Phytochemical screening, GC-MS analysis and antimicrobial evaluation of Ambrex (an herbal formulation)

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ABSTRACT

The present investigation was aimed to analyze the bioactive constituents present in Ambrex and evaluate its potentiality on distinct microorganisms. 'Ambrex' is a herboamber drug having therapeutic effects with a composition of four indigenous herbs *Withania somnifera* (Ashwagandha), *Orchi smascula* (Shalamishri), *Shorea robusta* (Roomimastagi), *Cycas circirnalis* (Madankamappu) with amber (a resin from *Pinus succinifera*). Qualitative phytochemical analysis was carried out by extracting 5 gm powdered drug with petroleum ether, chloroform, ethyl acetate and methanol successively. The presence or absence of different phytoconstituents was detected. Quantitative analysis of phenolic compounds, tannins, and flavonoids were determined in the aqueous Ambrex extract. Antimicrobial susceptibility assay was carried out on seven distinct microorganisms. Finally the aqueous Ambrex extract was subjected to GC-MS analysis. Preliminary phytochemical screening of Ambrex extract exhibited high content of flavanoids and carbohydrates. In the antimicrobial susceptibility assay maximum zone formation was observed with *Bacillus subtilis* and *Trichophytonrubrum*. GC-MS analysis of Ambrex extract revealed the presence of six different compounds. Ambrex demonstrated the presence of potential bioactive constituents and antimicrobial activity. The major component was found to be Methyl commate A in GC-MS analysis.

KEYWORDS: GC-MS; antimicrobial; phytochemical screening; Ambrex.

1. INTRODUCTION

The side effects of allopathic drugs have led to look for products of natural origin to cure many diseases. Almost 8,000 species of medicinal plants are being used in India for betterment of health (Anon, 1996). Plants as antimicrobials are rather effective in the treatment of infectious diseases while simultaneously avoiding many of the side effects that are associated with synthetic antimicrobial drugs (Chang, 2002). The therapeutic potential of medicinal plants relies on the presence of bioactive constituents constituting certain physiological and pharmacological activity. In recent years, the extensive use of antimicrobial drugs is considered to be the reason for multiple drug resistance in human pathogenic microorganisms (Chung, 1998; Evans, 2002). Over the past twenty years, interests are developed in the investigation of natural materials as sources of new antibacterial agents (Fluck, 1973). Many reports have showed the effectiveness of traditional herbs against microorganisms, as a result, plants are one of the bed roses for modern medicine to attain new principle (Girish and Satish, 2008).

A polyherbal formulation - Ambrex which is being used traditional medicine was chosen for our present study. Ambrex is a herboamber drug which is a cocktail of four indigenous herbs *Withania somnifera, Cycas circirnalis, Shorea robusta,* and *Orchis mascula* with pon amber (a resin from *Pinus succinifera*). *Withania somnifera* (Ashwagandha) has intense anti-fungal properties. Powdered root of *Cycas circirnalis* (Madanakamappu) is considered to be an invigorating and nutritive tonic for healthy men and for children emaciated by famine or disease. *Shorea robusta* (Roomimastagi) mastic gum shows an effective bactericidal as well as anti-fungal activity. *Orchis mascula* (Shalamishri) was used as a great restorative and invigorator, as a tonic. Therefore, Ambrex, which is combination of all the herbs above, might have good medicinal properties. The objective of this research is to investigate the phytochemicals and the bioactive constituents present in Ambrex and to evaluate its potentiality on distinct micro organisms.

2. MATERIALS AND METHODS

2.1. Selection of plant material: Ambrex, a standard Siddha propriety medicine duly certified from Quality Control Department was obtained as a gift sample from Care and Care Herbs Ltd, Chennai. Four indigenous herbs viz. *Withania somnifera, Cycas circirnalis, Shorea robusta,* and *Orchis mascula* with amber, a resin from *Pinus succinifera* were purchased by standard suppliers in open market, identified by a botanist and shade dried. All the above ingredients are separately powdered in micropulveriser and sieved through fine mesh and were used for the preparation of aqueous Ambrex extract.

2.2. Chemicals and Reagents: Folin's phenol reagent, Molisch's reagent, ferric chloride, sodium hydroxide chloroform, 10% ammonia solution, glacial acetic acid, ninhydrin, methanol, BHT, L-Ascorbic Acid, Libemann-Buchard reagent, sodium nitro prusside, sulphanilamide, DPPH (2,2-diphenyl-1-picrylhydrazyl) was purchased from Sigma, USA, and all other reagents were purchased from Sisco Research Laboratories Pvt. Ltd., India.

2.3. Preparation of Ambrex extract: Fine powder of Ambrex was extracted using aqueous extraction procedure. About 15 grams of powder was added to 90 ml of distilled water in a ratio 1:3 in a dry flask. The flask was then incubated for 1 hr in a water bath at 100˚C. After incubation, the extract was collected using Whatman no. 1 filter paper and evaporated below 50˚C. To the residual mixture, another 50 ml of distilled water was added and incubated in a water bath for 1 hr**.** The extract was collected again using Whatman filter paper no. 1 and evaporated below 40˚C, which was used for further phytochemical analysis. The extract obtained was then concentrated and the final extract was stored in desiccators for further analysis.

2.4. Qualitative and quantitative phytochemical analysis: For qualitative phytochemical analysis, 5gm powdered drug was extracted with petroleum ether, chloroform, ethylacetate and methanol successively. The extracts were dried and weighed. The

presence or absence of different phytoconstituentsviz, triterpenoids, alkaloids, steroids, sugar, tannin, glycosides and flavonoids etc. were detected by usual prescribed methods (Linuma et al., 1994). The amount of Phenolic compounds (Mallika and Shyamala, 2004), tannins (McDonald et al., 2001), and Flavanoids (Mohanasundari et al., 2007) were determined in the aqueous Ambrex extract.

2.5. Antimicrobial susceptibility assay: Seven distinct micro-organisms, namely, *Escherichia coli, Serratiamarcescens, Staphylococcus aureus, Aeromonas hydrophila, Pseudomonas aeruginosa, Bacillus subtilis* and *Trichophyton rubrum* were sub-cultured overnight to obtain fresh cultures. Antibacterial activity of aqueous extract was determined by well diffusion method on Mueller-Hinton agar medium (Prashant et al., 2011)**.** Plates were inoculated with test pathogens and three wells were punched, for extract, positive control and negative control. Ciproflaxin was used for positive control. Dimethyl Sulphoxide (DMSO) was used as negative control. The plates were tightly wrapped and incubated for 24 hours. Zone of inhibition if any around the wells was measured in mm.

2.6. GC- MS analysis: The aqueous ambrex extract was filtered in ashless filter paper with sodium sulphate (2 g) and the extract was concentrated to 1 ml by bubbling nitrogen into the solution. The Clarus 500 GC used in the analysis employed a column packed with Elite-1[100% Dimethyl poly siloxane, 30nm X 0.25 nm ID X 1µm df] and the components were separated using helium (1 ml / min) as carrier gas. The extract injected into the instrument was detected by the turbo mass gold mass detector. The injector temperature was set at 250°C (mass analyzer). The different parameters involved in the operation of Clarus 500 MS, were also standardized (Inlet line temperature: 200°C; Electron energy: 70eV; mass scan: (m/Z) 45-100). The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The aqueous extract components were identified by comparing their relative retention times and mass spectra with those of authentic sample (analytical standards from data base).

3. RESULTS

3.1. Qualitative and quantitative phytochemical analysis: The Ambrex extract was obtained through aqueous extraction. Yield of the extract was 0.246g/g of Ambrex powder. The preliminary phytochemical tests are performed to detect the presence of phytochemicals, if any. Ambrex, being a polyherbal drug, contains varied phytochemicals. From the tests it is seen that, aqueous extract of Ambrex exhibited high content of flavonoids and carbohydrates. Phenols, tannins, saponins, quinines, cardiac glycosides, terpenoids, coumarins, and steroids were present in small amounts; whereas glycosides, alkaloids, anthroquinones, phlobatannins, phytosteroids and triterpenoids were absent (Table 1).

Table 1: **Qualitative Phytochemical Analysis of Aqueous Ambrex Extract**

Phytochemicals	Observation	Inference
Carbohydrates	Purple color.	++
Tannins	Dark blue color initially.	+
Saponins	Layer of foam was formed.	+
Flavanoids	Yellow color.	++
Alkaloids	No green/white color precipitate.	_
Quinones	Red color.	+
Glycosides	No pink color formed.	_
Cardiac Glycosides	Brown ring at the surface.	+
Terpenoids	Reddish-brown color at interface.	+
Triterpenoids	Absence of blue-green color.	_
Phenols	Bluish black color.	+
Coumarins	Yellow color.	+
Steroids	Brown ring.	+
Phytosteroids	Absence of bluish-brown ring.	_
Ninhydrin test	Mild blue color.	+
Anthraquinone	Absence of pink color.	_
Phlobatannins	No red color precipitate.	_

+ Low, ++ Moderate, — Absence

It was evident from the test that the aqueous extract has good amount of phenolic compounds. Total phenolic content as determined in Ambrex extract was 129 µg gallic acid equivalent /g of dry weight of extract. Total tannin content of aqueous extract was found to be 16 µg tannic acid equivalent /g dry weight of extract. Since flavonoids are a class of secondary plant phenolics with powerful antioxidant properties, it would be valuable to determine the flavonoid contents of the aqueous extract under study. Total flavonoid content of aqueous extract was 149 µg quercitin equivalent /mg dry weight of extract (Fig. 1).

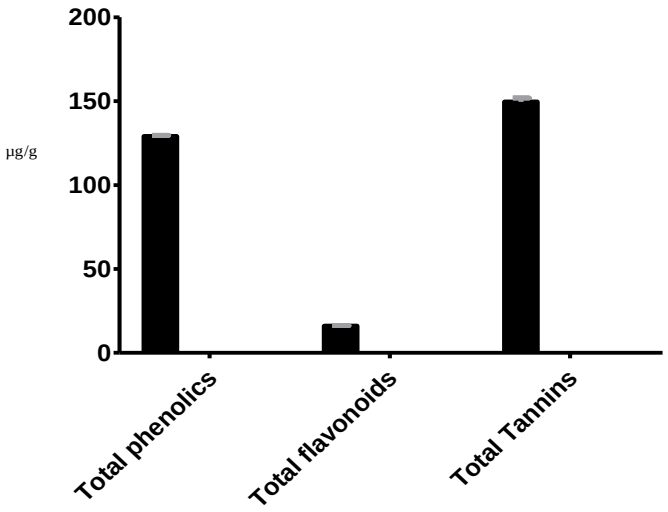

Figure 1: Quantitative Analysis Of Aqueous Ambrex Extract
Values are mean ± SEM, n=3

3.2. Antimicrobial activity of Ambrex extract: The antimicrobial activity of Ambrex was measured with seven distinct species. For 50mg/ml of the extract, all the species were considerably sensitive. Distinct, separate zone formation was observed and high amount of antimicrobial activity was observed when compared with positive control ciproflaxin. Negative control DMSO (Dimethyl sulfoxide) showed very meager zone formation. Maximum zone formation was observed with *Bacillus subtilis* and *Trichophyton rubrum* (Table 2).

Table 2: Antimicrobial Activity Of The Aqueous Ambrex Extract

Organism	Extract (mm) (Conc–50mg/ml)	Positive control (mm)	Negative control (mm)
Pseudomonas aeruginosa	20	32	2
Aeromonashydrophila	19	40	5
Escherichia coli	19	38	4
Staphylococcus aureus	17	36	2
Serratiamarcescens	20	37	3
Bacillus subtilis	21	38	2
Trichophytonrubrum	21	45	3

3.3. GC-MS analysis: The aqueous extract of Ambrex was further studied by GC-MS. GC-MS analysis of Ambrex extract revealed the presence of six different compounds namely Undecane; 1-Octanol, 3,7-dimethyl ester; Hexadecanoic acid, Methyl ester; Octadecanoic acid, Methyl ester; Oxirane, 2,2-dimethyl-3-[3,7-dimethyl-9-(phenylthio)-3,7-nonadienyl]; Methyl commate A. Retention times and relative percentage of the compounds present in aqueous extract were recorded in (Fig. 2). Among the identified compounds Methyl Commate A was present in an appreciable amount.

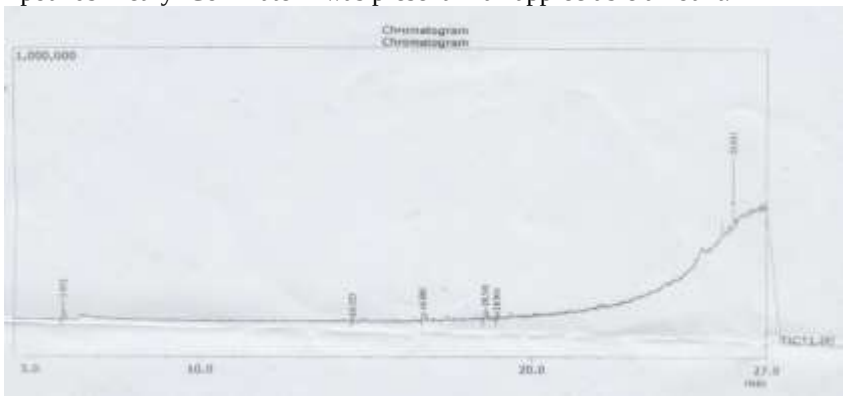

Figure 2: GC-MS analysis of aqueous Ambrex extract revealed the presence of six different compounds.
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Table 3: Peak report of Total ion chromatogram

Peak	Retention time	Area	Area % Name
1	5.952	128975	13.04 Undecane
2	14.523	9500	0.96 1- Octanol, 3, 7- dimethyl ester
3	16.681	115972	11.73 Hexadecanoic acid, methyl ester
4	18.591	93937	9.50 octadecanoic acid, methyl ester
5	18.941	47546	4.81 Oxirane 2,2 – dimethyl – 3-(3,7 – dimethyl -9-(phenylthio)-3,7- nonadienyl)
6	26.011	592776	59.95 Methyl commate

4. DISCUSSION

Ambrex is found to be nontoxic to the vital organs under the experimental conditions in rats. Ambrex exhibited no hepato, renal and nervous toxicities with the therapeutic dosage under experimental conditions in rats. It appears to be a safe compound. Preliminary studies with ambrex show that it exhibits a variety of beneficial effects with little or no associated toxicity (Johanna R, 2014).

In phytochemical screening, aqueous extract of Ambrex was found to contain saponins, tannins, quinones, terpenoids, phenols, coumarins, steroids, glycosides and flavanoids. The antimicrobial study by agar well diffusion method showed that the plant has an antimicrobial activity comparable to that of commercial antibiotic ciproflaxin. *Staphylococcus aureus* was found to be comparatively less resistance to antibacterial activity. This may be due to the fact that *Staphylococcus aureus*has intrinsic resistance from a restrictive outer membrane barrier and transenvelope multidrug resistance pumps (MDRs). All others microorganisms was found to be more resistant to Ambrex extract (Rastogi and Mehrotra, 2002).

The antimicrobial property was claimed to be conferred by phytochemicals present in the plant. Tannins have been reported to prevent the development of microorganisms by precipitating microbial protein and making nutritional proteins unavailable for them (Raveendra, 2007). The growth of many fungi, yeasts, bacteria and viruses was inhibited by tannins (Sadipo, 1991). Flavonoids displayed a remarkable array of biochemical and pharmacological actions viz. anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral and anticarcinogenic activities. Flavonoides are also shown to inhibit microbes which are resistant to antibiotics (Samy and Ignacimuthu, 2000). Saponins are a special class of glycosides which have soapy characteristics (Schanderl, 1970). It has also been shown that saponins are active antifungal agents (Raveendra, 2007). Natural quinones exhibited a biological or pharmacological activity, and some of them showed antitumoral activity. It possessed a number of biological properties, including some claims in herbal medicine. The applications include purgative, anti-micro-bacterial, anti-tumor, inhibition of PGE2 (prostaglandin E2) biosynthesis and anti-cardiovascular disease. Plant terpenoids played a role in traditional herbal remedies and are under investigation for antibacterial, antineoplastic, and other pharmaceutical functions. The steroids and sterols in animals are biologically produced from terpenoid precursors. Sometimes terpenoids are added to proteins to enhance their attachment to the cell membrane; this is known as isoprenylation. Phenolic compounds like phenolic acids, phenols, flavonoids, phenyl proponoids, phenolic quinines acted as antiseptic and anti-inflammatory.

The aqueous extract of Ambrex was analysed using GC-MS. The major component was found to be Methyl commate A. Further pure compound isolation of the extract can be performed. Methyl commate A can be studied in advance for its pharmacological activity and may be used for drug discovery.

An important characteristic of plant extracts and their components is their hydrophobicity, which enables them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable. Extensive leakage from bacterial cells or the exit of critical molecules and ions will lead to death (Vasantha et al., 2012). The results of the present investigation is successful in identifying the antibacterial activity and bioactive component of Ambrex, which will help in further finding the nature of the bioactive principle and its solubility, isolation and characterization of the active principle responsible for the activity.

REFERENCES

Anon, Pharmacopiea of India. Govt. of India, New Delhi, Ministry of Health and Family Welfare. 1996.

Chang C, Yang M, Wen H, Chern J, Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J. Food Drug Analysis, 10, 2002, 178-182.

Chung KT, Wong TY, Wei CI, Huang YW, Lin Y, Tannins and human health: A review. Crit. Rev. Food Science and Nutrition, 38, 1998, 421-464.

Evans CE, Banso A, Samuel OA, Efficacy of some nupe medicinal plants against Salmonella typhi: an in vitro study. J. Ethnopharmacol, 80, 2002, 21-24.

Fluck H, Medicinal plants and their uses. W. Feulshom and comp. Ltd. New York, 1973, 7-15.

Girish HV, Satish S, Antibacterial Activity of Important Medicinal Plants on Human Pathogenic Bacteria-a Comparative Analysis. World Applied Sciences Journal, 5, 2008, 267-271.

Linuma M, Tsuchiya H, Sato, Yokoyama J, Ohyama M, Ohkawa Y, Tanaka T, Fujiwara S, Fujii T, Flavanones with potent antibacterial activity against methicillin-resistant *Staphylococcus aureus*. J. Pharmacol, 46, 1994, 892-895.

Mallika J, Shyamala Devi CS, Effect of an amber-based formulation on gastric mucosal damage: Role of antioxidant enzymes and lipid profile. Indian J Physiol Pharmacol, 48, 2004, 343-347.
McDonald S, Prenzler PD, Antolovich M, Robards K, Phenolic content and antioxidant activity of olive extract. Food Chemistry, 73, 2001, 73-84.
Mohanasundari C, Natarajan D, Srinivasan K, Umamaheswari SA, Ramachandran A, Antibacterial properties of Passiflorafoetida L. a common exotic medicinal plant. African Journal of Biotechnology, 6, 2007, 2650-2653.
Prashant T, Bimlesh K, Mandeep K, Gurpreet K, Harleen K, Phytochemical screening and Extraction: A Review. Internationale pharmaceuticasciencia, 1, 2011, 98-106.
Johanna R, Jamuna Devi A, Rekha R, Evaluation of safety and toxicity of ambrex (an amber based herbal formulation) in rats. International Journal of Future Biotechnology, 3(1), 2014, 1-6.
Rastogi RP, Mehrotra BN, Glossary of Indian Medicinal Plants. National Institute of science communication, New Delhi, India, 2002, 20-25.
Raveendra Retnam K, John Debritto A, Antibacterial activity of a medicinal plant Hybanthusenneaspermus (Linn.) F. Muell, Natural Product radiance, 5, 2007, 366-368.
Sadipo OA, Akanji MA, Kolawole FB, Odutuga AA, Saponin is the active antifungal principle in Garcinia Kola, heckle seed. Biosci. Res. Commun, 3, 1991, 171.
Samy RP, Ignacimuthu S, Antibacterial activity of some folklore medicinal plants used by tribals in Western Ghats of India. J. Ethnopharmacol, 69, 2000, 63-71.
Schanderl SH, Tannins and related phenolics, In: Joslyn M.A. (ed.): Methods in Food Analysis, Academic Press, New York, 1970, 701-724.
Vasantha K, Priyavardhini S, Tresina SP, Mohan VR, Phytochemical analysis and antibacterial activity of kedrostisfoetidissima (jacq.), Cogn. Bioscience Discovery, 3, 2012, 6-16.