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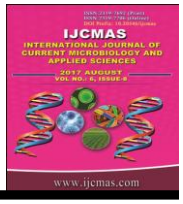

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Herbal Immersion Oil for Microscopic Identification of Malaria Parasites

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A B S T R A C T

Oils from thirty-two plants were extracted using solvent extraction/steam distillation methods. All oils along with 11 blended oil mixtures were tested for their immersion oil properties for the detection of malaria parasites. The physicochemical properties required for an immersion oil including density, viscosity, refractive index and acid value of five oils namely as *Ocimum basilicum, Pogostemon cablin, Papaver somniferum Ricinus communis* and *Valeriana jatamansi* and blended mixture 6 were determined. Stability of above stated plant oils and blended mixture 6, with time showed that all were stable under the observation period while non-dryness of these oils and blended mixture 6, represented very good criteria for the non-drying less than 1% variation in weight. GC-MS analysis of mixture 6 clearly identified compounds namely alpha copane, trans-Caryophyllene Linalool L, Estragole, hexadecanoic acid phenyl methyl ester and 9, 12-Octadecadienoic acid and besides other minor components peaks. No peak of castor oil was detected due to lack of sample character and detection. Summary of in-house validation of blended mixture 6 classified as very good immersion oil (range 67-81%) and good (19-33%) for the examination of malaria parasites while the results of external validation clearly revealed that the developed plant oil mixture 6 possessed very good property as immersion oil for the examination of malaria parasites.

Introduction

Conventional immersion oils typically contain polychlorinated biphenyls (PCBs) which when blended with mineral oil and viscosity adjusting compounds provide generally useful immersion oil having many of the ideal characteristic. In recent years, however, PCB's have been discovered to be carcinogenic, a hazard to the human environment, and are generally regarded as toxic. Furthermore, PCB's are difficult to dispose off after use since they are extremely stable and non -biodegradable (Liva, 1986).

Attempts were also made to use oils from natural resources like plants. Cedar wood oil having a refractive index of 1.495 to 1.510 (British Pharmacopeia, 1963) was widely used for many years as immersion oil. Cedar-wood oil was a mixture of organic compounds considered generally safe by the FDA as a food additive preservative and also used as an anti-bacterial, fungicide. However, studies have shown that prolonged exposure to high levels of cedar-wood oil can cause liver and pulmonary toxicity (FAO, 1995). In addition

to this the cost wise cedar wood oil was expensive. In India, Victor *et al.,* (2005) made an attempt to identify cost effective, but qualitative immersion oil for microscopy and photo microscopy. Pure castor oil and refined sunflower oil have been tried along with commercially available immersion oil on GTG banded chromosome preparations. Application of castor oil has been recommended as alternative to synthetic immersion oil. A review of the literature revealed that a limited work has been carried out to explore the possibility of using plant oils as an immersion oil for microscopy in spite of the fact that India has huge floura and fauna of plants. Attempts were made to screen different plants oils and combination of plant oils for their immersion oil property for microscopic identification of malaria parasites.

Materials and Methods

Thirty-two plants based on the physico - chemical properties reported in the literature were collected from various parts of India in consultation with Forest Research Institute, Dehradun. Scientific name, family and common names, raw material used, extraction method of the plants under investigation are given in table 1. Plants were dried in shades and oils were extracted from the plants using solvent extraction (Soxhlet Extraction) and steam distillation methods.

Microscopic examination of blood slides using plant oils as immersion oil

All oils were examined for their immersion oil properties for the microscopic identification of malaria parasites. Stained blood slides were examined under microscope at 100 x objective lens for detecting the malaria parasites. Different malaria parasite namely as *Plasmodium falciparum*/ *Plasmodium vivax* stages including gametocyte, rings, and schizont were examined by using immersion oil/plant oils.

Although microscopic examination is a manual qualitative approach, attempts were made to grade all plant oils for their performance based on clarity, sharpness and contrast of parasite stages. Based on microscopic examination of malaria parasite, only five plants oils showed good results as immersion oil. Eleven combinations of above five plants oils with different proportions were also prepared and microscopic tested of malaria parasites. The different compositions of five oils were as follow:

Mixture 1 Consisted of fifty percent each of *Ocimum basilicum* and *Papaver somniferum* (50:50), Mixture 2: Composed of fifty percent each of *Pogostemon cablin* and *Papaver somniferum* (50:50), Mixture 3: 25% *Ocimum basilicum* and 25% *Pogostemon cablin* oil and 50% oil *Papaver somniferum* (25:25:50), Mixture 4: *Ocimum basilicum* oil was added into *Ricinus communis* oil at the ratio of 50:50, Mixture 5: The mixture was made from fifty percent of *Pogostemon cablin* and *Ricinus communis oil* (50:50), Mixture 6: It consisted of twenty five percent of each four of *Pogostemon cablin, Ocimum basilicum, Papaver somniferum* and *Ricinus communis* (25:25:25:25), Mixture 7: *Papaver somniferum* was blended with *Ricinus communis* (50:50), Mixture 8: It was a combination of three different oils namely as *Ocimum basilicum, Pogostemon cablin* and *Ricinus communis* at the ratio of 33:33:34, Mixture 9: The thirty-five percent of *Ocimum basilicum, Pogostemon cablin* was blended with thirty percent of *Ricinus communis.*, Mixture 10: It is composed of twenty-five percent of each of four different oils namely as *Ocimum basilicum, Pogostemon cablin, Ricinus communis* and *Valeriana jatamansi* and Mixture 11. Thirty-four percent of *Valeriana jatamansi oil* was mixed with

thirty-three percent of *Papaver somniferum* and *Ricinus communis* oil.

Determination of physico-chemical properties of different oils

Density

The density of the microscopically selected plant oils/blended mixtures was measured by specific gravity bottles (relative density bottles) method. Experiments were conducted at room temperature.

Acid value

Acid value of different oils was determined as reported earlier (Barkatullah *et al.*, 2012).

Viscosity

The Viscosity of the microscopically selected plant oils/blended mixtures were measured at the shear rate by using a D.V-iii ultra-programmable rheometer (Brookfield Engineering Labs, U.S.A).The viscosity was determined by using different spindle no.18, 21 and 27 and different shear rates, ranging from 1.7-4.65s^{-1}. Nzikou *et al.,* (2007) method was used to measure the different oil viscosities.

Refractive index

The refractive index of microscopically selected immersion oils/blended mixtures oils were determined by using refractometer based on the principle of the critical angle using diffused daylight.

Non-dryness

The non-dryness was determined by performing a test at 30 C for 24 hours in accordance with JIS C 2201 Test of evaporation amount of "electrical insulating oils" (Fujioka *et al.*, 2009) and was evaluated based on the following two levels. Good (0): evaporation amount of less than 1% by mass Poor (x): evaporation amount of 1% by mass or more.

Stability

Microscopically selected plant oils/ blended mixtures were kept at 25 and37°C to investigate the stability of oils as an immersion oil. All the five plant oils/ blended mixtures were tested for the microscopic examination of malaria parasite at the intervals of five weeks.

Gas chromatography- mass spectrometry (GCMS) analysis of plant oils blended mixtures

GC-MS analysis for the separation and identification of plant oils /blended oils was performed at Department of Chemistry, Forest Research Institute, Dehradun. The sample was analyzed using a Shimadzu GC-2010 gas chromatograph coupled to a QP 2010 mass-selective detector with capillary column BP-20(30m in length, 0.25mm i.d, and 0.25µm in thickness). GCMS conditions were used as described by Dua *et al.,* (2013). Different peaks of gas chromatographic analysis were identified using NIST, WELY and SZTERP software library of mass spectra.

Validation

In –house validation

Four microscopist from National Institute of Malaria Research, field unit, BHEL, Haridwar were selected to examine the blood slides for malaria parasite using mixture 6. Each microscopist was provided 10 malaria positive blood slides with *Plasmodium falciparum* infection having ring/gametocyte and 10 *Plasmodium vivax* infections with ring, gametocytes and trophozoite stages.

External validation

Each participating institutes was provided mixture 6 as immersion oil for the evaluation report. It is suggested to examine minimum of ten blood slide each with *Plasmodium falciparum* and *Plasmodium vivax* infection having different stages of parasites for evaluation of particular oil.

Results and Discussion

The maximum % yield was obtained from *Junglans regia*, (51), followed by *Papaver somniferum* (45),*Michalia champaca* (37), *Terminellia belerica* (33), *Aleurites fordii* (32) *Sapindus mukrossi* (31), *Pongammia pinnata* (31), *Moringa oleferia* (29), *Ceiba pentandra* (28), *Sterculia foetida* (28), *Jatropa curcas* (28), *Ricinus communis* (25), *Terminelia chebulla* (22), *Bauhinia purpurea* (16), *Mimusops elengi* (15), *Bauhinia retusa* (15), *Pinus roxberghii* (15), *Pithecolobium dulci* (14), *Olea europaea* (14), *Psorylia coryfolia* (8). *Cesalpinia boundecella* (2), *Cymbopogon martini* (1.2), *Cedrus deodara* (1.2), *Melaleuca alternifolia* (1.1), *Cymbopogon citratus* (1.1), *Eucalyptus citriodora* (0.9), *Valeriana jatamansi* (0.87), *Cymbopogon nardus* (0.8), *Pogostemon cablin* (0.53), *Mentha piperita* (0.19), *Ocimum basilicum* (0.09), *and Pelargonium gravelens* (0.09).

A large variation in the extraction yield of oil from the plants may be due to variation in the plant parts, seasonal variation, and environmental factors for the growth of a particular plant.

Based on microscopic examination of malaria parasite only five plants oils namely Ocimum *basilicum, Pogostemon cablin, Papaver somniferum, Ricinus communis* and *Valeriana jatamansi* showed satisfactory results as immersion oil for the examination of malaria parasites.

Performance of different blended mixtures was in order of Mixture 6, Mixture 10, Mixture 4, Mixture 5, Mixture 8, Mixture 7, Mixture 11, Mixture 9, Mixture 1 and Mixture 2 respectively. Results revealed that the mixture 6 consisted of twenty-five percent of each four of *Pogostemon cablin, Ocimum basilicum, Papaver somniferum* and *Ricinus communis* (25:25:25:25) showed the best oil to be used for microscopic examination of malaria parasites. Figure 1 and 2 represented microscopic identification of *Plasmodium falciparum* ring and schizont stages of mixture 6 and synthetic immersion oil respectively.

The physiochemical properties required for an immersion oil including density, viscosity, refractive index and acid value of five oils namely as *Ocimum basilicum, Pogostemon cablin, Papaver somniferum, Ricinus communis* and *Valeriana jatamansi* and 11 blended mixtures were are summarized in table 2. Density, refractive index, viscosity and acid values from Ocimum *basilicum, Pogostemon cablin, Papaver somniferum, Ricinus communis, Valeriana jatamansi oils* and blended mixture 1, mixture 2, mixture 3, mixture 4, mixture 5, mixture 6, mixture 7, mixture 8, mixture 9, mixture 10 and mixture 11 ranged 0.916 – 0.967, 1.478 -1.501, 1.2 - 970 and 0.25 – 20.0 respectively. Refractive index and density of the *Ocimum basilicum* of present study was similar to the reports of Hussain *et al.,* (2008) who investigated the density and refractive index 0.95-0.97g/cm3 and1.4995-1.5045 respectively. Dev *et al* (2011) reported basil oil refractive index and density as 1.515 and 0.928 g/cm3 respectively. *Pogostemon cablin* oil density, refractive index and acid value were 0.919g/cm3, 1.499 and 0.25. Parganiha (2012) found that the density, refractive index and acid value of the *Pogostemon cablin* were 0.951-0.991, 1.5089, and1.68-3.93 respectively. Similarly, density, refractive

index, viscosity and acid value of *Papaver somniferum* oil were 0.921, 1.478, 65.4 and 20.3respectively. Ozcan and Atalay (2006) reported refractive index and acid value of the poppy seed oil as 1.4773 and 1.0-3.2repectively. *Ricinus communis* density, refractive index, viscosity and acid value were 0.961, 1.481, 970, and 0.3. respectively which were similar to earlier reports (Deligiannis *et al.*, 2009), *Valeriana jatamansi density*, refractive index, viscosity and acid value were 0.934, 1.491, 5.6, and 0.35 respectively.

Immersion liquids for light microscopy including Cargille immersion oils, currently comply by the ISO/German DIN (2015) which is specified for synthetic immersion oils. As per literature, no specifications have been given for the immersion oil obtained from the plants. Density, refractive index, acid value and viscosity of blended mixture 6 plant oil were 0.945, 1.488, 33.6 and 2.36 respectively which are slightly vary from the DIN specification due to the fact that mixture 6 is generated from the plants source. It is to point out that British Pharmacopeia has approved cedar wood plant oil as immersion oil and density, refractive index, viscosity and acid values were similar to the values found for mixture 6 oil.

Stability of oils from *Ocimum basilicum, Pogostemon cablin, Papaver somniferum, Ricinus communis, Valeriana jatamansi* and mixture 1, mixture 2, mixture 3, mixture 4, mixture 5, mixture 6, mixture 7, mixture 8, mixture 9, mixture 10 and mixture 11 with time showed that all were stable under the observation period while non-dryness of the oil extracted from *Ocimum basilicum, Pogostemon cablin, Papaver somniferum, Ricinus communis, Valeriana jatamansi* and blended mixture 1, mixture 2, mixture 3, mixture 4, mixture 5, mixture 6, mixture 7, mixture 8, mixture 9, mixture 10, mixture 11were represented very good criteria for the non-drying less than 1% variation in weight.

GC-MS analysis of patchouli oil revealed presence of alpha.-Copaene (79.13%), trans-Caryophyllene (13.74%) besides 3, 3, 5, 5, 3', 3', 5', 5'-Octamethyl-DI-(.DELT) and 7-Ethylidene-6b 7, 8,8a-tetrahydrocyclobut[a]. Luo *et al.*, (1999) identified pogostone (30.99%) in stems, 21.31% in leaves, patchouli alcohol (10.26%) in stems, 37.53% in leaves, deltaguaiene, alpha-guaiene (2.27%) in stems, 6.18% in leaves, seychellene (1.56%) in stems, 1.99% in leaves, alpha-patchoulene, aciphyllene, and trans-caryophyllene (4.92%) in stems, 6.75% in leaves as main constituents from leaves and stem of Pogostemonis (Patchouli) while Cheng *et al.*, (2010) identified patchouli alcohol and pogostone as chemical markers. GC-MS study of Patchouli oil carried out by Micheal (1992) and Daniel (2006), Baby *et al.*, (2007) showed presence of 74 compounds namely Patchouli alcohol, 3-octanone, Benzaldehyde, dimethyl phenol, octanoic-acid, Pogostol, 4-methyl-pentanoic-acid,b-elemene, epiguaipyridine, Ombuine, nor-patchoulinol, a-bulnesene, b-patchoulene, epoxycaryophyllen e, p-vinyl-phenol, seychellene, a-bulnesene oxide, b-pinene, Eugenol, pachypodol, nor-patchoulinol, a-bulnesone, Bulnesol, eugenol, cinnamic aldehyde, patchouli-alcohol, patchouli pyridine, a-guaiene, Cadinene, g-patchoulene, Patchoulipyridine, Methylchavicol, a-guaiene oxide, Camphene, guaiacol, pentanoic-acid, Limonene, a-patchoulene, caryophyllene, guaipyridine, phenol, Pinene, a-pinene, caryophyllene-oxide, heptanoic-acid, pogostol, p-methoxycinnamaldehyde, anethole, cinnamaldehyde, humulene, pogostone, 1,10-epoxy-alphabulnesene, anisaldehyde, cis-2-pentylcyclopropylcarboxylic –acid, Limonene, rhamnetin, 1-alpha,5-alpha-epoxyalpha-guaiene, Apigenin,

cycloseychellene, nonanoic-acid, seychellene, 1-beta,5-beta-epoxy-alphaguaiene, apigenin-7-o-beta-d-(-6"p-coumaroyl)-glucoside, d-patchoulene, nordehydropatcho ulol,tannin,2-methyl-butyric-acid,apigenin-7-o-beta-glucoside; benzaldehyde, dehydracetic-acid, norpatchoulenol, trans-2pentylcyclopropylcarboxylic-acid,2-methylhexanoic-acid, azulene, dhelwangin, o-cresol. Bunrathep (2006) identified Sesquiterpenes δ-elemene (t-trace), β-patchoulene (t), β-elemene, cis-thujopsene, trans-caryophyllene, α-guaiene, γ-patchoulene, α-humulene, α-patchoulene, seychellene, valencene, germacrene D, β-selinene, α-selinene, viridiflorene, germacrene A, α-bulnesene, 7-epi-α-selinene, oxygenated sesquiterpenes longipinanol, globulol, patchouli alcohol ,1-octen-3-ol. Among these, patchouli alcohol (60.30 %) was the major component, followed by germacrene A (11.73 %).

Bure and sellier (2004) carried out GC/MS study of Indonesian patchouli oil and showed the presence of the following compounds; α-pinene, δ-patchoulene, β-pinene, aciphyllene, limonene, δ-guaiene, δ-elemene, 7-epi-α-selinene, α-copaene, norpatchoulenol, α-patchoulene, 1,10-epoxy-11-bulnesene, β-elemene, caryophyllene oxide, cycloseychellene, nortetrapatchoulol, β-caryophyllene, patchouli alcohol, α-guaiene, patchoulenone, seychellene, 9-oxopatchoulol, α-humulene, pogostol, α-patchoulene, isopatchoulenone, γ-gurjunene, and germacrene D.

Our study revealed alpha copane and trans-Caryophyllene as a chemical marker for the presence of patchouli oil. It is to point out that alpha copaene and trans-Caryophyllene has been reported as a major constituent in patchouli oil by Feng and coworkers (1999), Luo *et al.,* (1999) and Silva *et al.,* (2004).

GC-MS analysis of basil oil revealed presence of Linalool L (24.07%), Estragole (73.47%), 3,3,5,5,3',3',5',5' Octamethyl -DI-(DELT) (1.55%),1,1'-bibicyclo (2.2.2) octyl1-4-carboxylic acid (0.51%), and 1,1'-bibicyclo (2.2.2) octyl1-4-carboxylic acid (0.4%). Kathirvel and Ravi (2012) reported chemical compositions of basil fresh leaves identified by GC-MS: 11 components were identified. The major constituents were found to be methyl cinnamate (70.1%), linalool (17.5%), β-elemene (2.6%) and camphor (1.52%). Linalool was the main constituent of *O. basilicum* essential oil (56.7-60.6%), followed by epi-a-cadinol (8.6-11.4%), a-bergamotene (7.4-9.2%), c-cadinene (3.3-5.4%), germacrene D (1.1-3.3%) and camphor (1.1-3.1%) (Hussain *et al.,* 2008).

Present study revealed that Linalool L and Estragole as chemical marker for the identification of basil oil. Estragol, linalool, methyl eugenol, geraniol, methyl cinnamate, bergamotene, α-cubebene, germacrene D, β-elemene, 1,8-cineole, methyl cinnamate, α-cadinol and limonene are considered as the main constituents and chemotypes of basil from different parts of the world (Koba *et al.,* 2009, Zhang and coworkers (2009), Abduelrahman *et al.,* (2009) and Vani and coworkers (2009). Jirovetz and Buchbauer (2001) found a high level of linalool (71.4%) in *O. basilicum* essential oil from Bulgaria. According to Gurbuz *et al.,* (2006), linalool (41.2%) was the main compound, identified in the hydro-distilled *O. basilicum* essential oil from Turkey. Hassanpouraghdam *et al.,* (2010) reported Linalool in leaves as major component in Basil oil.

GC-MS analysis of poppy seed revealed presence of hexadecanoic acid ethyl ester (1.40), 9-octadecanoic acid (3.55), 9,12,15–octadecatrienoic acid, (2-phenyl-1,3-dioxolan-4-YL) methyl E (0.22), hexadecanoic acid phenyl methyl ester (30.73), ethyl(9Z,12Z)-9,12-Octadecadienoate (11.23),9,12-Octadecadienoic acid (Z, Z) (41.59), and Heneicosyl pentafluoro-

propionate (7.82). Ozcan and Atalay (2006) reported linoleic acid high in all the samples, and varied between 52.6 to 71.50% while Rahimi *et al.,* (2011) studied opium poppy seed oils from Turkey where the main fatty acids were linoleic (56.4- 69.2%), oleic (16.1-19.4%) and palmitic acids (10.6- 16.3%). Singh *et al.,* (1990) stated poppy seeds contained up to 50% oil and Indian cultivars have high levels of oleic and linoleic acids. Similarly, some researchers have reported that the linoleic (C18:2), oleic (C18:1) and palmitic acids (C16:0) are major fatty acids in the poppy seed oil (Erinc *et al.,* 2009, Singh *et al.,* (1990), Sener *et al.,* (1999), Bezakova *et al.,* (1994), Bajpai (1999), Bozan and Temelli (2008) and Luthra and Singh, 1989).

Table.1 Scientific name, family and common names, raw plant parts used for extraction and method of extraction the plants under investigation

S.no.	Scientific name	Family	Common name	Raw material used for oil extraction	Method of extraction
1.	*Cymbopogon martini*	Graminae (Poaceae)	Palmarosa	Leaves	Steam Distillation
2.	*Pogostemon cablin*	Lamiaceae (Labiatae)	Patchouli	Leaves	Steam Distillation
3.	*Melaleuca alternifolia*	Myrtaceae	Tea Tree	Leaves	Steam Distillation
4.	*Cedrus deodara*	Pinaceae	Cedar wood	Cone	Steam Distillation
5.	*Pelargonium gravelens*	Geraniaceae	Geranium	Leaves	Steam Distillation
6.	*Cymbopogon nardus*	Graminae (Poaceae)	Citronella	Leaves	Steam Distillation
7.	*Pinus roxberghii*	Pinaceae	Pine	Needle	Steam Distillation
8.	*Ocimum basilicum*	Lamiaceae (Labiatae)	Basil	Leaves	Steam Distillation
9.	*Mentha piperita*	Lamiaceae (Labiatae)	Peppermint	Leaves	Steam Distillation
10.	*Cymbopogon citratus*	Graminae (Poaceae)	Lemongrass	Leaves	Steam Distillation
11.	*Eucalyptus citriodora*	Myrtaceae	Lemon gum	Leaves	Steam Distillation
12.	*Jatropa curcas*	Euphorbiaceae	Ratanjyot, Physic Nut	Seed	Solvent Extraction
13.	*Pongammia pinnata*	Leguminosae, (Fabaceae)	Karanja oil	Seed	Solvent Extraction
14.	*Terminelia chebulla*	Combretaceae	Harra	Seed	Solvent Extraction
15.	*Terminellia*	Combretaceace.	Bahera,	Seed	Solvent

	belerica		bastard myrobalan		Extraction
16.	*Sapindus mukrossi*	Sapindaceae	Reetha, soapnut	Seed	Solvent Extraction
17.	*Mimusops elengi*	Sapotaceae	Maulsari	Seed	Solvent Extraction
18.	*Bauhinia retusa*	Caesalpiniaceae	Devanagari	Seed	Solvent Extraction
19.	*Bauhinia purpurea*	Leguminosae	Orchid tree, purple bauhinia	Seed	Solvent Extraction
20.	*Pithecolobium dulci*	Fabaceae	Madras thorn, jungle jalebi	Seed	Solvent Extraction
21.	*Ceiba pentandra*	Malvaceae	Kapok	Seed	Solvent Extraction
22.	*Moringa oleferia*	Moringaceae	Senjana	Seed	Solvent Extraction
23.	*Sterculia foetida*	Malvaceae	Wild almond	Seed	Solvent Extraction
24.	*Junglans regia*	Juglandaceae.	Walnut	Seed	Solvent Extraction
25.	*Michalia champaca*	Magnolia	Champaca	Seed	Solvent Extraction
26.	*Cesalpinia boundecella*	Caesalpiniaceae	Gray Nicker, Kantkarej	Seed	Solvent Extraction
27.	*Aleurites fordii*	Euphorbiaceae	Tung	Seed	Solvent Extraction
28.	*Olea europaea*	Oleaceae	Olive	Seed	Solvent Extraction
29.	*Papaver somniferum*	Papaveraceae	Opium poppy	Seed	Solvent Extraction
30.	*Ricinus Communis*	Euphorbiaceae	Castor	Seed	Solvent Extraction
31.	*Valeriana jatamansi*	Caprifoliaceae	Jatamansi	Root	Solvent Extraction
32.	*Psoralea corylifolia*	Fabaceae	Babchi	Seed	Solvent Extraction

Table.2 Physicochemical properties of plant oils and blended mixtures used as immersion oil for microscopic examination of malaria parasites

S. No	Name of the oils/ Mixtures	Density (g/cm3)	Refractive Index (RI)	Viscosity at 5 RPM(cP)	Acid Value (mg/KOH)
1	*Ocimum basilicum* (Basil)	0.937	1.501	1.2	0.57
2	*Pogostemon cablin* (Patchouli)	0.919	1.499	9	0.25
3	*Papaver somniferum* (Poppy seed)	0.921	1.478	65.4	3.49
4	*Ricinus communis* (Castor)	0.961	1.481	970	0.3
5	*Valeriana jatamansi* (*Jatamansi*)	0.934	1.491	5.6	0.49
6	Mixture1	0.927	1.492	13.8	3.76
7	Mixture2	0.916	1.488	29	3.36
8	Mixture3	0.937	1.491	19.4	2.76
9	Mixture4	0.947	1.494	31.2	0.48
10	Mixture5	0.936	1.491	102	0.31
11	Mixture6	**0.945**	**1.488**	**33.6**	**1.70**
12	Mixture7	0.967	1.478	206	3.15
13	Mixture8	0.922	1.485	18.6	0.29
14	Mixture9	0.932	1.485	16.8	0.29
15	Mixture10	0.935	1.478	16	0.5
16	Mixture11	0.919	1.478	24.4	1.45

Table.3 Result of In-house validation

S. No.	Examiner Code	Total slides examined	Observations	
			Very Good ++++ (%)	Good +++ (%)
1	NC	20	16(80)	4(20)
2	MR	20	15 (75)	5(25)
3	AR	20	16 (80)	4(20)
4	AK	20	17 (85)	3(15)

Fig.1a&b Microscopic identification of *Plasmodium falciparum* ring stage using synthetic immersion oil and microscopic identification of *Plasmodium falciparum* ring stage using Mixture 6 immersion oil

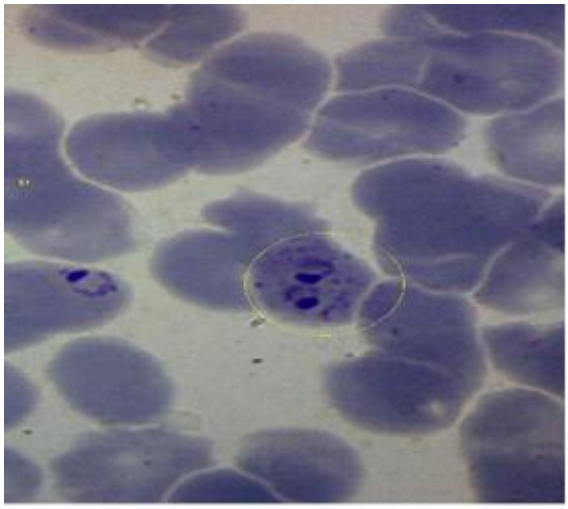

Figure 1a

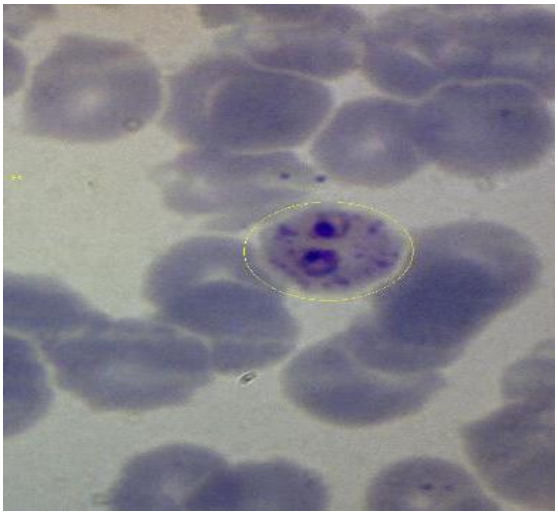

Figure 1b

Fig.2a&b Microscopic identification of *Plasmodium falciparum* schizont stage using synthetic immersion oil and Microscopic identification of *Plasmodium falciparum* schizont stage using Mixture 6 immersion oil

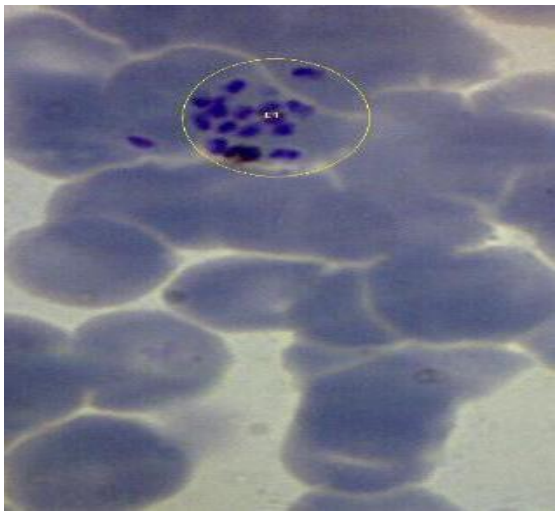

Figure 2a

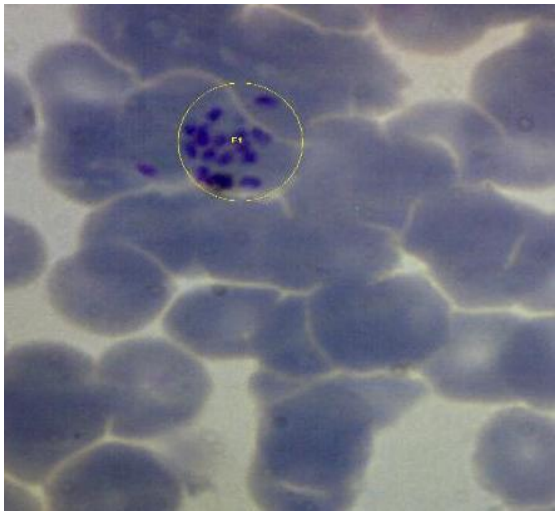

Figure 2b

Table.4 Result of external validation

S. No.	Examiner Code	Total slide examine	Observations	
			Very Good ++++ (%)	Good +++ (%)
1	HIM	20	17 (85)	3(15)
2	SG	20	16(80)	4(20)
3	MET	20	18(90)	2(10)
4	GH	20	17(85)	3(15)

HIM: Himalayan Hospital Jolly Grant, Dehradun, SG: Shri Guru Ram Rai Hospital Dehradun, MET: Metro Hospital SIDCUL, Hardwar and GH: Government Hospital Hardwar.

GC-MS analysis of mixture 6 clearly identified presence of alpha copane and trans-Caryophyllene as a chemical marker for the presence of patchouli oil, Linalool L and Estragole as chemical marker for basil oil and phenyl methyl ester, and 9,12-Octadecadienoic acid for the presence of poppy seed oil besides other minor components peaks as stated in results. It is to note that no peak was detected for castor oil due to lack of sample character and detection procedure.

Summary of in-house validation of blended mixture 6 carried out by four microscopists is given in table 3. It is to point out that all four microscopists have classified mixture 6 as very good immersion oil (range 67-81%) and good (19-33%) for the examination of malaria parasites while the results of external validation by four different hospitals/ laboratory are given in table 4. Certifications by four Head/Incharge of the hospitals clearly revealed that the developed plant oil mixture 6 possessed very good property as immersion oil for the examination of malaria parasites and may develop as an alternative of synthetic immersion oil.

Present study was aimed to find out plant oil based immersion oil as an alternative to synthetic immersion oil which showed airborne contact dermatitis, burning pruritus and urticarial-like lesions on the face and forearms and carcinogenic, a hazard to the human environment. A blended mixture consisted of twenty-five percent of *Pogostemon cablin*, *Ocimum basilicum*, *Papaver somniferum* and *Ricinus communis* (25:25:25:25) showed very good results for microscopic examination of malaria parasites.

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