicauda	Sa	Methanol	–	–	–	–	+	+	+	+
		Petroleum ether	–	–	–	+	+	+	+	+
	Pa	Methanol	–	–	–	–	+	+	+	+
		Petroleum ether	–	–	–	+	+	+	+	+
	Ca	Methanol	–	–	+	+	+	+	+	+
		Petroleum ether	–	–	–	+	+	+	+	+
C. burgensis	Sa	Methanol	–	–	–	–	+	+	+	+
		Petroleum ether	–	–	–	+	+	+	+	+
	Pa	Methanol	–	–	–	–	+	+	+	+
		Petroleum ether	–	–	–	+	+	+	+	+
	Ca	Methanol	–	–	+	+	+	+	+	+
		Petroleum ether	–	–	–	+	+	+	+	+

[–] Resistance (growth of bacteria/fungi or turbidity) [+] Concentrations show no turbidity (inhibition of bacterial/fungal growth)

TABLE 4: RESULTS OF THE PRELIMINARY PHYTOCHEMICAL SCREENING OF THE GRADIENT EXTRACTS

Plant species	Phytochemicals screened	Plant extracts		
		Methanol	*Water*	*Petroleum ether*
C. longicauda	Proteins	+	+	+
	Fixed oils	–	–	+
	Carbohydrates	+	+	+
	Tannins	+	+	–
	Saponins	+	+	–
	Alkaloids	–	–	–
	Flavonoids	+	+	+
	Cardiac glycosides	–	–	–
	Steroids	–	–	+
C. burgensis	Proteins	+	+	+
	Fixed oils	–	–	+
	Carbohydrates	+	+	+
	Tannins	+	+	–
	Saponins	+	+	–
	Alkaloids	–	–	–
	Flavonoids	+	+	+
	Cardiac glycosides	–	–	–
	Steroids	–	–	+

[+] Indicates Presence of phytochemicals [–] Indicates absence of phytochemicals

metabolites detected in methanol and petroleum ether gradient extracts could be responsible for the observed antimicrobial activity though the exact mode of action is not clearly understood. Polyphenolic compounds like flavonoids exhibit various biological activities. Polyphenols are strong antioxidants and free radical scavengers[21]. Flavonoids have also been reported to possess antibacterial activity, which could be attributed to their ability to form complex with extracellular, soluble proteins and bacterial cell wall[22]. Tannins, another class of polyphenolic compounds which are believed to have an astringent property, play a great role in healing of microbial associated inflamed surface of mouth and throat[21]. They result antimicrobial action by precipitating microbial protein[23]. Saponins, a special class of glycosides; have showed antibacterial activity against *S. aureus*[24]. Phytosterols, natural plant estrogens, are believed to have an abortifacient property and are expected to be new research areas in the development of safe and effective chemicals for legal medical abortion. Similar to previous phytochemical investigations done on Clematis species[25-33], the preliminary phytochemical screening of the gradient extracts of *C. longicauda* and *C. burgensis* leaves revealed the presence of saponins, and flavonoids. But unlike some studies done on the genus Clematis[33] alkaloids and cardiac glycosides were not present.

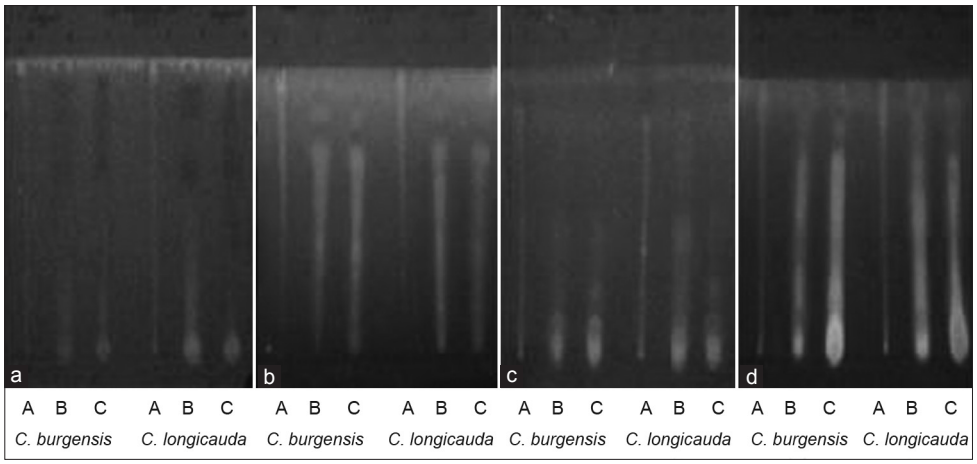

Fig. 3: TLC of *C. longicauda* and *C. burgensis* leaves methanol gradient extract fractions.
Mobile phase used was (a) ethyl acetate/pyridine/water (3:1:3), (b) n-butanol/acetic acid/water (4:1;5), (c) benzene/methanol/acetic acid (45:5:8) and (d) n-butanol/acetic acid/acetone (17:1:2)

TABLE 5: R_F VALUES OF SAPONINS AND PHENOLIC COMPOUNDS IN METHANOL GRADIENT EXTRACT FRACTIONS OF *C. LONGICAUDA* AND *C. BURGENSIS*

Plants	Solution A	Solution B	Solution C
C. longicauda	0.96[1], 0.93[2], 0.95[3], 0.77[3], 0.45[3], 0.93[4]	0.96[1], 0.63[1], 0.21[1], 0.95[2], 0.89[2], 0.69[2], 0.39[2], 0.45[3], 0.17[3], 0.08[3], 0.93[4], 0.69[4], 0.53[4], 0.25[4]	0.89[1], 0.63[1], 0.68[2], 0.6[2], 0.37[2], 0.45[3], 0.17[3], 0.08[3], 0.71[4], 0.64[4], 0.51[4], 0.23[4]
C. burgensis	0.96[1], 0.93[2], 0.93[3], 0.8[3], 0.45[3], 0.93[4]	0.96[1], 0.63[1], 0.95[2], 0.89[2], 0.69[2], 0.39[2], 0.45[3], 0.12[3], 0.93[4], 0.68[4], 0.4[4], 0.08[4]	0.89[1], 0.63[1] , 0.68[2], 0.29[2], 0.43[3], 0.12[3], 0.05[3], 0.93[4], 0.72[4], 0.63[4], 0.37[4], 0.08[4]

[1] Ethyl acetate/pyridine/water (3:1:3); [2] n-butanol/acetic acid/water (4:1:5); [3] benzene/methanol/acetic acid (45:5:8); [4] n-butanol/acetic acid/acetone (17:1:2)

The separation of saponins and phenolic compounds in methanol gradient extract fractions of both plants was performed on TLC plates; the numbers of spots along with their travelled distance are shown in (fig. 3 and Table 5). Saponins were best resolved in ethyl acetate/pyridine/water (3:1:3) and n-butanol/acetic acid/water (4:1:5) solvent systems, while phenolic compounds were resolved better in benzene/methanol/acetic acid (45:5:8) and n-butanol/acetic acid/acetone (17:1:2). The spots fluoresced blue, purple and red when observed under UV light (365 nm). The components could not be resolved in hexane/ethyl acetate (3:1) and petroleum ether/chloroform/acetic acid (7:2:1).

As the study is one of the first reports on the two indigenous species of Clematis; isolation, purification and characterization of the different primary and secondary metabolites may further yield alternative options to the microbial chemotherapy.

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Accepted 16 January 2012
Revised 06 January 2012
Received 10 August 2011
Indian J. Pharm. Sci., 2012, 74 (1): 29-35